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(54) **METHODS OF TREATMENT USING ANTI-ERBB ANTIBODY-MAYTANSINOID CONJUGATES**

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(57) **ABSTRACT**

The application concerns methods of treatment using anti-ErbB receptor antibody-maytansinoid conjugates, and articles of manufacture suitable for use in such methods. In particular, the invention concerns ErbB receptor-directed cancer therapies, using anri-ErbB receptor antibody-maytansinoid conjugates.

8 Claims, 46 Drawing Sheets

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VARIABLE HEAVY DOMAIN

```
                  10        20          30          40
2C4       EVQLQQSGPELVKPGTSVKISCKAS [GFTFTDYTMD] WVKQS
              **  **  *  * ***  *                  * *
574       EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTDYTMD] WVRQA
                                       ** * *
hum III   EVQLVESGGGLVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA

                     50 a      60          70          80
2C4       HGKSLEWIG [DVNPNSGGSIYNQRFKG] KASLTVDRSSRIVYM
          *  *   **                     *** *    **** *
574       PGKGLEWVA [DVNPNSGGSIYNQRFKG] RFTLSVDRSKNTLYL
                     ****** *** ****       * * *
hum III   PGKGLEWVA [VISGDGGSTYYADSVKG] RFTISRDNSKNTLYL

            abc      90         100ab         110
2C4       ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLTVSS
          ***  **                                **
574       QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGTLVTVSS
                             ********
hum III   QMNSLRAEDTAVYYCAR [GRVGYSLYDY] WGQGTLVTVSS
```

FIG. 1

Variable Light Domain

```
                10        20        30        40
2C4     DTVMTQSHKIMSTSVGDRVSITC [KASQDVSIGVA] WYQQRP
          **  ****  *         *                 *
574     DIQMTQSPSSLSASVGDRVTITC [KASQDVSIGVA] WYQQKP
                                  *  ** ***
hum KI  DIQMTQSPSSLSASVGDRVTITC [KASQDVSIGVA] WYQQKP

                50        60        70        80
2C4     GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTFTISSVQA
            **              *  *      *    * *
574     GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTLTISSLQP
              * *****
hum KI  GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISSLQP

                90       100
2C4     EDLAVYYC [QQYYIYPYT] FGGGTKLEIKRT
           * *               *  *
574     EDFATYYC [QQYYIYPYT] FGQGTKVEIKRT
                    *** *
hum KI  EDFATYYC [QQYNSLPWT] FGQGTKVEIKRT
```

FIG. 2

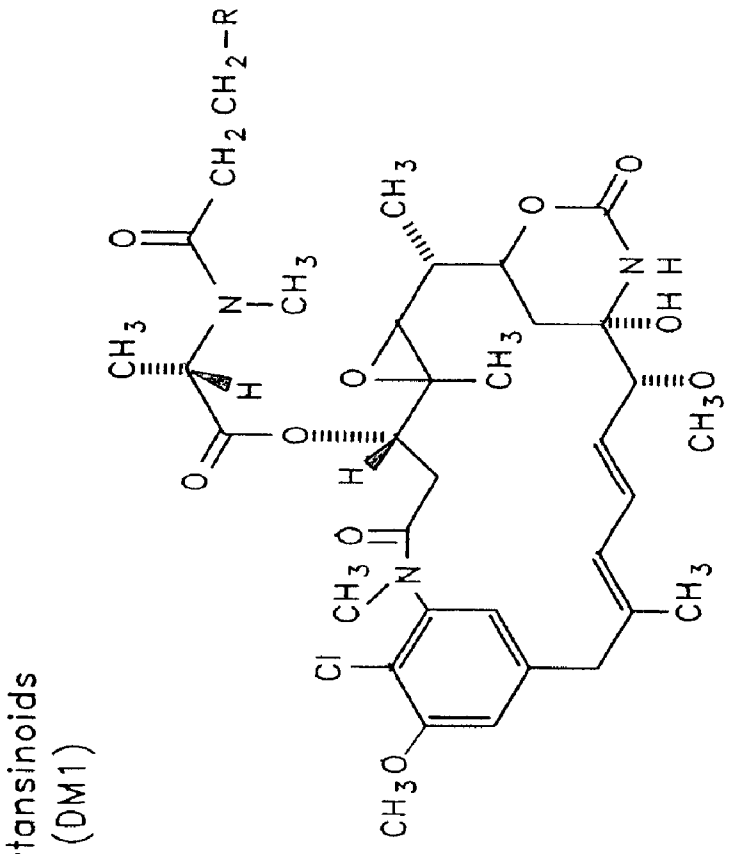

FIG. 3

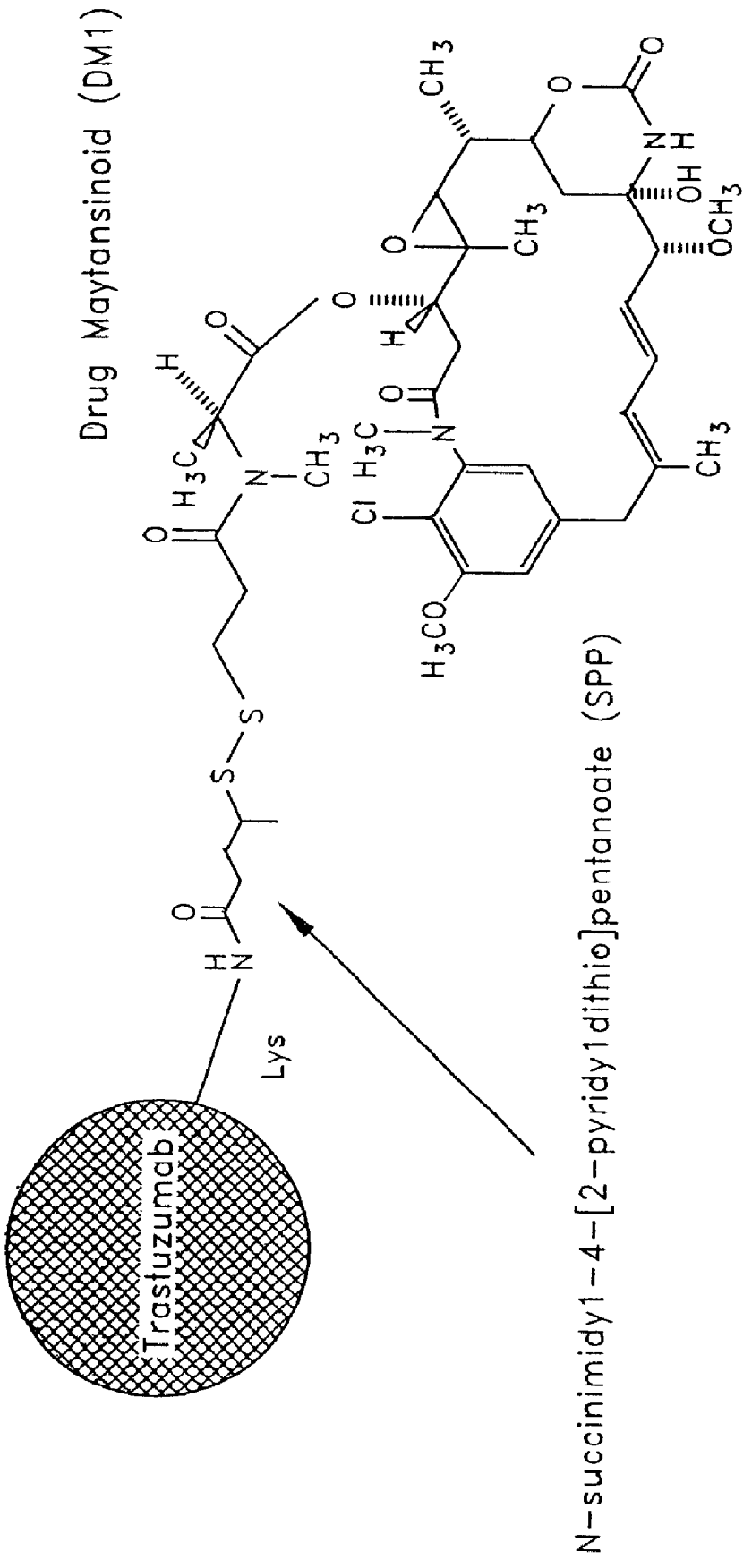
Drug Maytansinoid (DM1)
Trastuzumab
Lys
N-succinimidyl-4-[2-pyridyldithio]pentanoate (SPP)

FIG. 4

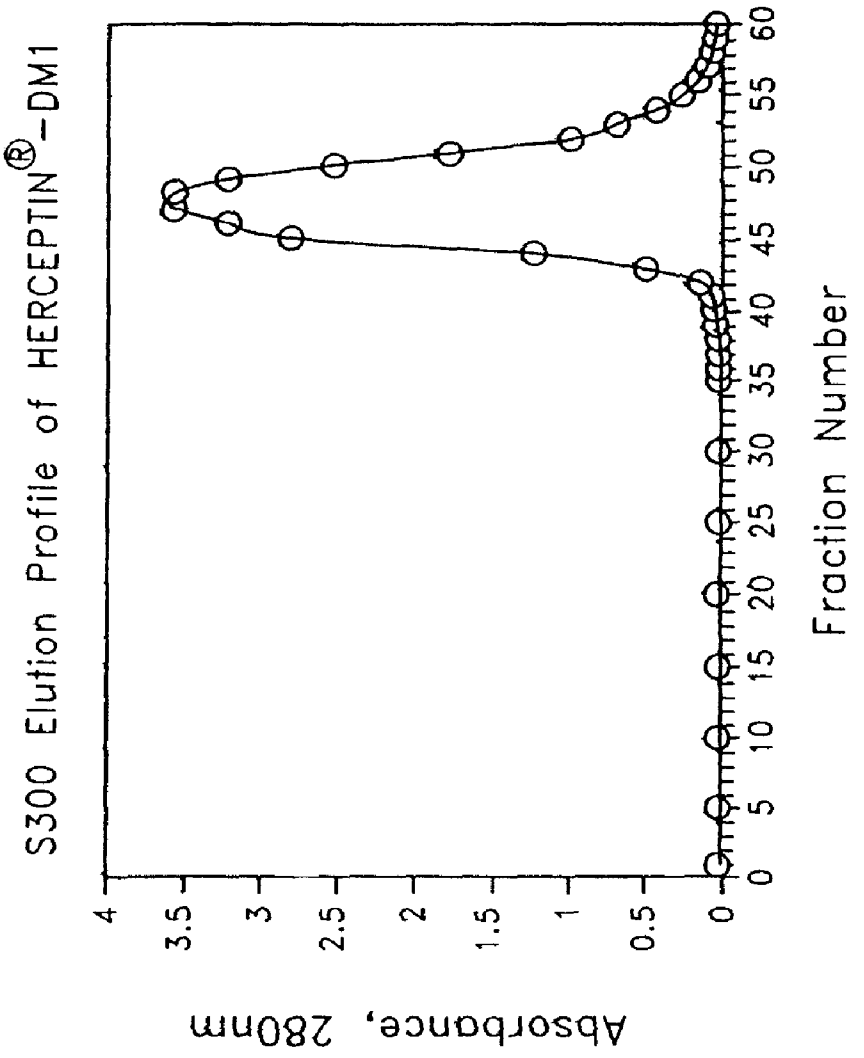
S300 Elution Profile of HERCEPTIN®-DM1
Absorbance, 280nm
Fraction Number
0
0.5
1
1.5
2
2.5
3
3.5
4
0
5
10
15
20
25
30
35
40
45
50
55
60

FIG. 5

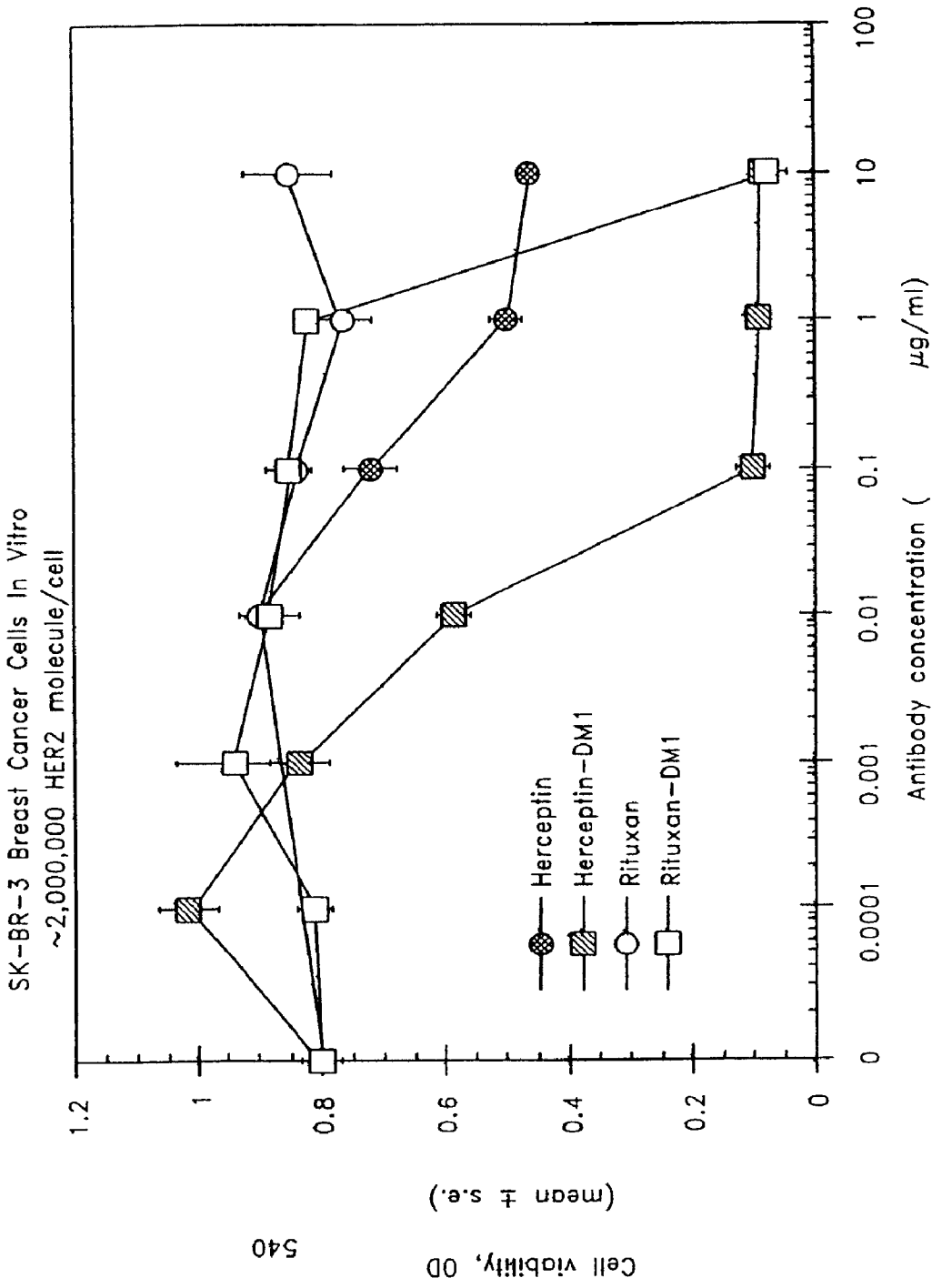
SK-BR-3 Breast Cancer Cells In Vitro
~2,000,000 HER2 molecule/cell
Herceptin
Herceptin-DM1
Rituxan
Rituxan-DM1
Cell viability, OD
540
(mean ± s.e.)
Antibody concentration (μg/ml)
0
0.2
0.4
0.6
0.8
1
1.2
0.0001
0.001
0.01
0.1
1
10
100

FIG. 6

```
                                           rmaI
                hgiAI/aspHI      sau3AI    maeI
          sau3AI                 mboI/ndeII
          mboI/ndeII   tru9I     dpnII    nheI         nlaIII  tseI                                     rmaI
          dpnII bsp1286          dpnI cac8I   tseI    sphI     fnu4HI/bsoFI                             maeI
         pvuI/bspCI    mseI   nlaIII thaI cac8I       nspHI    bbvI                                     bfaI
         mcrI   bsiHKAI   tsp509I    fnuDII/mvnI  cac8I      scfI                                      styI
         bsiEI  bmyI  aseI/asnI/vspI bstUI bfaI  aluI nspI   pstI                                      bsaJI
        taqI    apaLI/snoI   rcaI    bsh1236I fnu4HI/bsoFI cac8I                                       blnI
     aluI dpnI  alw44I/snoI  bspHI nruI  aluI bbvI    cac8I  bsgI                   avaI               avrII
  1 AAGCTCGATC GGTGCACATT AATTCATGAT CGCGAGCTAG CAGCTTGCAT GCCTGCAGCA GAAATGGTTG AACTCCCGAG AGTGTCCTAC ACCTAGGGGA
    TTCGAGCTAG CCACGTGTAA TTAAGTACTA GCGCTCGATC GTCGAACGTA CGGACGTCGT CTTTACCAAC TTGAGGGCTC TCACAGGATG TGGATCCCCT
     ^start of linker 1                          ^end of linker 1
                                                                   ^start of MMTV promoter

                                                                 hgiJII
           styI                                   hinPI          bsp1286
           bsaJI                                  hhaI/cfoI      bmyI                                  mwoI
       tseI                                      mstI            banII                             tseI
       fnu4HI/bsoFI            styI    bcgI      aviII/fspI  fokI                                  fnu4HI/bsoFI
       bbvI                    bsaJI ahdI/eam1105I           bstF5I                                bbvI
101 GAAGCAGCCA AGGGGTTGTT TCCCACCAAG GACGACCCGT CTGCGCACAA ACGGATGAGC CCATCAGACA AAGACATATT CATTCTCTGC TGCAAACTTG
    CTTCGTCGGT TCCCCAACAA AGGGTGGTTC CTGCTGGGCA GACGCGTGTT TGCCTACTCG GGTAGTCTGT TTCTGTATAA GTAAGAGACG ACGTTTGAAC

                                                     mwoI
                                                     cac8I   hgiJII
                                                   hgiAI/aspHI                              mboII
                                                   bsp1286   bsp1286                       earI/ksp632I
                                           mwoI    bsiHKAI   bmyI           pleI   tru9I sapI           mamI
        aluI                               aciI    bmyI      banII hphI     hinfI  mseI aluI            bsaBI
201 GCATAGCTCT GCTTTGCTGG GGCATTGGGG GAAGTTGCGG TTCGTGCTCG CAGGGCTCTC ACCCTTGACT CTTTTAATAG CTCTTCTGTG CAAGATTACA
    CGTATCGAGA CGAAACGACC CCGTAACCCC CTTCAACGCC AAGCACGAGC GTCCCGAGAG TGGGAACTGA GAAAATTATC GAGAAGACAC GTTCTAATGT
```

FIG. 7A

```
                                           mnlI       sau96I                                 claI/bsp106
                                          ddeI        avaII                                  bspDI
                                         eco81I       asuI                                  sfaNI
           tsp509I          taqI    mnlI bsu36I/mstII/sauI                                 aciI taqI
                                                                           mnlI          fnu4HI/bsoFI        eco57I
301 ATCTAAACAA TTCGGAGAAC TCGACCTTCC TCTCCTGAGG CAAGGACCAC AGCCAACTTC CTCTTACAAG CCGCATCGAT TTTGTCCTTC AGAAATAGAA
    TAGATTTGTT AAGCCTCTTG AGCTGGAAGG AGAGGACTCC GTTCCTGGTG TCGGTTGAAG GAGAATGTTC GGCGTAGCTA AAACAGGAAG TCTTTATCTT

           cac8I                                                                                                rsaI
       bsmI             tsp509I                                                       tru9I      tfiI           csp6I
                                                                           maeIII     mseI       hinfI          scaI
401 ATAAGAATGC TTGCTAAAAA TTATATTTTT ACCAATAAGA CCAATCCAAT AGGTAGATTA TTAGTTACTA TGTTAAGAAA TGAATCATTA TCTTTTAGTA
    TATTCTTACG AACGATTTTT AATATAAAAA TGGTTATTCT GGTTAGGTTA TCCATCTAAT AATCAATGAT ACAATTCTTT ACTTAGTAAT AGAAAATCAT

                                                         hgaI
               tsp509I                                  esp3I         tsp509I                                  haeIII/p
             apoI                                       bsmBI         munI/mfeI                                stuI
                                                        bsmAI       mnlI     mboII                             haeI
501 CTATTTTTAC TCAAATTCAG AAGTTAGAAA TGGGAATAGA AAATAGAAAG AGACGCTCAA CCTCAATTGA AGAACAGGTG CAAGGACTAT TGACCACAGG
    GATAAAAATG AGTTTAAGTC TTCAATCTTT ACCCTTATCT TTTATCTTTC TCTGCGAGTT GGAGTTAACT TCTTGTCCAC GTTCCTGATA ACTGGTGTCC

                                                                       bsmFI
                                                                      scrFI
                                                                      mvaI                sau96I
                                                                      ecoRII              avaII
                                                                      dsaV                asuI
                                                                      bstNI              ppuMI
     rmaI                                                             bssKI              nlaIV
     maeI                                                             bsaJI              eco0109I/draII
     bfaI                                            bsmAI            apyI               bsmFI        scfI
601 CCTAGAAGTA AAAAAGGGAA AAAAGAGTGT TTTTGTCAAA ATAGGAGACA GGTGGTGGCA ACCAGGGACT TATAGGGGAC CTTACATCTA CAGACCAACA
    GGATCTTCAT TTTTTCCCTT TTTTCTCACA AAAACAGTTT TATCCTCTGT CCACCACCGT TGGTCCCTGA ATATCCCCTG GAATGTAGAT GTCTGGTTGT

                                                                                                    mnlI
                                                                                                   sau3AI
                                                                                                   mboI/ndeII
                                                                                                   dpnII
                                          tsp509I                                                  dpnI
                                        tru9I                                                      alwI
    sfaNI       bslI       mboII        mseI                 maeIII                              bstYI/xhoII
701 GATGCCCCCT TACCATATAC AGGAAGATAT GACTTAAATT GGGATAGGTG GGTTACAGTC AATGGCTATA AAGTGTTATA TAGATCCCTC CCTTTTCGTG
    CTACGGGGGA ATGGTATATG TCCTTCTATA CTGAATTTAA CCCTATCCAC CCAATGTCAG TTACCGATAT TTCACAATAT ATCTAGGGAG GGAAAAGCAC
```

FIG. 7B

```
                      rmaI                                                                               nlaIII           rmaI
                      maeI        styI                                                                  rsaI               maeI
      pleI            bfaI        bsaJI             smlI                                                csp6I              bfaI        bsmI
      hinfI          aluI      mnlI              bsmAI    mboII             nlaIII
801 AAAGACTCGC CAGAGCTAGA CCTCCTTGGT GTATGTTGTC TCAAGAAGAA AAAGACGACA TGAAACAACA GGTACATGAT TATATTTATC TAGGAACAGG
    TTTCTGAGCG GTCTCGATCT GGAGGAACCA CATACAACAG AGTTCTTCTT TTTCTGCTGT ACTTTGTTGT CCATGTACTA ATATAAATAG ATCCTTGTCC

                                                   tspRI
                                                   alwNI
                                styI
                                bslI     bsmFI
                          bslI  bsaJI mnlI         alw26I/bsmAI                                       nlaIII
901 AATGCACTTT TGGGGAAAGA TTTTCCATAC CAAGGAGGGG ACAGTGGCTG GACTAATAGA ACATTATTCT GCAAAAACTT ATGGCATGAG TTATTATGAA
    TTACGTGAAA ACCCCTTTCT AAAAGGTATG GTTCCTCCCC TGTCACCGAC CTGATTATCT TGTAATAAGA CGTTTTTGAA TACCGTACTC AATAATACTT

                                            tru9I
               sau96I             styI      mseI                                            fokI
               haeIII/palI   nlaIV         smlI                                tru9I         bstF5I
               asuI        aciI    bsaJI  aflII/bfrI          maeIII            mseI        mnlI        bsmAI
001 TAGCCTTTAT TGGCCCAACC TTGCGGTTCC CAAGGCTTAA GTAAGTTTTT GGTTACAAAC TGTTCTTAAA ACGAGGATGT GAGACAAGTG CTTTGGTGAC
    ATCGGAAATA ACCGGGTTGG AACGCCAAGG GTTCCGAATT CATTCAAAAA CCAATGTTTG ACAAGAATTT TGCTCCTACA CTCTGTTCAC CAAAGGACTG

                                  sstI
                                  sacI
                                  hgiJII
                                  hgiAI/aspHI
                                  ecl136II
                                  bsp1286
                                  bsiHKAI
                                  bmyI
                                dde I
                             sau3AI
                             mboI/ndeII
                             dpnII aluI
                             dpnI banII ddeI                                       tsp509I
                                                                                  apoI
101 TTGGTTTGGT ATCAAAGGTT CTGATCTGAG CTCTGAGTGT TCTATTTTCC TATGTTCTTT TGGAATTTAT CCAAATCTTA TGTAAATGCT TATGTAAACC
    AACCAAACCA TAGTTTCCAA GACTAGACTC GAGACTCACA AGATAAAAGG ATACAAGAAA ACCTTAAATA GGTTTAGAAT ACATTTACGA ATACATTTGG
```

FIG. 7C

PHIGENIX
Exhibit 1001-15

```
                                                                                          bsmAI
                                                                                         esp3I
                                                          mnlI                       fokI bsmBI bsrBI tsp45I
                                                        hphI                         bstF5I    aciI  maeIII
201 AAGATATAAA AGAGTGCTGA TTTTTTGAGT AAACTTGCAA CAGTCCTAAC ATTCACCTCT TGTGTGTTTG TGTCTGTTCG CCATCCCGTC TCCGCTCGTC
    TTCTATATTT TCTCACGACT AAAAAACTCA TTTGAACGTT GTCAGGATTG TAAGTGGAGA ACACACAAAC ACAGACAAGC GGTAGGGCAG AGGCGAGCAG

                                             sau3AI                          fnu4HI/bsoFI
                          sau96I            alwI                            haeIII/palI
                          nlaIV          mspI       cac8I     bcgI         mcrI
                          avaII          hpaII   rmaI      tfiI            eagI/xmaIII/eclXI
                          asuI          scrFI    maeI      hinfI           eaeI
                         sanDI          nciI mboI/ndeII thaI claI/bsp106 notI
                         ppuMI          dsaV dpnII  aluI fnuDII/mvnI     fnu4HI/bsoFI  scfI
                         nlaIV          cauII    nheI   bstUI taqI   cac8I cfrI   tru9I
                         eco0109I/draII bssKI    cac8I  bsh1236I    aluI aciI aciI    pstI
                      mnlI bsmFI aciI  bsaJI dpnI bfaI nruI  bspDI hindIII bsiEI  mseI bsgI             mnlI
301 ACTTATCCTT CACTTTCCAG AGGGTCCCCC CGCAGACCCC GGATCGCTAG CTCGCGAATC GATAAGCTTG CGGCCGCTTA ACTGCAGAAG TTGGTCGTGA
    TGAATAGGAA GTGAAAGGTC TCCCAGGGGG GCGTCTGGGG CCTAGCGATC GAGCGCTTAG CTATTCGAAC GCCGGCGAAT TGACGTCTTC AACCAGCACT
                                                            ^start of BS intron insert at Cla
                                                                   ^bp820 in pCI
                                                                   ^start of BS insert at Hind111

                                                                                  pleI
                                                                                  hinfI
                                                                               mboII                 nlaIV
      bsrI                                tru9I bsmAI                    bsmAI bpuAI                 hgiCI
     tspRI bspMI               maeIII     mseI  bsaI       bsrI        taqI    bbsI                  banI
401 GGCACTGGGC AGGTAAGTAT CAAGGTTACA AGACAGGTTT AAGGAGACCA ATAGAAACTG GGCTTGTCGA GACAGAGAAG ACTCTTGCGT TTCTGATAGG
    CCGTGACCCG TCCATTCATA GTTCCAATGT TCTGTCCAAA TTCCTCTGGT TATCTTTGAC CCGAACAGCT CTGTCTCTTC TGAGAACGCA AAGACTATCC
                ^start of chimeric intron at pCI 857
```

FIG. 7D

```
                                                                                               fnu4HI/bsoFI
                                                                                            mcrI
                                                                                            eagI/xmaIII/eclXI
                                                                  scrFI                     eaeI
                                                                  mvaI                      cfrI
                                                                  ecoRII                   notI
                                                                  dsaV                     fnu4HI/bsoFI
                                                                  bstNI                tru9I  haeIII/palI        tsp509I
                                                                  bssKI                mseI bsiEI   hindIII     ecoRI
                       fokI                                       apyI            aluI smlI aciI aciI aluI       taqI
                       bstF5I                               bslI  bsaJI      tsp509I    aflII/bfrI  cac8I    ecoRV apoI
501  CACCTATTGG TCTTACTGAC ATCCACTTTG CCTTTCTCTC CACAGGTGTC CACTCCCAGG TTCAATTACA GCTCTTAAGC GGCCGCAAGC TTGATATCGA
     GTGGATAACC AGAATGACTG TAGGTGAAAC GGAAAGAGAG GTGTCCACAG GTGAGGGTCC AAGTTAATGT CGAGAATTCG CCGGCGTTCG AACTATAGCT
                                                      ^end of chimeric intron at pCI 989
                                                                           end of BS insert at Hind111^

                                                                      hinPI
                                                                      hhaI/cfoI
          scrFI                                                      thaI
          nciI                                                       fnuDII/mvnI
          mspI                                                       bstUI
          hpaII                                                      bsh1236I
          dsaV                                                      mwoI    sau96I
          cauII                                                     hinPI mspI
          bssKI                                                     hhaI/cfoI
          bsaJI sau3AI                                              cac8I hpaII
         xmaI/pspAI                                                 bssHII   nlaIV                    mwoI
         smaI   mboI/ndeII   sau3AI                                thaI   scrFI                       hinPI thaI
         scrFI  dpnII        mboI/ndeII                            fnuDII/mvnI                        hhaI/cfoI
         nciI   dpnI         dpnII                                 bstUI nciI                        thaI   fnuDII/
     tseI dsaV  alwI         dpnI                        mnlI  bstUI nciI                             fnuDII/mvnI
     fnu4HI/bsoFI       rmaI  alwI                     xhoI    bsh1236I haeIII/palI            mwoI   bstUI  bstUI
     bbvI cauII nlaIV   maeI nlaIV       tsp509I       smlI   hinPI   dsaV                     tseI   bsh1236I
    scfI  bssKI bstYI/xhoII  bstYI/xhoII               paeR7I hhaI/cfoI  asuI                  fnu4HI/bsoFI    bsh1236
    pstI  bsaJI bamHI  bfaI bamHI      ecoRI      aluI avaI   cac8I  cauII               mnlI bbvI     aciI   aciI
    bsgI  avaI  alwI   speI alwI       apoI     hindIII taqI  bssHII bssKI
601 ATTCCTGCAG CCCGGGGGAT CCACTAGTGG ATCCAAAGAA TTCAAAAAGC TTCTCGAGGG CGCGCGCCCG GCCCCCACCC CTCGCAGCAC CCCGCGCCCC
    TAAGGACGTC GGGCCCCCTA GGTGATCACC TAGGTTTCTT AAGTTTTTCG AAGAGCTCCC GCGCGCGGGC CGGGGGTGGG GAGCGTCGTG GGGCGCGGGG
                         ^end of BS intron insert at spe
                                                   ^start of human HER2 from BS at xhol
```

FIG. 7E

```
                    sau96I                                                                                            hgiAI/aspHI
                    avaII                                                                                  nlaIV      bsp1286
                    asuI                                                                                 mspI         bsiHKAI
                   nlaIV                                                                                 hpaII       cac8I
                   scrFI              nlaIII                                nlaIV                        scrFI        thaI
                   nciI              styI                                  hgiJII                        nciI         fnuDII/mvnI
                   mspI              ncoI                                  bsp1286                       dsaV         bstUI  mslI
        mnlI       hpaII             dsaI                         mspA1I/nspBII   mnlI                   cauII        bsh1236I
      mwoI         dsaV          nlaIV           fnu4HI/bsoFI aciI     bmyI   bseRI                      bssKI       aciI bmyI
     hinPI         cauII        mspI  bstXI  cac8I  haeIII/palI        banII mnlI                       bsaJI   fnu4HI/bsoFI
     hhaI/cfoI     bssKI        hpaII bsaJI aluI aciI         fnu4HI/bsoFI bseRI        mnlI
1701 GCGCCCTCCC AGCCGGGTCC AGCCGGAGCC ATGGAGCTGG CGGCCTTGTG CCGCTGGGGG CTCCTCCTCG CCCTCTTGCC CCCCGGAGCC GCGAGCACCC
     CGCGGGAGGG TCGGCCCAGG TCGGCCTCGG TACCTCGACC GCCGGAACAC GGCGACCCCC GAGGAGGAGC GGGAGAACGG GGGGCCTCGG CGCTCGTGGG
1                                     M  E  L  A   A  L  C   R  W  G   L  L  L  A   L  L  P   P  G  A   A  S  T  Q

                                                                                                            scrFI
                                                                                                            mvaI
                                                                                                 scrFI      ecoRII
       mspI                                                        scrFI                         mvaI       dsaV
      cfr10I/bsrFI                                                 mvaI                          ecoRII     bstNI
     hgiAI/aspHI                fnu4HI/bsoFI                       ecoRII                        dsaV       bssKI
     bsp1286                    aciI                     bsmAI     dsaV                          bstNI      apyI
     bsiHKAI    mslI         tseI                        bsaI      bstNI  nlaIII          mnlI   bssKI tseI
     bmyI hpaII              fnu4HI/bsoFI           bsmFI          bssKI  nspHI           ecoNI  bsaJI fnu4HI/bsoFI
     apaLI/snoI             aluI                  bsrI  avaI       apyI   nspI   aciI     bslI   apyI  bbvI
     alw44I/snoI     nlaIII bbvI  nlaIV
1801 AAGTGTGCAC CGGCACAGAC ATGAAGCTGC GGCTCCCTGC CAGTCCCGAG ACCCACCTGG ACATGCTCCG CCACCTCTAC CAGGGCTGCC AGGTGGTGCA
     TTCACACGTG GCCGTGTCTG TACTTCGACG CCGAGGGACG GTCAGGGCTC TGGGTGGACC TGTACGAGGC GGTGGAGATG GTCCCGACGG TCCACCACGT
25    V  C  T   G  T  D   M  K  L  R   L  P  A   S  P  E   T  H  L  D   M  L  R   H  L  Y   Q  G  C  Q   V  V  Q

                                                                     mnlI
        scrFI                                                       scrFI                         hgiAI/aspHI
        mvaI                                                        mvaI                          bsp1286
        ecoRII                                            scfI      ecoRII                        bsiHKAI
        dsaV                                              pstI      dsaV                          bmyI
        bstNI                                             bsgI      bstNI                        taiI
        bssKI                                            sse8387I   bssKI                        maeII
        apyI     hphI    bspMI         cac8I             sbfI   ecoRV apyI                      bsaAI
1901 GGGAAACCTG GAACTCACCT ACCTGCCCAC CAATGCCAGC CTGTCCTTCC TGCAGGATAT CCAGGAGGTG CAGGGCTACG TGCTCATCGC TCACAACCAA
     CCCTTTGGAC CTTGAGTGGA TGGACGGGTG GTTACGGTCG GACAGGAAGG ACGTCCTATA GGTCCTCCAC GTCCCGATGC ACGAGTAGCG AGTGTTGGTT
58    G  N  L   E  L  T  Y   L  P  T   N  A  S   L  S  F  L   Q  D  I   Q  E  V   Q  G  Y  V   L  I  A   H  N  Q
```

FIG. 7F

```
                                                                                     haaeIII/palI
                                                                                    eaeI
                                                                                    cfrI
                       alwNI                                                       scrFI
            bsmFI      alw26I/bsmAI                                                mvaI
           sau96I     mwoI                                                         ecoRII
           nlaIV      bstAPI                                                       dsaV
           avaII    scfI       mwoI                                                bstNI
           asuI     pstI    tseI           nlaIV                                   bssKI     rmaI
          ppuMI     bsgI    fnu4HI/bsoFI   hgiCI                                   apyI      maeI       bsmAI mspA1I/nspBII
        bspMI     tspRI     bbvI           banI                                   bsaJI      bfaI       bsaI  aciI
     mnlI eco0109I/draII mnlI  aciI       mnlI      aluI     mnlI
001  GTGAGGCAGG TCCCACTGCA GAGGCTGCGG ATTGTGCGAG GCACCCAGCT CTTTGAGGAC AACTATGCCC TGGCCGTGCT AGACAATGGA GACCCGCTGA
     CACTCCGTCC AGGGTGACGT CTCCGACGCC TAACACGCTC CGTGGGTCGA GAAACTCCTG TTGATACGGG ACCGGCACGA TCTGTTACCT CTGGGCGACT
91   V  R  Q  V   P  L  Q   R  L  R   I  V  R  G   T  Q  L   F  E  D   N  Y  A  L   A  V  L   D  N  G   D  P  L  N

                                   scrFI                 tseI
                                   mvaI                  fnu4HI/bsoFI
                                   ecoRII                bbvI
                                   dsaV                 scfI      taqI
                               mnlI bstNI               pstI     sfuI               sau3AI                  mspA1I/nspB
                             haeIII/palI     cac8I      bsgI     bstBI              mboI/ndeII             sau3AI
                 tsp45I    sau96I   bssKI  haeIII/palI tseI      bsiCI              dpnII                  mboI/ndeII
                 maeIII    asuI     apyI   stuI       fnu4HI/bsoFI                  dpnI                   dpnII
               ecoNI     nlaIV    bsaJI mnlI     mwoI  bbvI     asuII              bstYI/xhoII             dpnI    nlaIV
               bslI      eco0109I/draII haeI     aciI aluI   aluI       mnlI       bglII        mnlI       alwI  aciI
101  ACAATACCAC CCCTGTCACA GGGGCCTCCC CAGGAGGCCT GCGGGAGCTG CAGCTTCGAA GCCTCACAGA GATCTTGAAA GGAGGGGTCT TGATCCAGCG
     TGTTATGGTG GGGACAGTGT CCCCGGAGGG GTCCTCCGGA CGCCCTCGAC GTCGAAGCTT CGGAGTGTCT CTAGAACTTT CCTCCCCAGA ACTAGGTCGC
125   N  T  T   P  V  T   G  A  S  P   G  G  L   R  E  L   Q  L  R  S   L  T  E   I  L  K   G  G  V  L   I  Q  R

                          scrFI
                          mvaI
                          ecoRII
                          dsaV
                          bstNI                                        cac8I                                   haeIII/
                          bssKI                                       aluI                         bsrBI    sau96I
                aluI      apyI                                       pvuII                         bslI     asuI
                                                        mboII        mspA1I/nspBII tspRI           aciI avaI
201  GAACCCCCAG CTCTGCTACC AGGACACGAT TTTGTGGAAG GACATCTTCC ACAAGAACAA CCAGCTGGCT CTCACACTGA TAGACACCAA CCGCTCTCGG
     CTTGGGGGTC GAGACGATGG TCCTGTGCTA AAACACCTTC CTGTAGAAGG TGTTCTTGTT GGTCGACCGA GAGTGTGACT ATCTGTGGTT GGCGAGAGCC
158   N  P  Q   L  C  Y  Q   D  T  I   L  W  K   D  I  F  H   K  N  N   Q  L  A   L  T  L  I   D  T  N   R  S  R
```

FIG. 7G

hinPI
hhaI/cfoI
thaI
fnuDII/mvnI
bstUI
mwoI bsh1236I mspI
alwNI hgaI hpaII mwoI
mnlI alw26I/bsmAI tspRI cfr10I/bsrFI
ddeI
sau96I
nlaIV
haeIII/palI
sau96I
pspOMI/bsp120I haeIII/palI
nlaIV haeI
tseI hgiJII
mwoI scrFI
mspI eco0109I/draII mvaI
hpaII bsp1286 ecoRII
naeI/ngoMI bmyI dsaV
cfr10I/bsrFI asuI bstNI
cac8I banII bssKI
tseI fnu4HI/bsoFI apyI
fnu4HI/bsoFI asuI mwoI cac8I mnlI
bbvI bbvI apaI
mspAlI/nspBII
mwoI tseI
nlaIV fnu4HI/bsoFI
hgiJII bslI
bsp1286 bbvI
bmyI aciI
banII bslI
cac8I
tseI
fnu4HI/bsoFI
mspAlI/nspBII
bslI tspRI
aciI haeIII/palI
cac8I sau96I
bsp1286 asuI
bmyI bbvI nlaIV tspRI
tseI
fnu4HI/bsoFI
bbvI nlaIII tspRI
91 A C H P C S P M C K G S R C W G E S S E D C Q S L T R T V C A G G C
GCCTGCCACC CCTGTTCTCC GATGTGTAAG GGCTCCCGCT GCTGGGGAGA GAGTTCTGAG GATTGTCAGA GCCTGACGCG CACTGTCTGT GCCGGTGGCT
CGGACGGTGG GGACAAGAGG CTACACATTC CCGAGGGCGA CGACCCCTCT CTCAAGACTC CTAACAGTCT CGGACTGCGC GTGACAGACA CGGCCACCGA
101 GTGCCCGCTG CAAGGGGCCA CTGCCCACTG ACTGCTGCCA TGAGCAGTGT GCTGCCGGCT GCACGGGCCC CAAGCACTCT GACTGCCTGG CCTGCCTCCA
CACGGGCGAC GTTCCCCGGT GACGGGTGAC TGACGACGGT ACTCGTCACA CGACGGCCGA CGTGCCCGGG GTTCGTGAGA CTGACGGACC GGACGGAGGT
225 A R C K G P L P T D C C H E Q C A A G C T G P K H S D C L A C L H

FIG. 7H

bstEII
scrFI
mvaI
ecoRII
dsaV
bstNI
tseI
fnu4HI/bsoFI
bbvI
tspRI sfaNI
aluI tspRI bslI bsaJI maeIII
2501 CTTCAACCAC AGTGGCATCT GTGAGCTGCA CTGCCCAGCC CTGGTCACCT ACAACACAGA CACGTTTGAG TCCATGCCCA ATCCCGAGGG CCGGTATACA
GAAGTTGGTG TCACCGTAGA CACTCGACGT GACGGGTCGG GACCAGTGGA TGTTGTGTCT GTGCAAACTC AGGTACGGGT TAGGGCTCCC GGCCATATGT
258 F N H S G I C E L H C P A L V T Y N T D T F E S M P N P E G R Y T
cac8I
hinPI
hhaI/cfoI
nlaIV
narI
kasI
hinlI/acyI
hgiCI
haeII aluI ahdI/eam1105I
eheI pvuII tsp45I
banI mspAlI/nspBII
ahaII/bsaHI maeIII bsmFI
2601 TTCGGCGCCA GCTGTGTGAC TGCCTGTCCC TACAACTACC TTTCTACGGA CGTGGGATCC TGCACCCTCG TCTGCCCCCT GCACAACCAA GAGGTGACAG
AAGCCGCGGT CGACACACTG ACGGACAGGG ATGTTGATGG AAAGATGCCT GCACCCTAGG ACGTGGGAGC AGACGGGGGA CGTGTTGGTT CTCCACTGTC
291 F G A S C V T A C P Y N Y L S T D V G S C T L V C P L H N Q E V T A
sau3AI
mboI/ndeII
dpnII
dpnI
alwI
nlaIV
bstYI/xhoII
taiI bamHI
maeII alwI mnlI
tsp45I
maeIII
hphI
mnlI
hgiAI/aspHI
bsp1286
bsiHKAI
bmyI
nlaIII
hphI
mnlI mnlI maeIII
bsrI
cac8I
tseI avaI
fnu4HI/bsoFI bsp1286
bbvI bmyI
2701 CAGAGGATGG AACACAGCGG TGTGAGAAGT GCAGCAAGCC CTGTGCCCGA GTGTGCTATG GTCTGGGCAT GGAGCACTTG CGAGAGGTGA GGGCAGTTAC
GTCTCCTACC TTGTGTCGCC ACACTCTTCA CGTCGTTCGG GACACGGGCT CACACGATAC CAGACCCGTA CCTCGTGAAC GCTCTCCACT CCCGTCAATG
325 E D G T Q R C E K C S K P C A R V C Y G L G M E H L R E V R A V T
fokI aciI
bstF5I mspAlI/nspBII
mnlI mslI

FIG. 7I

```
801 CAGTGCCAAT ATCCAGGAGT TTGCTGGCTG CAAGAAGATC TTTGGGAGCC TGGCATTTCT GCCGGAGAGC TTTGATGGGG ACCCAGCCTC CAACACTGCC
    GTCACGGTTA TAGGTCCTCA AACGACCGAC GTTCTTCTAG AAACCCTCGG ACCGTAAAGA CGGCCTCTCG AAACTACCCC TGGGTCGGAG GTTGTGACGG
358 S  A  N  I  Q  E  F  A  G  C  K  K  I  F  G  S  L  A  F  L  P  E  S  F  D  G  D  P  A  S  N  T  A

901 CCGCTCCAGC CAGAGCAGCT CCAAGTGTTT GAGACTCTGG AAGAGATCAC AGGTTACCTA TACATCTCAG CATGGCCGGA CAGCCTGCCT GTCGGACGGA CTGGAGTCGC
    GGCGAGGTCG GTCTCGTCGA GGTTCACAAA CTCTGAGACC TTCTCTAGTG TCCAATGGAT ATGTAGAGTC GTACCGGCCT GTCGGACGGA CTGGAGTCGC
391 P  L  Q  P  E  Q  L  Q  V  F  E  T  L  E  E  I  T  G  Y  L  Y  I  S  A  W  P  D  S  L  P  D  L  S  V

001 TCTTCCAGAA CCTGCAAGTA ATCCGGGGAC GAATTCTGCA CAATGGCGCC TACTCGCTGA CCCTGCAAGG GCTGGGCATC AGCTGGCTGG GGCTGCGCTC
    AGAAGGTCTT GGACGTTCAT TAGGCCCCTG CTTAAGACGT GTTACCGCGG ATGAGCGACT GGGACGTTCC CGACCCGTAG TCGACCGACC CCGACGCGAG
425  F  Q  N  L  Q  V  I  R  G  R  I  L  H  N  G  A  Y  S  L  T  L  Q  G  L  G  I  S  W  L  G  L  R  S
```

FIG. 7J

sau96I
avaII
asuI
nlaIV
scrFI
mvaI
draIII
mwoI bsp1286 bsmFI
bstAPI bmyI ecoRII
hgiAI/aspHI dsaV
bsp1286 nlaIV bstNI
bsiHKAI hgiCI bssKI
bmyI banI bsaJI
apaLI/snoI apyI
alw44I/snoI bsaJI aluI nlaIV aciI
mnlI
haeIII/palI
asuI bstF5I
sau96I fokI
haeIII/palI
mnlI bsrI tspRI
ddeI bsrI tspRI
101 ACTGAGGGAA CTGGGCAGTG GACTGGCCCT CATCCACCAT AACACCCACC TCTGCTTCGT GCACACGGTG CCCTGGGACC AGCTCTTTCG GAACCCGCAC
TGACTCCCTT GACCCGTCAC CTGACCGGGA GTAGGTGGTA TTGTGGGTGG AGACGAAGCA CGTGTGCCAC GGGACCCTGG TCGAGAAAGC CTTGGGCGTG
458 L R E L G S G L A L I H H N T H L C F V H T V P W D Q L F R N P H
sau96I
pspOMI
nlaIV
hgiJII
bsp1286
bmyI
banII
scrFI
mvaI
ecoRII
dsaV
bstNI
bssKI
bsaJI
apyI
sau96I
avaII asuI
asuI apaI
nlaIV eco0109I
haeIII/palI
scrF
mvaI
ecoRII
dsaV
bstNI
bssKI
apyI
haeIII/palI
sau96I
asuI haeI
eco0109I/draII
mnlI mwoI cac8I
haeIII/palI
eaeI
cfrI
mspI
hpaII
bslI mnlI
bcgI
bsaJI
avaI bsp1286
hinPI bmyI
aluI hhaI/cfoI
pvuII
mspA1I/nspBII mnlI tspRI
aluI
tspRI cfr10I/bsrFI
201 CAAGCTCTGC TCCACACTGC CAACCGGCCA GAGGACGAGT GTGTGGGCGA GGGCCTGGCC TGCCACCAGC TGTGCGCCCG AGGGCACTGC TGGGGTCCAG
GTTCGAGACG AGGTGTGACG GTTGGCCGGT CTCCTGCTCA CACACCCGCT CCCGGACCGG ACGGTGGTCG ACACGCGGGC TCCCGTGACG ACCCCAGGTC
491 Q A L L H T A N R P E D E C V G E G L A C H Q L C A R G H C W G P G
sequence change from Coussens to Yamamoto^

FIG. 7K

```
                                         scrFI
                                         mvaI
                                         ecoRII
                                         dsaV                                                     scrFI
                                         bstNI                                                    mvaI
                          tseI           bssKi                                                    ecoRII          scrFI
                          fnu4HI/bsoFI   apyI                                                     dsaV            mvaI
                          bbvI          mwoI                                 scfI     nlaIV       bstNI           ecoRII
     draIII              scfI           haeIII/palI                          pstI     hgiJii      bssKI           dsaV
   sau96I  tspRI         pstI          sau96I                                bsgI     bsp1286     bsaJI           bstNI
   haeIII/palI           bsgI   bsrI   asuI                 bsmI           rsaI       bmyI        apyI            bssKI
   asuI   bsrI         hincII/hindII   nlaIV              mnlI             csp6I      banII      bsaJI       bsmI apyI
                                                                           scaI
01 GGCCCACCCA GTGTGTCAAC TGCAGCCAGT TCCTTCGGGG CCAGGAGTGC GTGGAGGAAT GCCGAGTACT GCAGGGGCTC CCCAGGGAGT ATGTGAATGC
   CCGGGTGGGT CACACAGTTG ACGTCGGTCA AGGAAGCCCC GGTCCTCACG CACCTCCTTA CGGCTCATGA CGTCCCCGAG GGGTCCCTCA TACACTTACG
525  P  T  Q  C  V  N  C  S  Q  F  L  R  G  Q  E  C  V  E  E  C  R  V  L  Q  G  L  P  R  E  Y  V  N  A

                                                                      mspI
                                                                      hpaII
   tspRI                                                              bsaWI      tspRI
   mwoI                                             tsp45I            sau961     pf1MI        bsp1286
   bstAPI                                           maeIII            avaII      bsrI      haeIII/palI
 alwNI                                             tspRI              asuI mnlI  bslI     haeI  bmyI bslI
 alw26I/bsmAI           ddeI        bslI          ddeI
401 CAGGCACTGT TTGCCGTCGG ACCCTGAGTG TCAGCCCCAG AATGGCTCAG TGACCTGTTT TGGACCGGAG GCTGACCAGT GTGTGGCCTG TGCCCACTAT
    GTCCGTGACA AACGGCACGG TGGGACTCAC AGTCGGGGTC TTACCGAGTC ACTGGACAAA ACCTGGCCTC CGACTGGTCA CACACCGGAC ACGGGTGATA
558  R  H  C  L  P  C  H  P  E  C  Q  P  Q  N  G  S  V  T  C  F  G  P  E  A  D  Q  C  V  A  C  A  H  Y

                                                                                                       mwoI
                       tseI                                                                           nlaIII
                       mwoI                                                                           sphI
                       fnu4HI/bsoFI                                                                   nspHI
     mnlI             mspA1I/nspBII                                                                   nspI
   sau96I              aciI                                                                   mnlI    cac8I
   nlaIV              cac8I                                                    nlaIII       mnlI     hinPI cac8I
   avaII              sau96I                                                   nspHI        bseRI    hhaI/cfoI
   asuI               haeIII/palI         aciI                         mnlI    nspI
   ppuMI              asuI bbvI         mspA1I/nspBII
   eco0109I/draII  mwoI
501 AAGGACCCTC CCTTCTGCGT GGCCCGCTGC CCCAGCGGTG TGAAACCTGA CCTCTCCTAC ATGCCCATCT GGAAGTTTCC AGATGAGGAG GGCGCATGCC
    TTCCTGGGAG GGAAGACGCA CCGGGCGACG GGGTCGCCAC ACTTTGGACT GGAGAGGATG TACGGGTAGA CCTTCAAAGG TCTACTCCTC CCGCGTACGG
591 K  D  P  P  F  C  V  A  R  C  P  S  G  V  K  P  D  L  S  Y  M  P  I  W  K  F  P  D  E  E  G  A  C  Q
```

FIG. 7L

```
                                                 scrFI
                                                 mvaI
                                                 ecoRII
                                                 dsaV
                                                 bstNI
                                                 bssKI                                                          taiI
                                                 apyI                                                           maeII     esp3I
                                              sau96I              tseI                                          hinlI/acyI bsmAI
                                              avaII fokI          fnu4HI/bsoFI                                  ahaII/bsaHI
                                  mslI        asuI  bstF5I        bbvI  aciI              cac8I mnlI  aatII       bsmBI  aciI
3601 AGCCTTGCCC CATCAACTGC ACCCACTCCT GTGTGGACCT GGATGACAAG GGCTGCCCCG CCGAGCAGAG AGCCAGCCCT CTGACGTCCA TCGTCTCTGC
     TCGGAACGGG GTAGTTGACG TGGGTGAGGA CACACCTGGA CCTACTGTTC CCGACGGGGC GGCTCGTCTC TCGGTCGGGA GACTGCAGGT AGCAGAGACG
625   P  C  P   I  N  C   T  H  S  C   V  D  L   D  D  K   G  C  P  A   E  Q  R   A  S  P   L  T  S  I   V  S  A

                                                                                    mspI
                                                    mnlI                            mroI
                                                  sau3AI                            bspMII
                                                  mboI/ndeII                        bspEI
                                                  mamI                              bsaWI
                                                  dpnII                           sau3AI
                                                  dpnI                            mboI/ndeII
                                                  bsaBI                           dpnII
                                                  alwI                            dpnI
                                                 nlaIV                            alwI
                                                 bstYI/xhoII          tseI       bstYI/xhoII                 mwoI     tseI
                                                 bamHI                fnu4HI/bsoFI hpaII  rsaI               aciI     fnu4HI/
               bsmI                              alwI       mwoI      bbvI  mboII accIII  csp6I sfaNI  bsmAI bbvI
3701 GGTGGTTGGC ATTCTGCTGG TCGTGGTCTT GGGGGTGGTC TTTGGGATCC TCATCAAGCG ACGGCAGCAG AAGATCCGGA AGTACACGAT GCGGAGACTG
     CCACCAACCG TAAGACGACC AGCACCAGAA CCCCCACCAG AAACCCTAGG AGTAGTTCGC TGCCGTCGTC TTCTAGGCCT TCATGTGCTA CGCCTCTGAC
658   V  V  G   I  L  L  V   V  V  L   G  V  V   F  G  I  L   I  K  R   R  Q  Q   K  I  R  K   Y  T  M   R  R  L
```

FIG. 7M

```
                                                                                                        sau3AI
                                                                                          hinPI         mboI/ndeII
                                                                                          hhaI/cfoI     dpnII
                                                                                      scrFI           nlaIV
                                                                                      mvaI            bstYI/xhoII
                                                          rmaI                        ecoRII          bamHI
                                                 mspA1I/nspBII                        dsaV          aciI
     scfI                                        aciI        maeI                     bstNI     sfaNI dpnI        esp3I       mnlI
     pstI           mwoI                        fnu4HI/bsoFI   aciI                   bssKI     mamI alwI         bsmBI     ddeI
     bsgI          aluI      nlaIV                 bfaI        sfaNI                  apyI      bsaBI alwI        bsmAI    aluI       hphI
801  CTGCAGGAAA CGGAGCTGGT GGAGCCGCTG ACACCTAGCG GAGCGATGCC CAACCAGGCG CAGATGCGGA TCCTGAAAGA GACGGAGCTG AGGAAGGTGA
     GACGTCCTTT GCCTCGACCA CCTCGGCGAC TGTGGATCGC CTCGCTACGG GTTGGTCCGC GTCTACGCCT AGGACTTTCT CTGCCTCGAC TCCTTCCACT
691  L  Q  E  T   E  L  V   E  P  L   T  P  S  G   A  M  P   N  Q  A   Q  M  R  I   L  K  E   T  E  L   R  K  V  R

                                                                          bslI
                                                                       sau3AI
                                                                       mboI/ndeII
                                                                       dpnII
              sau3AI mwoI                                              dpnI                                     mscI/balI
              mboI/ndeII                                               alwI                                     haeI
              dpnII  hinPI                                            nlaIV                                     eaeI
              dpnI   hhaI/cfoI                                        bstYI/xhoII                            tspRI
             bstYI/xhoII                                              bamHI                            tsp509I cfrI mslI
             alwI   haeII             accI       sfaNI                alwI                           apoI  bsrI haeIII/palI    mnlI
901  AGGTGCTTGG ATCTGGCGCT TTTGGCACAG TCTACAAGGG CATCTGGATC CCTGATGGGG AGAATGTGAA AATTCCAGTG GCCATCAAAG TGTTGAGGGA
     TCCACGAACC TAGACCGCGA AAACCGTGTC AGATGTTCCC GTAGACCTAG GGACTACCCC TCTTACACTT TTAAGGTCAC CGGTAGTTTC ACAACTCCCT
725   V  L  G   S  G  A   F  G  T  V   Y  K  G   I  W  I   P  D  G  E   N  V  K   I  P  V   A  I  K  V   L  R  E

                                                                                        nlaIV
                                                                                       hgiJII                 mwoI
                                                                     taiI              bsp1286             bslI
          fokI                                                       maeII             bmyI                aciI
          bstF5I                                   ddeI             bsaAI              banII   ndeI  bsmAI  bglI      sfaNI
001  AAACACATCC CCCAAAGCCA ACAAAGAAAT CTTAGACGAA GCATACGTGA TGGCTGGTGT GGGCTCCCCA TATGTCTCCC GCCTTCTGGG CATCTGCCTG
     TTTGTGTAGG GGGTTTCGGT TGTTTCTTTA GAATCTGCTT CGTATGCACT ACCGACCACA CCCGAGGGGT ATACAGAGGG CGGAAGACCC GTAGACGGAC
758  N  T  S   P  K  A  N   K  E  I   L  D  E   A  Y  V  M   A  G  V   G  S  P   Y  V  S  R   L  L  G   I  C  L
```

FIG. 7N

```
                                                                                                          sau96I
                                                                                           scrFI          avaII
                                                                                           mvaI           asuI
                                                                                           ecoRII        ppuMI
                                                                                     aciI  dsaV          scrFI
                                                                                   thaI    bstNI         mvaI
                                                                      scrFI        fnuDII/mvnI  hgiJII alwNI
                                                                      nciI         bstUI   bssKI         ecoRII
                                                                      mspI         bsh1236I bsaJI        dsaV
              aluI                                                    hpaII       sacII/sstII  bsp1286 eco0109I/draII
       xcmI  pvuII tsp45I                                             dsaV        mspA1I/nspBII bmyI  bstNI
       dsaI  mspA1I/nspBII                           mnlI          nlaIII         kspI        apyI nlaIV alw26I/bsmAI
       bsaJI tseI    maeIII                      tseI         tth111I/aspI        dsaI   hinlI/acyI      bssKI
     fokI    fnu4HI/bsoFI                        fnu4HI/bsoFI  bslI   cauII       bsaJI  hgaI    banII apyI
     bstF5I  bbvI  hphI       aluI               bbvI    ddeI pflFI   bssKI       aciI   ahaII/bsaHI   bsaJI  bspMI
4101 ACATCCACGG TGCAGCTGGT GACACAGCTT ATGCCCTATG GCTGCCTCTT AGACCATGTC CGGGAAAACC GCGGACGCCT GGGCTCCCAG GACCTGCTGA
     TGTAGGTGCC ACGTCGACCA CTGTGTCGAA TACGGGATAC CGACGGAGAA TCTGGTACAG GCCCTTTTGG CGCCTGCGGA CCCGAGGGTC CTGGACGACT
 791 T  S  T  V   Q  L  V   T  Q  L   M  P  Y  G   C  L  L   D  H  V   R  E  N  R   G  R  L   G  S  Q   D  L  L  N

                                             mnlI
                                            bpmI/gsuI
                                           scrFI
                                           mvaI
                                           ecoRII
                                           dsaV                                  bsrBI
                                           bstNI                                 aciI
                                           bssKI      fnu4HI/bsoFI              fnu4HI/bsoFI
                                   styI  fokI aluI   apyI  fokI  aciI           haeIII/palI
     bsrI                         bsaJI  bstF5I                 rsaI           eaeI         taiI        pleI
                                                   bstF5I       csp6I    bsmFI cfrI         maeII       hinfI
4201 ACTGGTGTAT GCAGATTGCC AAGGGGATGA GCTACCTGGA GGATGTGCGG CTCGTACACA GGGACTTGGC CGCTCGGAAC GTGCTGGTCA AGAGTCCCAA
     TGACCACATA CGTCTAACGG TTCCCCTACT CGATGGACCT CCTACACGCC GAGCATGTGT CCCTGAACCG GCGAGCCTTG CACGACCAGT TCTCAGGGTT
 825  W  C  M   Q  I  A   K  G  M  S   Y  L  E   D  V  R   L  V  H  R   D  L  A   A  R  N   V  L  V  K   S  P  N

                                                                                          bsp1286
                                                                  nlaIII                  bmyI
                                            tseI                  bstXI                   nlaIV  xcmI      hinPI
                               mwoI         fnu4HI/bsoFI        rsaI  mslI                hgiCI  pflMI     haeII
     nlaIII   tsp509I          cac8I        bbvI         bsmAI  csp6I                    banI   bslI   fokI hhaI/cfoI
                                                                                                        bstF5I
4301 CCATGTCAAA ATTACAGACT TCGGGCTGGC TCGGCTGCTG GACATTGACG AGACAGAGTA CCATGCAGAT GGGGGCAAGG TGCCCATCAA GTGGATGGCG
     GGTACAGTTT TAATGTCTGA AGCCCGACCG AGCCGACGAC CTGTAACTGC TCTGTCTCAT GGTACGTCTA CCCCCGTTCC ACGGGTAGTT CACCTACCGC
 858  H  V  K   I  T  D  F   G  L  A   R  L  L   D  I  D  E   T  E  Y   H  A  D   G  G  K  V   P  I  K   W  M  A
```

FIG. 70

PHIGENIX
Exhibit 1001-27

```
                         mspI
                         hpaII
                        naeI/ngoMI
                        cfr10I/bsrFI
                        cac8I                                                                        haeIII/palI
         pleI          sgrAI                                                                        sau96I
         hinfI                                                     tsp45I                            asuI
      bpmI/gsuI       aciI      aciI hphI  mslI                    maeIII         aluI               nlaIV      bslI
4401 CTGGAGTCCA TTCTCCGCCG GCGGTTCACC CACCAGAGTG ATGTGTGGAG TTATGGTGTG ACTGTGTGGG AGCTGATGAC TTTTGGGGCC AAACCTTACG
     GACCTCAGGT AAGAGGCGGC CGCCAAGTGG GTGGTCTCAC TACACACCTC AATACCACAC TGACACACCC TCGACTACTG AAAACCCCGG TTTGGAATGC
 891 L  E  S  I  L  R  R  R  F  T  H  Q  S  D  V  W  S  Y  G  V  T  V  W  E  L  M  T  F  G  A  K  P  Y  D

                  scrFI
                  nciI
                  mspI
                  hpaII
                  dsaV
                  cauII
                  bssKI
                 xmaI/pspAI
          bslI   smaI
       sau3AI    scrFI
       mboI/ndeII
       dpnII     nciI                                   tseI                                           sau3AI
       dpnI      dsaV    sau3AI                         mwoI                                           mboI/ndeII
       alwI      cauII   mboI/ndeII                     fnu4HI/bsoFI                                   dpnII
      nlaIV      bssKI   dpnII                          bbvI                                           dpnI
      bstYI/xhoII        dpnI                        fnu4HI/bsoFI                                     bclI
      bamHI      bsaJI   alwI     bspMI              aciI                                            nlaIII
      alwI       avaI   bstYI/xhoII                 bsrBI                                        accI mslI   nlaIII
4501 ATGGGATCCC AGCCCGGGAG ATCCCTGACC TGCTGGAAAA GGGGGAGCGG CTGCCCCAGC CCCCCATCTG CACCATTGAT GTCTACATGA TCATGGTCAA
     TACCCTAGGG TCGGGCCCTC TAGGGACTGG ACGACCTTTT CCCCCTCGCC GACGGGGTCG GGGGGTAGAC GTGGTAACTA CAGATGTACT AGTACCAGTT
 925  G  I  P  A  R  E  I  P  D  L  L  E  K  G  E  R  L  P  Q  P  P  I  C  T  I  D  V  Y  M  I  M  V  K
```

FIG. 7P

sau96I
sanDI
scrFI
mvaI nlaIV
ecoRII
dsaV avaII
bstNI
bssKI
bsaJI
apyI asuI
haeIII/palI
mscI/balI
hinPI
haeI ppuMI
hhaI/cfoI
tsp509I
eaeI nlaIV
haeII
bslI
ecoRI
nlaIII eco0109I/draII
fokI
apoI
aciI cfrI bsmFI
afeI/eco47III bstF5I
4601 ATGTTGGATG ATTGACTCTG AATGTCGGCC AAGATTCCGG GAGTTGGTGT CTGAATTCTC CCGCATGGCC AGGGACCCCC AGCGCTTTGT GGTCATCCAG
958 C W M I D S E C R P R F R E L V S E F S R M A R D P Q R F V V I Q
haeIII/palI
fokI pleI
eaeI tfiI
bstF5I hinfI cfrI hinfI
TACAACCTAC TAACTGAGAC TTACAGCCGG TTCTAAGGCC CTCAACCACA GACTTAAGAG GGCGTACCGG TCCCTGGGGG TCGCGAAACA CCAGTAGGTC
scrFI
mvaI
ecoRII
dsaV
bstNI
bssKI
apyI
sexAI
sau96I
avaII
ppuMI
nlaIV sfaNIU
eco0109I/draII mnlI
bsmFI fokI ddeI
nlaIII asuI bstF5I bseRI
4701 AATGAGGACT TGGGCCCAGC CAGTCCCTTG GACAGCACCT TCTACCGCTC ACTGCTGGAG GACGATGACA TGGGGGACCT GGTGGATGCT GAGGAGTATC
991 N E D L G P A S P L D S T F Y R S L L E D D D M G D L V D A E E Y L
sau96I
haeIII/palI
asuI
sau96I
pspOMI/bsp120I
nlaIV
hgiJII
bsp1286
bmyI
banII bsmFI
asuI bstXI styI
mnlI apaI bsrI bsaJI
bsrBI
mnlI
aciI tspRI bpmI/gsuI

FIG. 7Q

scrFI
nciI
mspI
hpaII
dsaV
cauII
bssKI
bslI
xmaI/pspAI
smaI
scrFI
nciI
dsaV
cauII
bssKI
bsaJI
mwoI
bslI bsaJI
alwNI bslI avaI
mboII alw26I/bsmAI
4801 TGGTACCCCA GCAGGGCTTC TTCTGTCCAG ACCCTGCCCC GGGCGCTGGG
ACCATGGGGT CGTCCCGAAG AAGACAGGTC TGGGACGGGG CCCGCGACCC
1025 V P Q Q G F F C P D P A P G A G G
bsmAI
xcmI
scrFI
mvaI
ecoRII
sau96I bsaI
nlaIV dsaV
hgiJII
bsp1286
bmyI
sau96I rmaI
avaII maeI
asuI bfaI
4901 GGACCTGACA CTAGGGCTGG AGCCCTCTGA AGAGGAGGCC CCCAGGTCTC
CCTGGACTGT GATCCCGACC TCGGGAGACT TCTCCTCCGG GGGTCCAGAG
1058 D L T L G L E P S E E E A P R S P
mwoI sau96I
bslI bsaJI avaII
alwNI bslI avaI hhaI/cfoI asuI
mboII alw26I/bsmAI haeII nlaIII
GGCGCTGGG GGCATGGTCC ACCACAGGCA
CCGCGACCC CCGTACCAGG TGGTGTCCGT
G A G G G M V H H R H R S S
aluI tseI
nlaIV fnu4HI/bsoFI
hgiCI bbvI
banI aciI
aluI
nlaIV fnu4HI/bsoFI bstNI
hgiCI bbvI bssKI
banI aciI apyI
CCGCAGCTCA TCTACCAGGA
GGCGTCGAGT AGATGGTCCT
S T R SS G G G
scrFI
mvaI
ecoRII
dsaV
ppuMI
nlaIV
eco0109
aciI bsmFI
GTGGCGGTGG
CACCGCCACC
nlaIV
cac8I
nlaIV
hgiCI
banI mnlI
bsrI bslI
tspRI bslI
GCTGGCTCCG ATGTATTTGA
GAGGCTTCCC CGACCGAGGC
A G S D V F D G D L
scrFI
mvaI
ecoRII
dsaV
bstNI
bssKI
bslI
bsaJI
tsp45I
maeIII
hphI apyI
bstEII
TGGTGACCTG
ACCACTGGAC

FIG. 7R

sau96I
nlaIV
avaII
asuI
rsaI
csp6I mnlI rsaI
scfI aciI tspRI ppuMI csp6I ddeI
mnlI mspAlI/nspBII eco0109I/draII mnlI bsmAI
nlaIII
CCTCTACAG CGGTACAGTG AGGACCCCAC AGTACCCCTG CCCTCTGAGA
GGGAGATGTC GCCATGTCAC TCCTGGGGTG TCATGGGGAC GGGAGACTCT
P L Q R Y S E D P T V P L P S E T
sau96I
haeIII/palI
asuI
sau96I
pspOMI/bsp120I
nlaIV
hgiJII
eco0109I/draII
bsp1286
bmyI
banII
asuI
apaI
eco0109I/draII
avaI
bslI mnlI mnlI
asuI mwoI
AACCAGCCAG ATGTTCGGCC CCAGCCCCCT TCGCCCCGAG AGGGCCCTCT
TTGGTCGGTC TACAAGCCGG GGTCGGGGGA AGCGGGGCTC TCCCGGGAGA
N Q P D V R P Q P P S P R E G P L
scrFI
mvaI
ecoRII
dsaV
bstNI
bssKI
dsaI
bsaJI
bsaJI
nlaIV
apyI
hgiCI
taiI
banI
bsaJI mboII
maeII
GTCAAAGACG TTTTTGCCTT TGGGGGTGCC
CAGTTCTGC AAAAACGGAA ACCCCCACGG
G K N G V V K D V F A F G G A
haeIII/palI
sau96I pleI
asuI hinfI
GGAAAGGGCC AAGACTCTCT CCCCAGGGAA GAATGGGGTC
GGGGTCCCTT CTTACCCCAG
tseI styI tseI
fnu4HI/bsoFI fnu4HI/bsoFI
bbvI bsaJI bbvI mnlI nlaIII
GGAATGGGGG CAGCCAAGGG GCTGCAAAGC CTCCCCACAC ATGACCCCAG
CCTTACCCCC GTCGGTTCCC CGACGTTTCG GAGGGGTGTG TACTGGGGTC
G M G A A K G L Q S L P T H D P S
tseI
fnu4HI/bsoFI
bbvI
scfI
pstI
bsgI
taiI
maeII
bspMI
CTGACCTGCA GCCCCCAGCC TGAATATGTG
GACTGGACGT CGGGGGTCGG ACTTATACAC
D G Y V A P L T C S P Q P E Y V
tseI
fnu4HI/bsoFI
bbvI
cac8I mwoI bspMI banI
nlaIV
hgiCI
haeIII/palI
sau96I pleI
asuI hinfI
GCCTGCTGCC CGACCTGCTG GTGCCACTCT GGAAAGGGCC AAGACTCTCT
CGGACGACGG GCTGGACGAC CACGGTGAGA CCTTTCCCGG TTCTGAGAGA
P A A R P A G A T L E R A K T L S

FIG. 7S

```
                                     scrFI
                                     mvaI
                                     ecoRII
                                     dsaV
                                     bstNI
                                     bssKi         tseI
                                     bsaJI         fnu4HI/bsoFI
                 rsaI                apyI         aluI
                 csp6I              bsaJi  mnlI  bbvI
            avaI scaI              bslI   bseRI
01  GTGGAGAACC CCGAGTACTT GACACCCCAG GGAGGAGCTG CCCCTCAGCC CCACCCTCCT CCTGCCTTCA GCCCAGCCTT CGACAACCTC TATTACTGGG
    CACCTCTTGG GGCTCATGAA CTGTGGGGTC CCTCCTCGAC GGGGAGTCGG GGTGGGAGGA GGACGGAAGT CGGGTCGGAA GCTGTTGGAG ATAATGACCC
191 V  E  N  P  E  Y  L  T  P  Q  G  G  A  A  P  Q  P  H  P  P  P  A  F  S  P  A  F  D  N  L  Y  Y  W  D
```

(above 01 line, continued: ddeI, mnlI; mnlI, bseRI, mnlI; eco57I; taqI; mnlI; bsrI; sau96I, avaII, asuI, nlaIV, bsmFI)

```
     sau96I
     nlaIV
     avaII
     asuI
     ppuMI
     eco0109I/draII
     scrFI
     mvaI
     ecoRII                 nlaIV
     dsaV                   hgiJII
     bstNI                  bsp1286
     bssKi        bsrBI     bmyI
     apyI      bslI aciI    banII      bslI      bsmFI
1   ACCAGGACCC ACCAGAGCGG GGGGCTCCAC CCAGCACCTT CAAAGGGACA CCTACGGCAG AGAACCCAGA GTACCTGGGT CTGCACGTGC CAGTGTGAAC
    TGGTCCTGGG TGGTCTCGCC CCCCGAGGTG GGTCGTGGAA GTTTCCCTGT GGATGCCGTC TCTTGGGTCT CATGGACCCA GACCTGCACG GTCACACTTG
225 Q  D  P  P  E  R  G  A  P  P  S  T  F  K  G  T  P  T  A  E  N  P  E  Y  L  G  L  D  V  P  V  O
```

(above 1 line, continued: scrFI, mvaI, ecoRII, dsaV, bstNI, bssKI, bslI, bsaJI, apyI, rsaI, csp6I; taiI, maeII; bsrI, tspRI)

FIG. 7T

```
                                                                                                                                rmaI
                                                                                                                                maeI
                                                                                                                               styI
                                                                                                                               bsaJI
                                                                                                                               blnI
                                                                                                                               avrII
                                                                                                                             haeIII/pal
                                          ddeI                     mnlI    aluI                                    mnlI       stuI
        mwoI                              mnlI                   haeIII/palI                                   mnlI           haeI
       haeIII/palI                        eco81I    mwoI        fnu4HI/bsoFI                                   bseRI         mnlI bfaI
      haeI      aciI                      bsu36I/mstII/sauI     aciI    ddeI
501 CAGAAGGCCA AGTCCGCAGA AGCCCTGATG TGTCCTCAGG GAGCAGGGAA GGCGGCCTCT GAGCTATTCC AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC
    GTCTTCCGGT TCAGGCGTCT TCGGGACTAC ACAGGAGTCC CTCGTCCCTT CCGCCGGAGA CTCGATAAGG TCTTCATCAC TCCTCCGAAA AAACCTCCGG

                                                                  sau96I
                                                                 sau96I
                                                                 pspOMI/bsp120I
                                                                 nlaIV
                                                                 hgiJII
                                                             thaI haeIII/palI
                                                             fnuDII/mvnI
                                                            sacII/sstII
                                                            mspA1I/nspBII
                                                            kspI bsp1286
                                                            dsaI bmyI
                                                            bsaJI asuI
                                                           fnu4HI/bsoFI
                                                          haeIII/palI
                                                         mcrI    banII
                                          xhoI           eagI/smaIII/eclXI
                                          smlI           eaeI cac8I
                                         pleI            cfrI aciI
                                       taqI taqI         bsiEI   asuI
                                     salI  paeR7I     rmaI  notI bstUI
                               claI/bsp106  hinfI    rsaI  maeI  fnu4HI/bsoFI                                   mnlI
                           aluI  bspDI    hincII/hindII csp6I bfaI  aciI bsh1236I                   taiI        ecoNI
                          hindIII taqI    accI  avaI   scaI  xbaI bsrBI aciI apaI      mnlI drdI    maeII       bslI
601 TAGGCTTTTG CAAAAAGCTT ATCGATACCG TCGACTCGAG AGTACTTCTA GAGCGGCCGC GGGCCCATCG CCTCTGACAG CAACGTCTAT GACCTCCTAA
    ATCCGAAAAC GTTTTTCGAA TAGCTATGGC AGCTGAGCTC TCATGAAGAT CTCGCCGGCG CCCGGGTAGC GGAGACTGTC GTTGCAGATA CTGGAGGATT
                ^start of BS insert of HER2 xba-hind111            ^start of hgh ex 4 (cla/nar)
                              ^end of human HER2 insert fropm BS at xho1                          ^TG PCR 5' pri
```

FIG. 7U

PHIGENIX
Exhibit 1001-33

```
                                                                 mspI
                                                                  hpaII
                                                                 scrFI
         rmaI                                                    nciI
         maeI                                                    dsaV              mboII
         bfaI                                                    cauII           sau3AI
     sau96I                                                      bssKI           mboI/ndeII
     avaII                                                       bslI            dpnII
     asuI            fokI                                        bslI            dpnI
    ppuMI   mnlI     bstF5I                                tseI  fnu4HI/bsoFI  bstYI/xhoII
    eco0109I/draII  sfaNI              mnlI      mboII      bbvI  bsaJI bsrI    bglII          scfI       taqI
701 AGGACCTAGA GGAAGGCATC CAAACGCTGA TGGGGAGGCT GGAAGATGGC AGCCCCCGGA CTGGGCAGAT CTTCAAGCAG ACCTACAGCA AGTTCGACAC
    TCCTGGATCT CCTTCCGTAG GTTTGCGACT ACCCCTCCGA CCTTCTACCG TCGGGGGCCT GACCCGTCTA GAAGTTCGTC TGGATGTCGT TCAAGCTGTG
                                     ^end of ex 4/ start ex 5

801 AAACTCACAC AACGATGACG CACTACTCAA GAACTACGGG CTGCTCTACT GCTTCAGGAA GGACATGGAC AAGGTCGAGA CATTCCTGCG CATCGTGCAG
    TTTGAGTGTG TTGCTACTGC GTGATGAGTT CTTGATGCCC GACGAGATGA CGAAGTCCTT CCTGTACCTG TTCCAGCTCT GTAAGGACGC GTAGCACGTC
```

FIG. 7V

```
                                           scrFI
                                           nciI
                                           mspI
                                           hpaII
                                           dsaV
                                          xmaI/pspAI
                                          smaI
                                          scrFI
                                          nciI                                          sau96I
                                          dsaV                                          haeIII/palI
                                          cauII                                         asuI
                                          bssKI                                      scrFI  scrFI
                       aluI           tseI cauII                                     mvaI   mvaI
                      pvuII           fnu4HI/bsoFI                                   ecoRII ecoRII
                     tseI             bbvI  bssKI                                    dsaV   dsaV                bsp1286
      bsrBI          mwoI          rmaI    bsaJI     mslI                            bstNI  bstNI               bmyI
      bslI           fnu4HI/bsoFI  maeI   mwoI       fokI          mnlI              bssKI  bssKI            bsrI
      aciI       mnlI mspAlI/nspBII   aluI  avaI     bstF5I  tsp45I       tspRI      bslI   apyI           bpmI/gsuI
     fnu4HI/bsoFI    bbvI          bfaI    bglI     sfaNI    maeIII      bsrI  mnlI  apyI bsaJI          mslI tspRI
901 TGCCGCTCTG TGGAGGGCAG CTGTGGCTTC TAGCTGCCCG GGTGGCATCC CTGTGACCCC TCCCCAGTGC CTCTCCTGGC CCTGGAAGTT GCCACTCCAG
    ACGGCGAGAC ACCTCCCGTC GACACCGAAG ATCGACGGGC CCACCGTAGG GACACTGGGG AGGGGTCACG GAGAGGACCG GGACCTTCAA CGGTGAGGTC
                                           ^end of spe-sma pBK-CMV/hgh insert to replace intron
                                                                                           ^TG PCR 3' primer


                                                         rmaI
                                tru9I                    maeI
                                mseI                     bfaI
                              tsp509I   sfaNI          ahdI/eam1105I       sspI           mnlI
001 TGCCCACCAG CCTTGTCCTA ATAAAATTAA GTTGCATCAT TTTGTCTGAC TAGGTGTCCT TCTATAATAT TATGGGGTGG AGGGGGGTGG TATGGAGCAA
    ACGGGTGGTC GGAACAGGAT TATTTTAATT CAACGTAGTA AAACAGACTG ATCCACAGGA AGATATTATA ATACCCCACC TCCCCCCACC ATACCTCGTT
                                          ^end of hgh exon5
```

FIG. 7W

PHIGENIX
Exhibit 1001-35

```
      bslI
     sau96I
     haeIII/palI
     asuI
    sau96I
    pspOMI/bsp120I
    nlaIV
    hgiJII
    bsp1286
    bmyI
    banII                           cac8I
    asuI                          haeIII/palI
    apaI          mboII          sau96I                                                                         mnlI
   nlaIV          bpuAI         eco0109I/draII                  bpmI/gsuI                           mwoI      bslI
   eco0109/draII  bbsI      scfI asuI    aciI            nlaIV    aluI      tspRI                tspRI       aciI
101 GGGGCCCAAG TTGGGAAGAC AACCTGTAGG GCCTGCGGGG TCTATTCGGG AACCAAGCTG GAGTGCAGTG GCACAATCTT GGCTCACTGC AATCTCCGCC
    CCCCGGGTTC AACCCTTCTG TTGGACATCC CGGACGCCCC AGATAAGCCC TTGGTTCGAC CTCACGTCAC CGTGTTAGAA CCGAGTGACG TTAGAGGCGG

                                                                      sphI
                                                                      nspHI
                                                                      nspI      scrFI
    scrFI                                                         scrFI         mvaI
    mvaI                                                          mvaI          ecoRII
    ecoRII                                                        ecoRII        dsaV
    dsaV                                                          dsaV     nlaIII    celII/espI
    bstNI                                                         bstNI ppu10I  bstNI
    bssKI        tfiI                                             bssKI nsiI/avaIII  blpI/bpu1102I              esp3I
    bsaJI        hinfI        ddeI       avaI                 tfiI apyI nlaIII  bssKI    aluI                 bsmBI
    apyI        bcgI        mnlI     mnlI                     hinfI    cac8I    apyI ddeI    tsp509I          bsmAI
201 TCCTGGGTTC AAGCGATTCT CCTGCCTCAG CCTCCCGAGT TGTTGGGATT CCAGGCATGC ATGACCAGGC TCAGCTAATT TTTGTTTTTT TGGTAGAGAC
    AGGACCCAAG TTCGCTAAGA GGACGGAGTC GGAGGGCTCA ACAACCCTAA GGTCCGTACG TACTGGTCCG AGTCGATTAA AAACAAAAAA ACCATCTCTG
```

FIG. 7X

```
                  scrFI
                  mvaI
                  ecoRII
                  ecoRII
                  dsaV
                  bstNI
                  bssKI
                  apyI
                haeIII/palI
               mscI/balI
               haeI                                    sau3AI
               eaeI         bsmAI                      mboI/ndeII
     hphI      cfrI        bsaI                        dpnII      haeI    bstXI
                                             hphI          styI haeIII/palI
                                        ddeI    dpnI       bsaJI  mnlI   tsp509I              tspRI
301 GGGGTTTCAC CATATTGGCC AGGCTGGTCT CCAACTCCTA ATCTCAGGTG ATCTACCCAC CTTGGCCTCC CAAATTGCTG GGATTACAGG CGTGAACCAC
    CCCCAAAGTG GTATAACCGG TCCGACCAGA GGTTGAGGAT TAGAGTCCAC TAGATGGGTG GAACCGGAGG GTTTAACGAC CCTAATGTCC GCACTTGGTG

                                                                                            mspI
                                                                                           cfr10I/bsrFI
                                                                                           bsaWI
                                                  taiI                             styI sau96I hpaII
                                                 hinlI/acyI                        ncoI haeIII/palI
                  tru9I                          ahaII/bsaHI                       dsaI asuI  ageI
                  mseI                           aatII                             bsaJI   pflMI
                 ahaIII/draI     bslI        mnlI maeII                        bspMI nlaIII bslI      bsmFI
401 TGCTCCCTTC CCTGTCCTTC TGATTTTAAA ATAACTATAC CAGCAGGAGG ACGTCCAGAC ACAGCATAGG CTACCTGCCA TGGCCCAACC GGTGGGACAT
    ACGAGGGAAG GGACAGGAAG ACTAAAATTT TATTGATATG GTCGTCCTCC TGCAGGTCTG TGTCGTATCC GATGGACGGT ACCGGGTTGG CCACCCTGTA

                                                                                 hgiAI/aspHI
                                                                            sau3AI
                                                                            mboI/ndeII   tru9I
                                                                            dpnII bsp1286
                                                                            dpnI  bsiHKAI   tsp509I
                                                  sau96I                   pvuI/bspCI    mseI    nlaIII
                                                  avaII                    mcrI    bmyI  aseI/asnI/vspI
       mwoI                                       asuI                     bsiEI   apaLI/snoI   rcaI apoI
       cac8I       tspRI   mnlI nlaIII           nlaIV     ddeI     sfaNI      tsp509I     alw44I/snoI  bspHI  tsp509I
501 TTGAGTTGCT TGCTTGGCAC TGTCCTCTCA TGCGTTGGGT CCACTCAGTA GATGCCTGTT GAATTACGAT CGGTGCACAT TAATTCATGA AATTCGTAAT
    AACTCAACGA ACGAACCGTG ACAGGAGAGT ACGCAACCCA GGTGAGTCAT CTACGGACAA CTTAATGCTA GCCACGTGTA ATTAAGTACT TTAAGCATTA
                                                                      ^start of linker 2               ^end of linker 2
```

FIG. 7Y

```
                                                                                                scrFI
                                                                                                mvaI
                                                                                                ecoRII
                                                                                                dsaV nlaIV
                                                                                                bstNI
                                                                                                bssKI
                                                                                                bsaJI
                                          bsrBI                          mspI                   apyI hgiCI
    nlaIII   aluI             tsp509I     aciI     tsp509I               hpaII                 mwoI  banI
601 CATGGTCATA GCTGTTTCCT GTGTGAAATT GTTATCCGCT CACAATTCCA CACAACATAC GAGCCGGAAG CATAAAGTGT AAAGCCTGGG GTGCCTAATG
    CTACCAGTAT CGACAAAGGA CACACTTTAA CAATAGGCGA GTGTTAAGGT GTGTTGTATG CTCGGCCTTC GTATTTCACA TTTCGGACCC CACGGATTAC

                                                                                                           aciI
                                                                                                          thaI
                                                                                                          fnuDII/mvnI
                                                                                                          bstUI
                                                                                                          bsh1236I
                                                                                                         hinPI
                                                                                                         hhaI/cfoI
                                                                                                eaeI    thaI
                                                                              aluI    tru9I     cfrI    fnuDII/mvnI
                       tsp509I   mwoI                                         pvuII   mseI  tfiI haeIII/palI
                     tru9I       hinPI                                        mspA1I/nspBII hinfI  bslI bstUI
          maeIII     mseI        hhaI/cfoI cac8I                            cac8I    aseI/asnI/vspI  bsh1236I
       mnlI         aseI/asnI/vspI    tspRI aciI    bsrI
701 AGTGAGGTAA CTCACATTAA TTGCGTTGCG CTCACTGCCC GCTTTCCAGT CGGGAAACCT GTCGTGCCAG CTGGATTAAT GAATCGGCCA ACGCGCGGGG
    TCACTCCATT GAGTGTAATT AACGCAACGC GAGTGACGGG CGAAAGGTCA GCCCTTTGGA CAGCACGGTC GACCTAATTA CTTAGCCGGT TGCGCGCCCC

                                                                                    mwoI
                                                                             fnu4HI/bsoFI
                              earI/ksp632I                    jinPI          aciI   aciI
                             sapI                             hhaI/cfoI    tseI    bsrBI
                           hinPI   aciI             pleI  tseI    mcrI     fnu4HI/bsoFI
        aciI               hhaI/cfoI                hinfI fnu4HI/bsoFI
     mnlI       mwoI      haeII mboII     mnlI     tspRI  bbvI    bsiEI    bbvI    cac8I       aluI          aciI
801 AGAGGCGGTT TGCGTATTGG GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC GGTATCAGCT CACTCAAAGG
    TCTCCGCCAA ACGCATAACC CGCGAGAAGG CGAAGGAGCG AGTGACTGAG CGACGCGAGC CAGCAAGCCG ACGCCGCTCG CCATAGTCGA GTGAGTTTCC
```

FIG. 7Z

```
                                                                                        scrFI
                                                                                        mvaI                        mwoI
                                                                                        ecoRII                      thaI
                                                                                        dsaV                        fnuDII/mvnI
                                                                                        bstNI                       bstUI
                                                          nlaIII          bslI          bssKI                       bsh1236I
                                                          nspHI           cac8I         apyI    bslI                aciI   cac8I
                               tfiI                       nspI          haeIII/palI   HaeIII/palI                 fnu4HI/bsoFI
                               hinfI                      aflIII        haeI         haeI    nlaIV               haeIII/palI
901 CGGTAATACG GTTATCCACA GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT
    GCCATTATGC CAATAGGTGT CTTAGTCCCC TATTGCGTCC TTTCTTGTAC ACTCGTTTTC CGGTCGTTTT CCGGTCCTTG GCATTTTTCC GGCGCAACGA

                                                                                                        scrFI
                                                                                                        mvaI
                                                                                                        ecoRII
                                                                                                        dsaV
                                                           hgaI                                         bstNI
                            aciI                           drdI                                         bssKI
                         nlaIV              sfaNI        taqI     smlI       mnlI                       apyI
001 GGCGTTTTTC CATAGGCTCC GCCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG
    CCGCAAAAAG GTATCCGAGG CGGGGGGACT GCTCGTAGTG TTTTTAGCTG CGAGTTCAGT CTCCACCGCT TTGGGCTGTC CTGATATTTC TATGGTCCGC

      scrFI
      mvaI
      ecoRII
      dsaV                                                      bciVI
      bstNI                                                   mspI
      bssKI                                          bslI     hpaII                                  hinPI
      apy             bssSI hinPI                    aciI    bsaWI                                   hhaI/cfoI
     bsaJI    aluI mnlI    hhaI/cfoI                fnu4HI/bsoFI         aciI                      haeII
101 TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC
    AAAGGGGGAC CTTCGAGGGA GCACGCGAGA GGACAAGGCT GGGACGGCGA ATGGCCTATG GACAGGCGGA AAGAGGGAAG CCCTTCGCAC CGCGAAAGAG

                                                                 hgiAI/aspHI                tseI
                                                                 bsp1286                    fnu4HI/bsoFI
                                                                 bsiHKAI                   mspAlI/nspBII  mspI
                                                                 bmyI                      aciI  hinPI    hpaII
                                                                 apaLI/snoI              mcrI bbvI        bsaWI
          scfI     ddeI                            aluI          alw44I/snoI             bsiEI   hhaI/cfoI
201 AATGCTCACG CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG CCCGACCGCT GCGCCTTATC
    TTACGAGTGC GACATCCATA GAGTCAAGCC ACATCCAGCA AGCGAGGTTC GACCCGACAC ACGTGCTTGG GGGGCAAGTC GGGCTGGCGA CGCGGAATAG
```

FIG. 7AA

alwNI
alw26I/bsmAI
mspI
tseI
hpaII
fnu4HI/bsoFI
scrFI
bbvI
nciI
tseI bsrI
pleI
dsaV
bsrI fnu4HI/bsoFI maeIII
hinfI
cauII
mnlI aciI
tspRI bbvI tspRI
maeIII
sml1 bssKI
301 CGGTAACTAT CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT
GCCATTGATA GCAGAACTCA GGTTGGGCCA TTCTGTGCTG AATAGCGGTG ACCGTCGTCG GTGACCATTG TCCTAATCGT CTCGCTCCAT ACATCCGCCA
sau3AI
mspI hinPI sau3AI mboI/nde
hpaII thaI mboI/ndeII dpnII
sau3AI tseI fnuDII/mvnI dpnII dpnI
mboI/ndeII fnu4HI/bsoFI hhaI/cfoI dpnI alwI
dpnII bbvI bstUI bstYI/xhoII bstYI/xhc
dpnI mspAlI/nspBII cac8I mwoI bsh1236I alwI sml1 mboII
aluI alwI aciI aciI
501 TTGGTAGCTC TTGATCCGGC AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA AAAAAAGGAT CTCAAGAAGA
AACCATCGAG AACTAGGCCG TTTGTTTGGT GGCGACCATC GCCACCAAAA AAACAAACGT TCGTCGTCTA ATGCGCGTCT TTTTTTCCTA GAGTTCTTCT
sau3AI
mboI/ndeII
mboII dpnII
sau3AI dpnI
mboI/ndeII alwI
dpnII rmaI
dpnI maeI
tru9I nlaIII bstYI/xhoII bstYI/xhoII
mseI rcaI alwI hphI bfaI
taiI bspHI
maeII
sau3AI
mboI/ndeII
dpnII ddeI
dpnI hgaI tspRI
601 TCCTTTGATC TTTTCTACGG GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC
AGGAAACTAG AAAAGATGCC CCAGACTGCG AGTCACCTTG CTTTTGAGTG CAATTCCCTA AAACCAGTAC TCTAATAGTT TTTCCTAGAA GTGGATCTAG

FIG. 7BB

```
          tru9I
        tsp509I
     tru9I                 tru9I                                                                      nlaIV
     mseI mseI             mseI                                                                       hgiCI
    ahaIII/draI           ahaIII/draI                                          maeIII       tru9I     mnlI
                                                                                            mseI   tspRI banI      ddeI
701 CTTTTAAATT AAAAATGAAG TTTTAAATCA ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT CAGTGAGGCA CCTATCTCAG
    GAAAATTTAA TTTTTACTTC AAAATTTAGT TAGATTTCAT ATATACTCAT TTGAACCAGA CTGTCAATGG TTACGAATTA GTCACTCCGT GGATAGAGTC

                                                                                              bsrI tseI
    sau3AI                                                                                 sau96I   fnu4HI/bsoFI
    mboI/ndeII                                  pleI                                       nlaIV    bbvI
    dpnII             fokI                      hinfI                                      haeIII/palI
    dpnI              bstF5I                    ahdI/eam1105I               mnlI           asuI tspRI   bsrDI
801 CGATCTGTCT ATTTCGTTCA TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATAACTACGA TACGGGAGGG CTTACCATCT GGCCCCAGTG CTGCAATGAT
    GCTAGACAGA TAAAGCAAGT AGGTATCAAC GGACTGAGGG GCAGCACATC TATTGATGCT ATGCCCTCCC GAATGGTAGA CCGGGGTCAC GACGTTACTA

       bsmAI
       bsai
    thaI
    fnuDII/mvnI       mspI bpmI/gsuI                              mspI
    bstUI             hpaII                                      mwoI     haeIII/palI      sau96I               fokI
    bsh1236I         cfr10I/bsrFI                                bglI   sau96I  hinPI      avaII              mnlI
    aciI           hphI  nlaIV                               cac8I hpaII asuI   hhaI/cfoI  asuI             aciI   bstF5I
901 ACCGCGAGAC CCACGCTCAC CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCTCC
    TGGCGCTCTG GGTGCGAGTG GCCGAGGTCT AAATAGTCGT TATTTGGTCG GTCGGCCTTC CCGGCTCGCG TCTTCACCAG GACGTTGAAA TAGGCGGAGG

                      scrFI
                      nciI
                      mspI                                                 taiI
                      hpaII                                              psp1406I
             tsp509I  dsaV     rmaI                                  hinPI maeII        tseI     mslI
           tru9I      cauII    maeI                       tru9I      hhaI/cfoI          fnu4HI/bsoFI
           mseI       bssKI    bfaI                       mseI     mstI  aclI         bsrDI  cac8I           tsp45I
    bsrI  aseI/asnI/vspI   aluI                       bsrI         aviII/fspI       mwoI  bbvI    sfaNI      maeIII
001 ATCCAGTCTA TTAATTGTTG CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGCCATTGC TGCTGGCATC GTGGTGTCAC
    TAGGTCAGAT AATTAACAAC GGCCCTTCGA TCTCATTCAT CAAGCGGTCA ATTATCAAAC GCGTTGCAAC AACGGTAACG ACGACCGTAG CACCACAGTG
```

FIG. 7CC

```
                                                            sau3AI
                                                            mboI/ndeII
                          nlaIV       sau3AI                dpnII
                        mspI          mboI/ndeII            dpnI                                sau96I
                      bsaWI           dpnII             nlaIII                                  avaII
                   aluI hpaII         dpnI          maeIII  nlaIII                 aciI  aluI   asuI
101 GCTCGTCGTT TGGTATGGCT TCATTCAGCT CCGGTTCCCA ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG
    CGAGCAGCAA ACCATACCGA AGTAAGTCGA GGCCAAGGGT TGCTAGTTCC GCTCAATGTA CTAGGGGGTA CAACACGTTT TTTCGCCAAT CGAGGAAGCC

      sau3AI
      mboI/ndeII
      dpnII              aciI
     pvuI/bspCI         fnu4HI/bsoFI
     mcrI              haeIII/palI                      tspRI
     bsiEI            eaeI                 nlaIII     tseI                             fokI
   mnlI dpnI          cfrI   tspRI         mslI       fnu4HI/bsoFI                     bstF5I
                                                      bbvI          tsp509I        nlaIII       sfaNI
201 TCCTCCGATC GTTGTCAGAA GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG TCATGCCATC CGTAAGATGC
    AGGAGGCTAG CAACAGTCTT CATTCAACCG GCGTCACAAT AGTGAGTACC AATACCGTCG TGACGTATTA AGAGAATGAC AGTACGGTAG GCATTCTACG

                                                                      hgaI
                                                                      hinlI/acyI
                                                                      ahaII/bsaHI
                                                                     mspI                  hinPI
                                                                     hpaII                 hhaI/cfoI
                                                       mcrI         scrFI                  thaI
                                                       bsiEI        nciI                   fnuDII/mvnI
       bsrI     rsaI                                   bcgI         dsaV                   bstUI
      tsp45I   scaI                                  fnu4HI/bsoFI   cauII                  bsh1236I
      maeIII hphI csp6I                ddeI          aciI           bssKI                 aciI
301 TTTTCTGTGA CTGGTGAGTA CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC ATCACGGGAT AATACCGCGC
    AAAAGACACT GACCACTCAT GAGTTGGTTC AGTAAGACTC TTATCACATA CGCCGCTGGC TCAACGAGAA CGGGCCGCAG TAGTGCCCTA TTATGGCGCG

                                                                                      sau3AI
                                          taiI                   sau3AI               mboI/ndeII
                     hgiAI/aspHI         maeII                   mboI/ndeII           dpnII
                     bsp1286            psp1406I                 dpnII                dpnI
               tru9I bsiHKAI            aclI                     dpnI     mspAlI/nspBII bsrI
               mseI  bmyI             xmnI                      bstYI/xhoII           alwI    taqI
             ahaIII/draI              asp700    mboII       smlI alwI      aciI       bstYI/xhoII  maeIII
401 CACATAGCAG AACTTTAAAA GTGCTCATCA TTGGAAAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC
    GTGTATCGTC TTGAAATTTT CACGAGTAGT AACCTTTTGC AAGAAGCCCC GCTTTTGAGA GTTCCTAGAA TGGCGACAAC TCTAGGTCAA GCTACATTGG
```

FIG. 7DD

```
         hgiAI/aspHI
         bsp1286         eco57I
         bsiHKAI        mboII
         bmyI          sau3AI
         apaLI/snoI    mboI/ndeII
         alw44IsnoI    dpnII                                                              aciI
      bssSI            dpnI      sfaNI         hphI          hphI                      fnu4HI/bsoFI
501 CACTCGTGCA CCCAACTGAT CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG CCGCAAAAAA GGGAATAAGG
    GTGAGCACGT GGGTTGACTA GAAGTCGTAG AAAATGAAAG TGGTCGCAAA GACCCACTCG TTTTTGTCCT TCCGTTTTAC GGCGTTTTTT CCCTTATTCC

                                                                                 nlaIII
                                                                                rcaI     bciVI
                               mboII                                            bspHI aciI
       mslI                  earI/ksp632I   sspI                             bsmAI   bsrBI
601 GCGACACGGA AATGTTGAAT ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG CGGATACATA TTTGAATGTA
    CGCTGTGCCT TTACAACTTA TGAGTATGAG AAGGAAAAAG TTATAATAAC TTCGTAAATA GTCCCAATAA CAGAGTACTC GCCTATGTAT AAACTTACAT

                           hinPI
                          thaI
                          fnuDII/mvnI
                          bstUI                                 taiI
                          bsh1236I                              maeII
                         aciI                                  hinlI/acyI            nlaIII
                     nnaIV hhaI/cfoI                           ahaII/bsaHI          rcaI     tru9I
                                                               aatII ddeI           bspHI    mseI
701 TTTAGAAAAA TAAACAAATA GGGGTTCCGC GCACATTTCC CCGAAAAGTG CCACCTGACG TCTAAGAAAC CATTATTATC ATGACATTAA CCTATAAAAA
    AAATCTTTT ATTTGTTTAT CCCCAAGGCG CGTGTAAAGG GGCTTTTCAC GGTGGACTGC AGATTCTTTG GTAATAATAG TACTGTAATT GGATATTTT

                                             thaI                                              mspI
                                             fnuDII/mvnI                                      scrFI
                                             bsh1236I                                         nciI esp3I
                                            hinPI                                             dsaV bsmBI
              sau96I    mboII               thaI                                         tseI  cauII
              haeIII/palI                   fnuDII/mvnI                                  fnu4HI/bsoFI
             eco0109I/draII                 bstUI                                      nlaIII   bssKI
            mnlI       bpuAI                bsh1236I                                  nspHI  aluI hpaII    tsp45I
         bssSI asuI    bbsI               mnlI hhaI/cfoI hphI     hphI    mnlI        nspI bbvI  bslI bsmAI maeIII
801 TAGGCGTATC ACGAGGCCCT TTCGTCTTCA AGAATACTGC CTCGCGCGTT TCGGTGATGA CGGTGAAAAC CTCTGACACA TGCAGCTCCC GGAGACGGTC
    ATCCGCATAG TGCTCCGGGA AAGCAGAAGT TCTTATGACG GAGCGCGCAA AGCCACTACT GCCACTTTTG GAGACTGTGT ACGTCGAGGG CCTCTGCCAG
```

FIG. 7EE

```
                    scrFI
                    nciI
                    mspI                              hgaI
                    hpaII                            thaI
                    dsaV                             fnuDII/mvnI
                  sfaNI                              bstUI   aciI
                 fokI  cauII                         bsh1236I
                 bstF5I                             hinPI  mspAlI/nspBII
  aluI        aciI    bssKI    drdI                 hhaI/cfoI
ACAGCTTGTC TGTAAGCGGA TGCCGGGAGC AGACAAGCCC GTCAGGGCGC GTCAGCGGGT GTTGGCGGGT GTCGGGGCGC AGCCATGACC CAGTCACGTA
TGTCGAACAG ACATTCGCCT ACGGCCCTCG TCTGTTCGGG CAGTCCCGCG CAGTCGCCCA CAACCGCCCA CAGCCCCGCG TCGGTACTGG GTCAGTGCAT

                                                         mwoI
                                                          bstAPI
                                                        hgiAI/aspHI
                                                        bsp1286
                                                        bsiHKAI
                           sfaNI               ddeI     bmyI  ndeI
      mwoI       tru9I    fnu4HI/bsoFI       rsaI       apaLI/snoI
      aciI       mseI     aciI               csp6I      alw44I/snoI aciI        aciI     sfaNI
001 GCGATAGCGG AGTTGGCTTA ACTATGCGGC ATCAGAGCAG ATTGTACTGA GAGTGCACCA TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA
    CGCTATCGCC TCAACCGAAT TGATACGCCG TAGTCTCGTC TAACATGACT CTCACGTGGT ATACGCCACA CTTTATGGCG TGTCTACGCA TTCCTCTTTT

      mwoI
      hinPI
      hhaI/cfoI
     nlaIV
     narI
     kasI
     hinlI/acyI                                         sau3AI
     hgiCI                                              mboI/ndeII
     haeII                                              dpnII
     sfaNI  eheI      mwoI                              dpnI       haeIII/palI           cac8I
     mwoI   banI      bglI                             pvuI/bspCI sau96I                 aluI
    aciI    ahaII/bsaHI                                mcrI        asuI mboII            pvuII
                                                       bsiEI    cac8I  earI/ksp632I      mspAlI/nspBII   fokI
                                                      mwoI      aciI  mnlI            cac8I              bstF5I
101 TACCGCATCA GGCGCCATTC GCCATTCAGG CTACGCAACT GTTGGGAAGG GCGATCGGTG CGGGCCTTCTT CGCTATTACG CCAGCTGGCG AAGGGGGGT
    ATGGCGTAGT CCGCGGTAAG CGGTAAGTCC GATGCGTTGA CAACCCTTCC CGCTAGCCAC GCCCGGAGAA GCGATAATGC GGTCGACCGC TTCCCCCCTA
```

FIG. 7FF

```
                                                 scrFI
                                                 mvaI
                                                 ecoRII
                                                 dsaV
                                                 bstNI                                     tspRI
                                                 bssKI                     taiI            bsrI
      tseI                                       bsaJI               tsp45I              haeIII/palI
      fnu4HI/bsoFI tru9I                         apyI                maeIII              eaeI
      bbvI         mseI        maeIII                           bsrI       maeII         cfrI
201 GTGCTGCAAG GCGATTAAGT TGGGTAACGC CAGGGTTTTC CCAGTCACGA CGTTGTAAAA CGACGGCCAG TGCC
    CACGACGTTC CGCTAATTCA ACCCATTGCT GTCCCAAAAG GGTCAGTGCT GCAACATTTT GCTGCCGGTC ACGG
```

FIG. 7GG

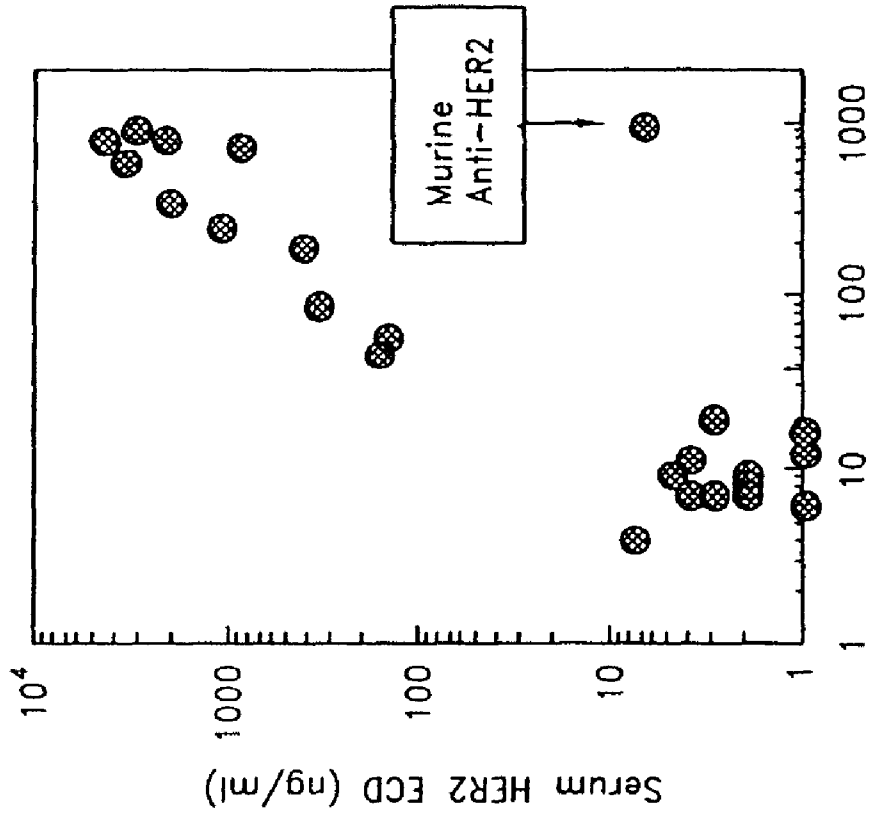
Murine
Anti-HER2
Serum HER2 ECD (ng/ml)
10^4
1000
100
10
1
Tumor weight in mg
1000
100
10
1

FIG. 8B

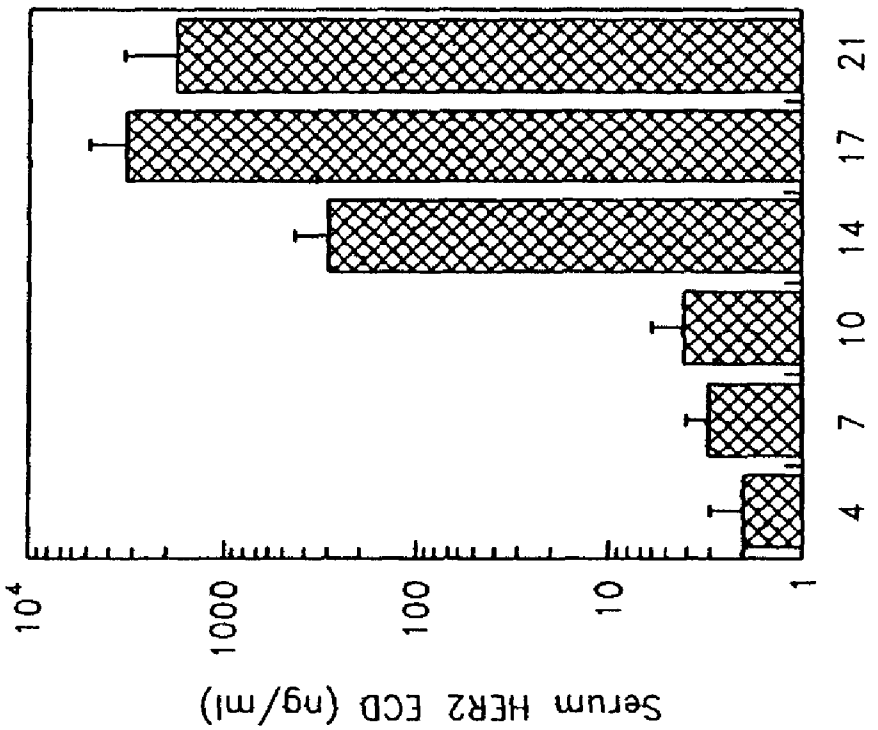
Serum HER2 ECD (ng/ml)
10^4
1000
100
10
1
Day post-transplant
4
7
10
14
17
21

FIG. 8A

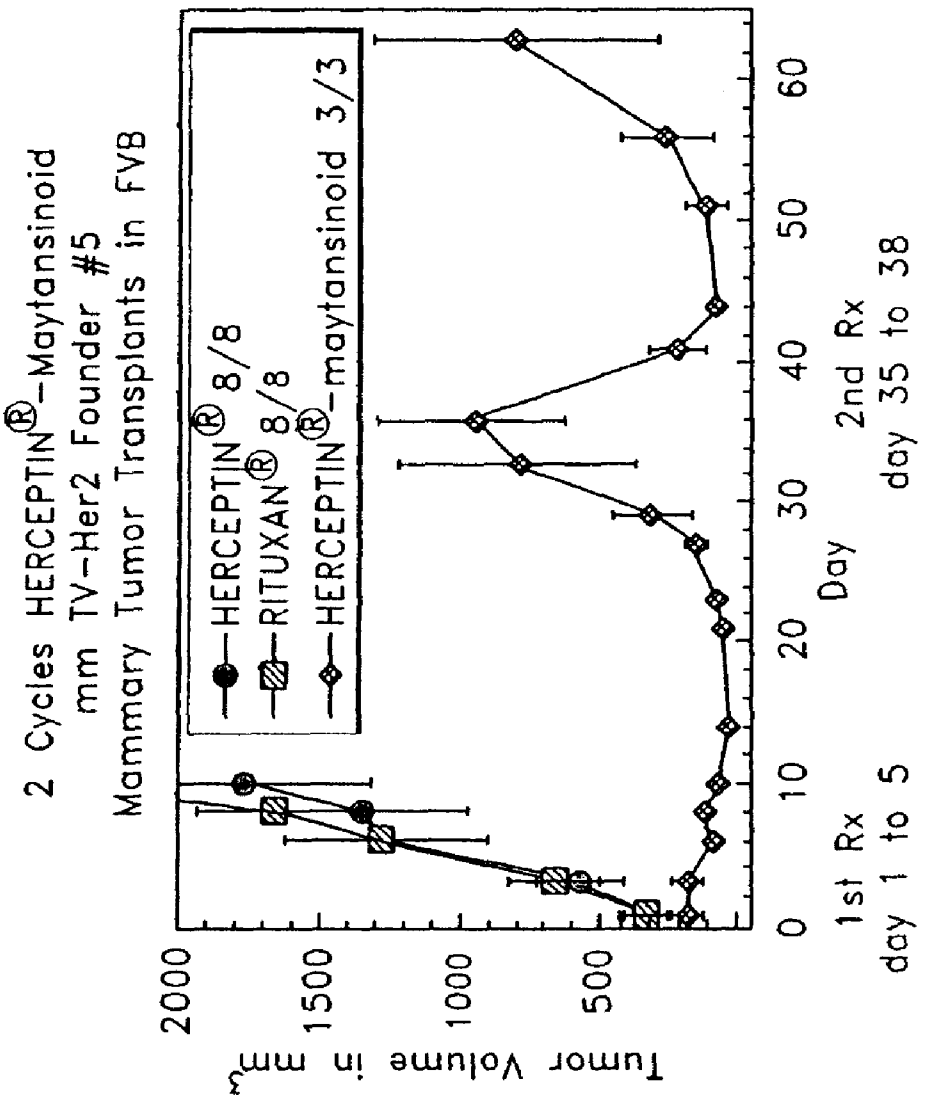
2 Cycles HERCEPTIN®-Maytansinoid
mm TV-Her2 Founder #5
Mammary Tumor Transplants in FVB
HERCEPTIN® 8/8
RITUXAN® 8/8
HERCEPTIN®-maytansinoid 3/3
Tumor Volume in mm³
2000
1500
1000
500
0
10
20
30
40
50
60
Day
1st Rx
day 1 to 5
2nd Rx
day 35 to 38

FIG. 9

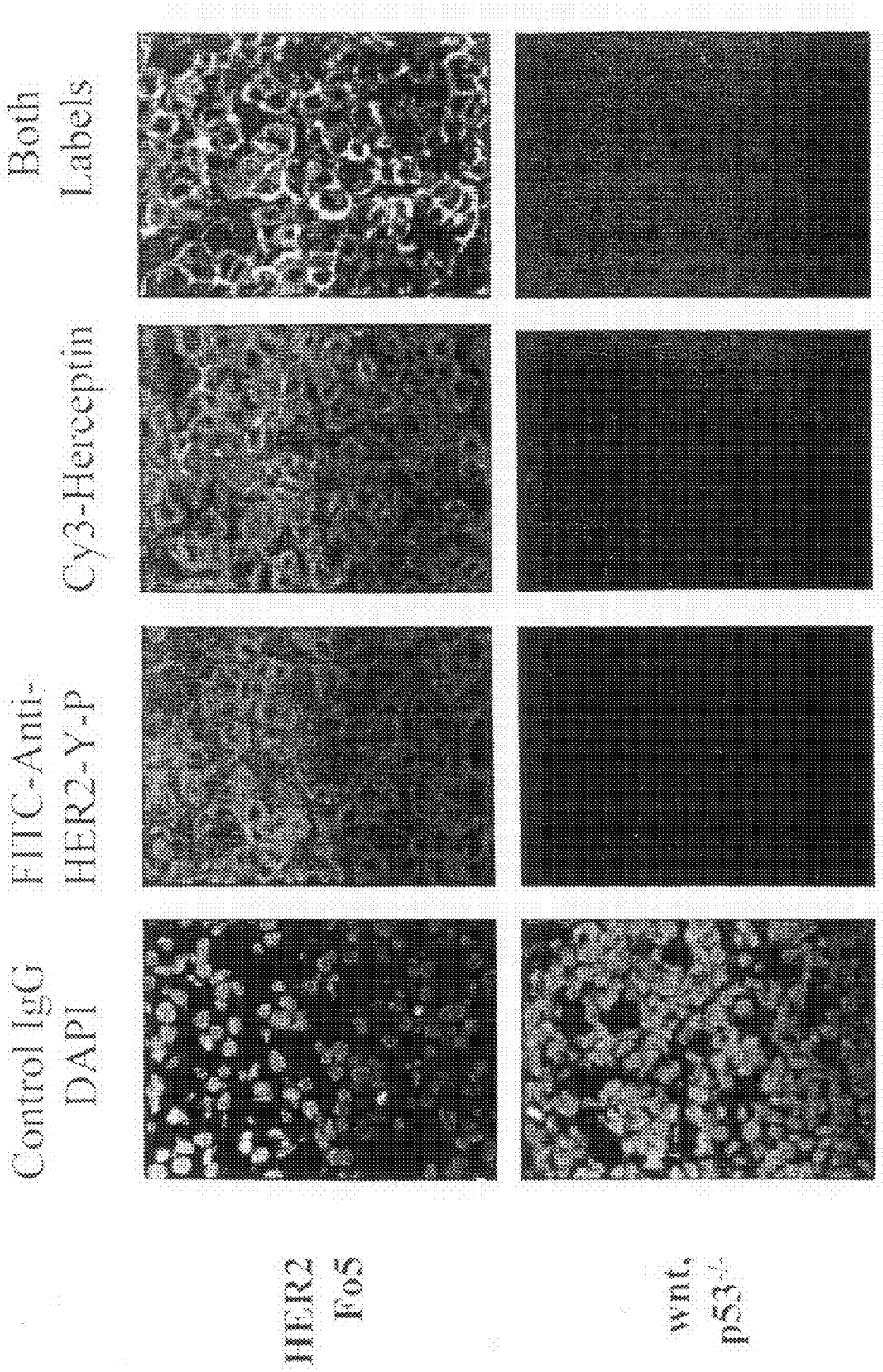
Control IgG
DAPI
FITC-Anti-HER2-Y-P
Cy3-Herceptin
Both Labels
HER2 Fo5
wmt, p53-/-

FIG. 10

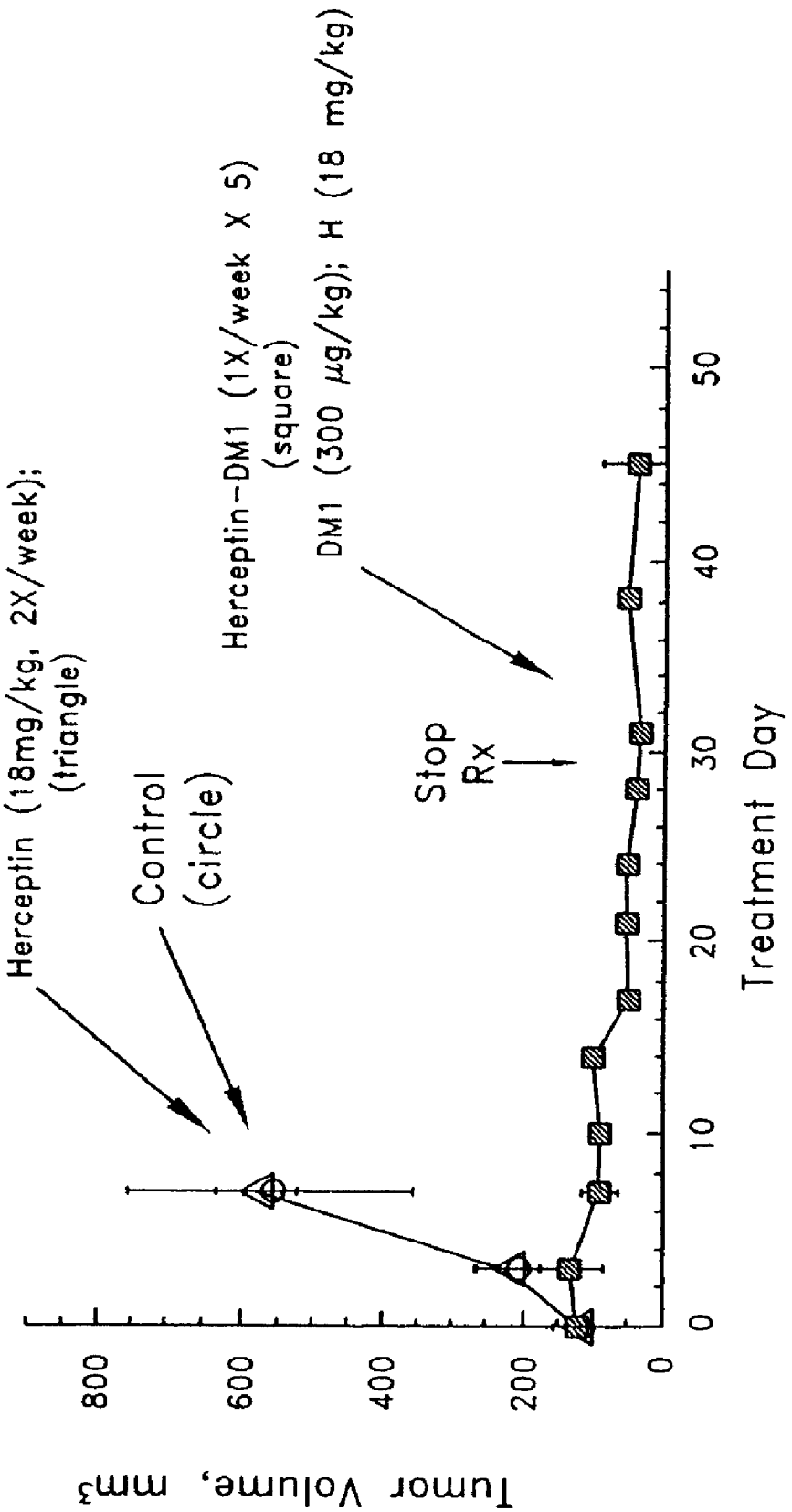
Herceptin (18mg/kg, 2X/week); (triangle)
Control (circle)
Herceptin-DM1 (1X/week X 5) (square)
DM1 (300 μg/kg); H (18 mg/kg)
Stop Rx
Tumor Volume, mm3
Treatment Day
0
200
400
600
800
0
10
20
30
40
50

FIG. 11

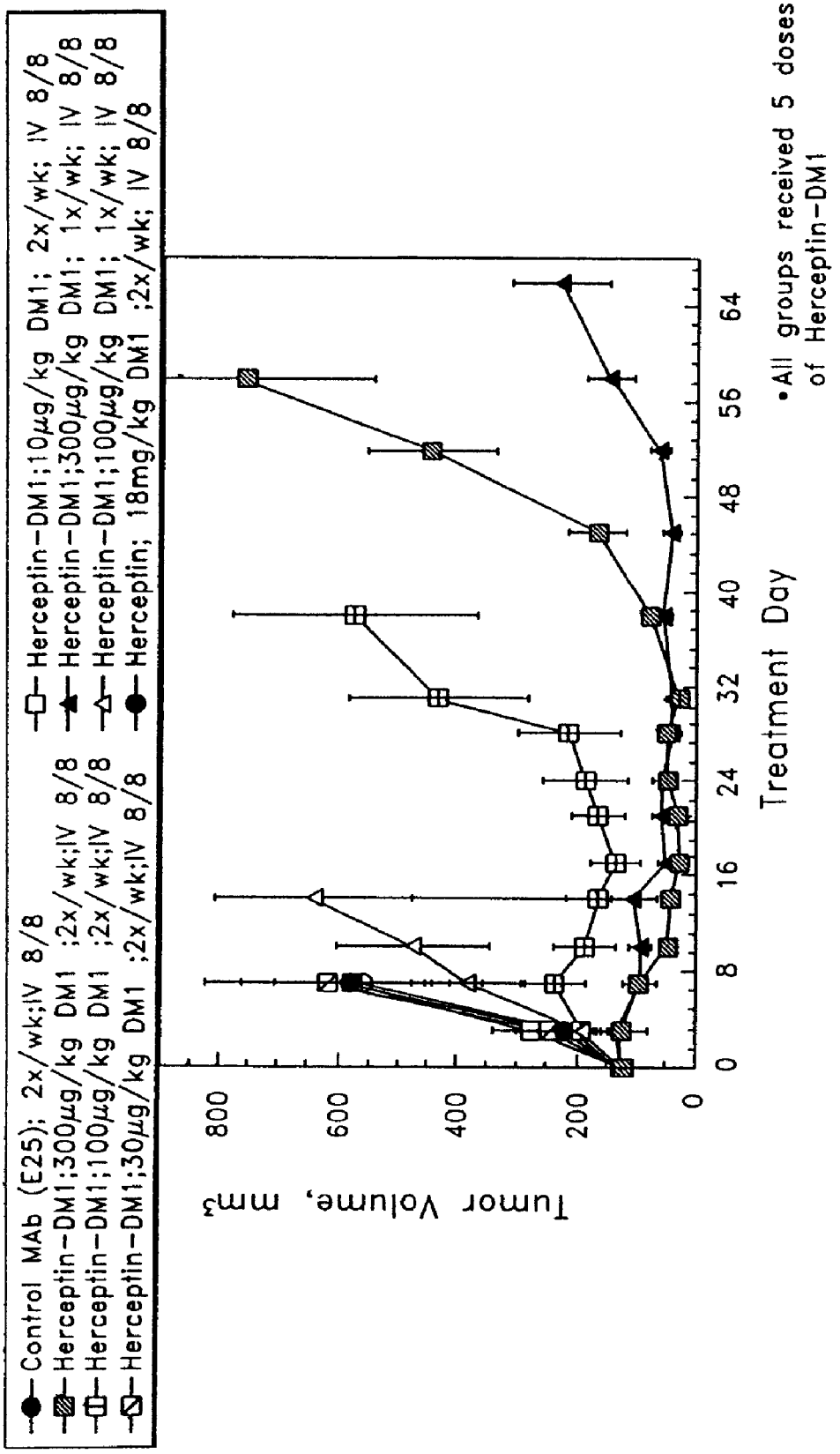
Control MAb (E25); 2x/wk;IV 8/8
Herceptin-DM1;300μg/kg DM1 ;2x/wk;IV 8/8
Herceptin-DM1;100μg/kg DM1 ;2x/wk;IV 8/8
Herceptin-DM1;30μg/kg DM1 ;2x/wk;IV 8/8
Herceptin-DM1;10μg/kg DM1; 2x/wk; IV 8/8
Herceptin-DM1;300μg/kg DM1; 1x/wk; IV 8/8
Herceptin-DM1;100μg/kg DM1; 1x/wk; IV 8/8
Herceptin; 18mg/kg DM1 ;2x/wk; IV 8/8
Tumor Volume, mm3
0
200
400
600
800
0
8
16
24
32
40
48
56
64
Treatment Day
•All groups received 5 doses of Herceptin-DM1

FIG. 12

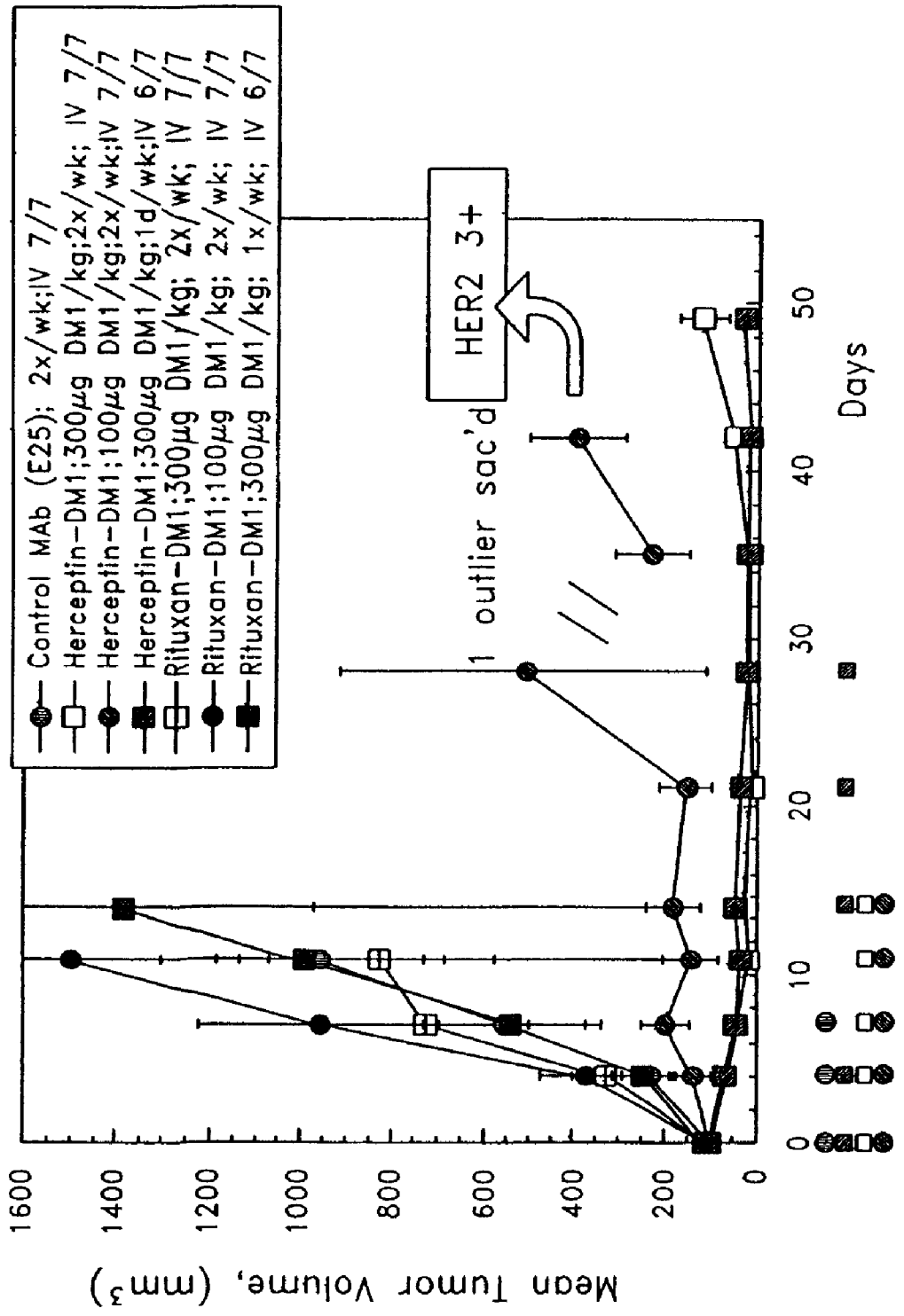
Control MAb (E25); 2x/wk;IV 7/7
Herceptin-DM1;300μg DM1/kg;2x/wk; IV 7/7
Herceptin-DM1;100μg DM1/kg;2x/wk;IV 7/7
Herceptin-DM1;300μg DM1/kg;1d/wk;IV 6/7
Rituxan-DM1;300μg DM1/kg; 2x/wk; IV 7/7
Rituxan-DM1;100μg DM1/kg; 2x/wk; IV 7/7
Rituxan-DM1;300μg DM1/kg; 1x/wk; IV 6/7
HER2 3+
1 outlier sac'd
Mean Tumor Volume, (mm³)
Days
0
200
400
600
800
1000
1200
1400
1600
10
20
30
40
50

FIG. 13

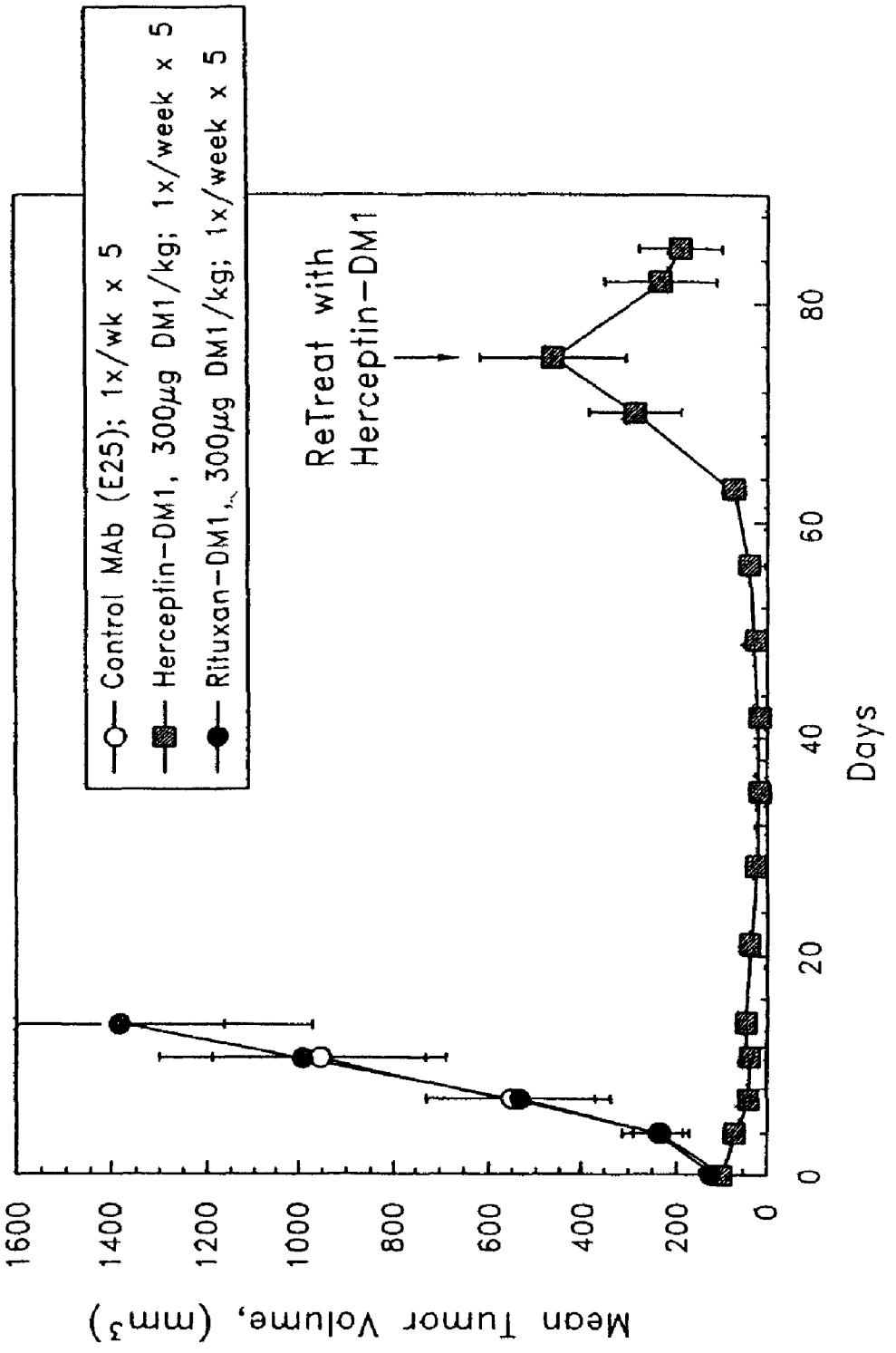
Control MAb (E25); 1x/wk x 5
Herceptin-DM1, 300μg DM1/kg; 1x/week x 5
Rituxan-DM1, 300μg DM1/kg; 1x/week x 5
ReTreat with Herceptin-DM1
Mean Tumor Volume, (mm³)
Days
0
200
400
600
800
1000
1200
1400
1600
0
20
40
60
80

FIG. 14

METHODS OF TREATMENT USING ANTI-ERBB ANTIBODY-MAYTANSINOID CONJUGATES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional application of U.S. Ser. No. 11/488,545, filed Jul. 7, 2006 now U.S. Pat. No. 7,575,748 which is a continuation application of U.S. Ser. No. 09/811,123 filed Mar. 16, 2001 now U.S. Pat. No. 7,097,840 which application claims priority to U.S. Provisional Application Nos. 60/238,327, filed Oct. 5, 2000, 60/189,844 filed Mar. 16, 2000, and 60/327,563 filed Jun. 23, 2000 (converted from U.S. Application No. 09/602,530) under 35 USC §119(e).

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention concerns methods of treatment, especially ErbB receptor-directed cancer therapies, using anti-ErbB receptor antibody-maytansinoid conjugates, and articles of manufacture suitable for use in such methods.

2. Description of the Related Art

1. Maytansine and Maytansinoids

Maytansine was first isolated from the east African shrub *Maytenus serrata* (U.S. Pat. No. 3,896,111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (U.S. Pat. No. 4,151,042). Synthetic maytansinol and maytansinol analogues are disclosed, for example, in U.S. Pat. Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533, the disclosures of which are hereby expressly incorporated by reference.

Maytansine and maytansinoids are highly cytotoxic but their clinical use in cancer therapy has been greatly limited by their severe systemic side-effects primarily attributed to their poor selectivity for tumors. Clinical trials with maytansine had been discontinued due to serious adverse effects on the central nervous system and gastrointestinal system (Issel et al., *Can. Trtmnt. Rev.* 5:199-207 [1978]).

2. The ErbB Family of Receptor Tyrosine Kinases and anti-ErbB Antibodies

Members of the ErbB family of receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes four distinct members, including epidermal growth factor receptor (EGFR or ErbB1), HER2 (ErbB2 or p185^{neu}), HER3 (ErbB3) and HER4 (ErbB4 or tyro2).

p185^{neu}, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the neu proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homologue of neu is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon et al., *Science,* 235:177-182 (1987); Slamon et al., *Science,* 244:707-712 (1989); and U.S. Pat. No. 4,968,603). To date, no point mutation analogous to that in the neu proto-oncogene has been reported for human tumors. Overexpression of ErbB2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder. See,

among others, King et al., *Science,* 229:974 (1985); Yokota et al., *Lancet:* 1:765-767 (1986); Fukushigi et al., *Mol Cell Biol.,* 6:955-958 (1986); Geurin et al., *Oncogene Res.,* 3:21-31 (1988); Cohen et al., *Oncogene,* 4:81-88 (1989); Yonemura et al., *Cancer Res.,* 51:1034 (1991); Borst et al., *Gynecol. Oncol.,* 38:364 (1990); Weiner et al., *Cancer Res.,* 50:421-425 (1990); Kern et al., *Cancer Res.,* 50:5184 (1990); Park et al., *Cancer Res.,* 49:6605 (1989); Zhau et al., *Mol. Carcinog.,* 3:354-357 (1990); Aasland et al. *Br. J. Cancer* 57:358-363 (1988); Williams et al. *Pathobiology* 59:46-52 (1991); and McCann et al., *Cancer,* 65:88-92 (1990). ErbB2 may be overexpressed in prostate cancer (Gu et al. *Cancer Lett.* 99:185-9 (1996); Ross et al *Hum. Pathol.* 28:827-33 (1997); Ross et al. *Cancer* 79:2162-70 (1997); and Sadasivan et al. *J. Urol.* 150:126-31 (1993)).

A spliced form of erbB2 oncogen encoding a constitutively tyrosine phosphorylated ErbB2 receptor is disclosed in PCT publication WO 00/20579, published on Apr. 13, 2000. The erbB2 protein encoded by the splice variant has an in frame deletion of 16 amino acids (CVDLDDKGCPAEQRAS (SEQ ID NO: 11)), two of which are conserved cysteine residues.

Antibodies directed against the rat p185^{neu} and human ErbB2 protein products have been described. Drebin and colleagues have raised antibodies against the rat neu gene product, p185^{neu}. See, for example, Drebin et al., *Cell* 41:695-706 (1985); Myers et al., *Meth. Enzym.* 198:277-290 (1991); and WO94/22478. Drebin et al. *Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions of p185^{neu} result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. See also U.S. Pat. No. 5,824,311 issued Oct. 20, 1998.

Other anti-ErbB2 antibodies with various properties have been described in Tagliabue et al. *Int. J. Cancer* 47:933-937 (1991); McKenzie et al. *Oncogene* 4:543-548 (1989); Maier et al. *Cancer Res.* 51:5361-5369 (1991); Bacus et al. *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al. *PNAS (USA)* 88:8691-8695 (1991); Bacus et al. *Cancer Research* 52:2580-2589 (1992); Xu et al. *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al. *Cancer Research* 52:2771-2776 (1992); Hancock et al. *Cancer Res.* 51:4575-4580 (1991); Shawver et al. *Cancer Res.* 54:1367-1373 (1994); Arteaga et al. *Cancer Res.* 54:3758-3765 (1994); Harwerth et al. *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al. *Oncogene* 14:2099-2109 (1997).

Hudziak et al., Mol. Cell. Biol. 9(3): 1165-1172 (1989) describe the generation of a panel of anti-ErbB2 antibodies which were characterized using the human breast tumor cell line SK-BR-3. Relative cell proliferation of the SK-BR-3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize ErbB2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also U.S. Pat. No. 5,677,171 issued Oct. 14, 1997. The anti-ErbB2 antibodies discussed in Hudziak et al. are further characterized in Fendly et al. *Cancer Research* 50:1550-1558 (1990); Kotts et al. *In Vitro* 26(3):59A (1990); Sarup et al. *Growth Regulation* 1:72-82 (1991); Shepard et al. *J. Clin. Immunol.* 11(3):117-127 (1991); Kumar et al. *Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras et al. *Oncogene* 9:1829-1838 (1994); Vitetta et al. *Cancer Research* 54:5301-5309 (1994); Sli-

wkowski et al. *J. Biol. Chem.* 269(20): 14661-14665 (1994); Scott et al. *J. Biol. Chem.* 266:14300-5 (1991); D'souza et al. *Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis et al. *Cancer Research* 56:1457-1465 (1996); and Schaefer et al. *Oncogene* 15:1385-1394 (1997).

The murine monoclonal anti-HER2 antibody inhibits the growth of breast cancer cell lines that overexpress HER2 at the 2+ and 3+ level, but has no activity on cells that express lower levels of HER2 (Lewis et al., *Cancer Immunol. Immunother.* [1993]). Based on this observation, antibody 4D5 was humanized (Carter et al., *Proc. Natl. Acad. Sci. USA* 89: 4285-4289 [1992]). The humanized version designated HERCEPTIN® (huMAb4D5-8, rhuMAb HER2, U.S. Pat. No. 5,821,337) was tested in breast cancer patients whose tumors overexpress HER2 but who had progressed after conventional chemotherapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 [1996]); Cobleigh et al., *J. Clin. Oncol.* 17: 2639-2648 [1999]). Most patients in this trial expressed HER2 at the 3+ level, though a fraction was 2+ tumors. Remarkably, HERCEPTIN® induced clinical responses in 15% of patients (complete responses in 4% of patients, and partial responses in 11%) and the median duration of those responses was 9.1 months. HERCEPTIN® received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the ErbB2 protein.

Homology screening has resulted in the identification of two other ErbB receptor family members; ErbB3 (U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS* (*USA*) 86:9193-9197 (1989)) and ErbB4 (EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA,* 90:1746-1750 (1993); and Plowman et al., *Nature,* 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

3. Maytansinoid-Antibody Conjugates

In an attempt to improve their therapeutic index, maytansine and maytansinoids have been conjugated to antibodies specifically binding to tumor cell antigens. Immunoconjugates containing maytansinoids are disclosed, for example, in U.S. Pat. Nos. 5,208,020; 5,416,064 and European Patent EP 0 425 235 B1, the disclosures of which are hereby expressly incorporated by reference. Liu et al., *Proc. Natl. Acad. Sci. USA* 93:8618-8623 (1996) described immunoconjugates comprising a maytansinoid designated DM1 linked to the monoclonal antibody C242 directed against human colorectal cancer. The conjugate was found to be highly cytotoxic towards cultured colon cancer cells, and showed antitumor activity in an in vivo tumor growth assay. Chari et al. *Cancer Research* 52:127-131 (1992) describe immunoconjugates in which a maytansinoid was conjugated via a disulfide linker to the murine antibody A7 binding to an antigen on human colon cancer cell lines, or to another murine monoclonal antibody TA.1 that binds the HER-2/neu oncogene. The cytotoxicity of the TA.1-maytansonoid conjugate was tested in vitro on the human breast cancer cell line SK-BR-3, which expresses 3×10^5 HER-2 surface antigens per cell. The drug conjugate achieved a degree of cytotoxicity similar to the free maytansinoid drug, which could be increased by increasing the number of maytansinoid molecules per antibody molecule. The A7-maytansinoid conjugate showed low systemic cytotoxicity in mice.

Although HERCEPTIN® is a breakthrough in treating patients with ErbB2-overexpressing breast cancers that have received extensive prior anti-cancer therapy, generally approximately 85% of the patients in this population fail to respond, or respond only poorly, to HERCEPTIN® treatment, and in the clinical trial preceding marketing approval, the median time to disease progression in all treated patients was only 3.1 months. Therefore, there is a significant clinical need for developing further HER2-directed cancer therapies for those patients with HER2-overexpressing tumors or other diseases associated with HER2 expression that do not respond, or respond poorly, to HERCEPTIN® treatment.

SUMMARY OF THE INVENTION

The present invention is based on the unexpected experimental finding that HERCEPTIN®-maytansinoid conjugates are highly effective in the treatment of HER2 (ErbB2) overexpressing tumors that do not respond, or respond poorly, to HERCEPTIN® therapy. The anti-ErbB2-maytansinoid conjugates of the present invention are expected to have superior clinical activity compared to treatment with HERCEPTIN® alone, including a better objective response rate and/or longer duration of response and/or increased survival.

In one aspect, the present invention concerns a method for the treatment of a tumor in a mammal, wherein the tumor is characterized by the overexpression of an ErbB receptor and does not respond or responds poorly to treatment with a monoclonal anti-ErbB antibody, comprising administering to the mammal a therapeutically effective amount of a conjugate of the anti-ErbB antibody with a maytansinoid.

In a preferred embodiment, the patient is human. In another preferred embodiment, the ErbB receptor is (human) ErbB2 (HER2). The method is not limited by the mechanism of action of the anti-ErbB antibody used. Thus, the anti-ErbB antibody may, for example, have growth inhibitory properties and/or may induce cell death and/or apoptosis. In a particularly preferred embodiment, the method concerns the treatment of cancer including, without limitation, breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, colorectal, thyroid, pancreatic, prostate and bladder cancer. Preferably the cancer is breast cancer, in particular, breast cancer which overexpresses ErbB2 at a 2+ level or above, more preferably at a 3+ level. A preferred group of antibodies has a biological characteristic of a 4D5 monoclonal antibody, or binds essentially the same epitope as a 4D5 monoclonal antibody, a humanized form of the murine monoclonal antibody 4D5 (ATCC CRL 10463) being particularly preferred.

The maytansinoid used in the conjugates of the present invention may be maytansine or, preferably, maytansinol or a maytansinol ester. The antibody and maytansinoid may be conjugated by a bispecific chemical linker, such as N-succinimidyl-4-(2-pyridylthio)propanoate (SPDP) or N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP). The linking group between the antibody and the maytansinoid may, for example, be a disulfide, thioether, acid labile, photolabile, peptidase labile, or esterase labile group.

In another aspect, the invention concerns an article of manufacture comprising a container and a composition contained therein, wherein the composition comprises an anti-ErbB antibody-maytansinoid conjugate, and further comprising a package insert or label indicating that the composition can be used to treat cancer characterized by overexpression of an ErbB receptor, preferably at a 2+ level or above.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the heavy chain variable region sequence of a humanized anti-HER2 antibody 2C4 (SEQ ID NO: 1) aligned with the heavy chain variable region sequences of antibody 574 (SEQ ID NO: 2) and the human subgroup V_HIII upon which the humanized sequences are based (SEQ ID NO: 3).

FIG. **2** shows the light chain variable region sequence of a humanized anti-HER2 antibody 2C4 (SEQ ID NO: 4) aligned with the light chain variable region sequences of antibody 574 (SEQ ID NO: 5) and the sequence of the human subgroup upon which the humanized sequences are based (SEQ ID NO: 6).

FIG. **3** shows the structure of the maytansinoid, designated "DM1." In the structure of DM1, "R" can be occupied by a variety of groups capable of forming a chemical bond with a selected linker. Preferably, "R" is an SH group or a protected derivative thereof, which forms an S—S bond with a linker, such as N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP).

FIG. **4** illustrates the structure of a HERCEPTIN®-DM1 conjugate.

FIG. **5** is the elution profile of HERCEPTIN®-DM1 conjugate on a Sephacryl S300 gel filtration column.

FIG. **6** shows the anti-proliferative effect of HERCEPTIN® and HERCEPTIN®-DM1 conjugate on SK-BR3 cells in vitro. As control, the unrelated monoclonal antibody RITUXAN® or RITUXAN®-DM1 conjugate was used.

FIG. **7**A-GG shows the nucleotide sequence of a HER2 transgene plasmid construct (SEQ ID NO: 7) directing the expression of native human HER2 (ErbB2) in the mammary gland of a transgenic mouse. The reverse strand is also depicted (SEQ ID NO: 10). The figure includes the nucleotide sequence of HER2 (ErbB2) cDNA insert (SEQ ID NO: 8) as well as the deduced amino acid sequence of HER2 (ErbB2) (SEQ ID NO: 9), including the signal sequence. Within SEQ ID NO: 9, residues from about 22 to about 645, inclusive represent the HER2 (ErbB2) extracellular domain.

FIGS. **8**A and B show that the amount of HER2 extracellular domain (ECD) shed into serum increases following transplant (FIG. **8**A) and is proportional to the weight of the resulting tumor (FIG. **8**B).

FIG. **9** illustrates the effect of HERCEPTIN®-DM1 on HER2-transgenic tumors. Two mm^3 pieces of MMTV-HER2-transgenic tumors were transplanted into the mammary fat pad of FVB mice. When tumors reached 250 mm^3, groups of 8 mice were injected i.v. on 5 consecutive days with a HERCEPTIN®-DM1 conjugate. Two other groups of mice were treated IP twice per week with 10 mg/kg of either HERCEPTIN® or RITUXAN®.

FIG. **10** shows that tumor cells originating from Founder 5 show binding to cy3-HERCEPTIN® and to an anti-tyrosine-phosphorylated-HER2 antibody. Antibodies were injected intravenously into transgenic mice and the next day tumors were collected and sectioned. Antibody binding was visualized by fluoresence microscopy.

FIG. **11** shows the effect of HERCEPTIN® and HERCEPTIN®-DM1 conjugate on the growth of HER2 transgenic tumor transplant. HERCEPTIN®-DM1 was administered once a week for 5 weeks at 300 μg DM1/kg or 18 mg/kg of HERCEPTIN®. HERCEPTIN® or a control mAb (RITUXAN®) was administered twice a week at 18 mg/kg.

FIG. **12** shows evaluation of doses and schedule of treatment with HERCEPTIN®-DM1 conjugate in HER2 transgenic tumor transplant in nude mice. HERCEPTIN® -DM1 conjugate was administered either twice a week at various doses (300, 100, 30 or 10 μg DM1/kg) or once a week at various doses (300 or 100 μg DM1/kg) for 5 weeks. HERCEPTIN® or a control mAb E25 (RITUXAN®) was administered twice a week at 18 mg/kg.

FIG. **13** shows the effect of different HERCEPTIN®-DM1 dosing regimens on HER2-transgenic tumors compared to matching doses of RITUXAN®-DM1. Mice with 100 mm^3 tumors were injected i.v. with HERCEPTIN®-DM1 or RITUXAN®-DM1 at doses of 100 or 300 μg DM1/kg twice a week or 300 μg DM1/kg once a week. All animals received 5 doses.

FIG. **14** is a comparison of the most effective observed dose of HERCEPTIN®-DM1 and RITUXAN®-DM1. A second dose of HERCEPTIN®-DM1 following the beginning of tumor regrowth was effective in shrinking the tumors again.

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton et al., *Dictionary of Microbiology and Molecular Biology* 2*nd ed.*, J. Wiley & Sons (New York, N.Y. 1994). One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

An "ErbB receptor" or "ErbB" is a receptor protein tyrosine kinase which belongs to the ErbB receptor family and includes ErbB1 (EGFR), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) receptors and other members of this family to be identified in the future. The definition specifically includes ErbB receptors encoded by spliced forms of the corresponding erbB oncogens, including, without limitation, the deletion variant of ErbB2 disclosed in PCT publication No. WO 00/20579 (published on Apr. 13, 2000). The ErbB receptor will generally comprise an extracellular domain, which may bind an ErbB ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The ErbB receptor may be a "native sequence" ErbB receptor or a functional derivative, such as an "amino acid sequence variant" thereof. Preferably the ErbB receptor is native sequence human ErbB receptor.

The terms "ErbB1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to native sequence EGFR as disclosed, for example, in Carpenter et al. *Ann. Rev. Biochem.* 56:881-914 (1987), including naturally occurring mutant forms thereof (e.g. a deletion mutant EGFR as in Humphrey et al. *PNAS (USA)* 87:4207-4211 (1990)), and its functional derivatives, such as amino acid sequence variants. erbB1 refers to the gene encoding the EGFR protein product.

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to native sequence human HER2 protein described, for example, in Semba et al., *PNAS (USA)* 82:6497-6501 (1985) and Yamamoto et al. *Nature* 319:230-234 (1986) (Genebank accession number X03363), and functional derivatives, such as amino acid sequence variants thereof. The term erbB2 refers to the gene encoding human HER2 and neu refers to the gene encoding rat $p185^{neu}$. Preferred HER2 is native sequence human HER2. Examples of antibodies which bind HER2 include MAbs 4D5 (ATCC CRL 10463), 2C4 (ATCC HB-12697), 7F3 (ATCC HB-12216), and 7C2 (ATCC HB 12215) (see, U.S. Pat. No. 5,772,997; WO98/77797; and U.S. Pat. No. 5,840,525, expressly incorporated herein by reference). Humanized anti-HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 (HERCEPTIN®) as described in Table 3 of U.S. Pat. No. 5,821,337 expressly

incorporated herein by reference; humanized 520C9 (WO93/21319). Human anti-HER2 antibodies are described in U.S. Pat. No. 5,772,997 issued Jun. 30, 1998 and WO 97/00271 published Jan. 3, 1997.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. *PNAS (USA)* 86:9193-9197 (1989), and functional derivatives, including amino acid sequence variants thereof. Examples of antibodies which bind HER3 are described in U.S. Pat. No. 5,968,511 (Akita and Sliwkowski), e.g. the 8B8 antibody (ATCC HB 12070) or a humanized variant thereof.

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman et al., *Proc. Natl. Acad. Sci. USA,* 90:1746-1750 (1993); and Plowman et al., *Nature,* 366:473-475 (1993), and functional derivatives, including amino acid sequence variants thereof such as the HER4 isoforms disclosed in WO 99/19488.

A "native" or "native sequence" EGFR, HER2, HER3 or HER4 polypeptide may be isolated from nature, produced by techniques of recombinant DNA technology, chemically synthesized, or produced by any combinations of these or similar methods.

"Functional derivatives" include amino acid sequence variants, and covalent derivatives of the native polypeptides as long as they retain a qualitative biological activity of the corresponding native polypeptide. Amino acid sequence variants generally differ from a native sequence in the substitution, deletion and/or insertion of one or more amino acids anywhere within a native amino acid sequence. Deletional variants include fragments of the native polypeptides, and variants having N— and/or C-terminal truncations. Ordinarily, amino acid sequence variants will possess at least about 70% homology, preferably at least about 80%, more preferably at least about 90% homology with a native polypeptide.

"Homology" is defined as the percentage of residues in the amino acid sequence variant that are identical after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. One such computer program is "Align 2", authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, D.C. 20559, on Dec. 10, 1991.

By "ErbB ligand" is meant a polypeptide which binds to and/or activates an ErbB receptor. The ErbB ligand of particular interest herein is a native sequence human ErbB ligand such as Epidermal Growth Factor (EGF) (Savage et al., *J. Biol. Chem.* 247:7612-7621 (1972)); Transforming Growth Factor alpha (TGF-alpha) (Marquardt et al., *Science* 223:1079-1082 (1984)); amphiregulin also known as schwanoma or keratinocyte autocrine growth factor (Shoyab et al. *Science* 243:1074-1076 (1989); Kimura et al. *Nature* 348:257-260 (1990); and Cook et al. *Mol. Cell Biol.* 11:2547-2557 (1991)); betacellulin (Shing et al., *Science* 259:1604-1607 (1993); and Sasada et al. *Biochem. Biophys. Res. Commun.* 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama et al., *Science* 251:936-939 (1991)); epiregulin (Toyoda et al., *J. Biol. Chem.* 270:7495-7500 (1995); and Komurasaki et al. *Oncogene* 15:2841-2848 (1997)), a heregulin (see below); neuregulin-2 (NRG-2) (Carraway et al., *Nature* 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang et al., *Proc. Natl. Acad. Sci.* 94:9562-9567 (1997)); or cripto (CR-1) (Kannan et al. *J. Biol. Chem.* 272(6):3330-3335 (1997)). ErbB ligands which bind EGFR include EGF, TGF-alpha, amphiregulin, betacellulin, HB-EGF and epiregulin. ErbB ligands which bind HER3 include heregulins. ErbB ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3 and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide which activates the ErbB2-ErbB3 and ErbB2-ErbB4 protein complexes (i.e. induces phosphorylation of tyrosine residues in the complex upon binding thereto). Various heregulin polypeptides encompassed by this term are disclosed in Holmes et al., *Science* 256:1205-1210 (1992); WO 92/20798; Wen et al., *Mol. Cell. Biol.* 14(3):1909-1919 (1994) and Marchionni et al., *Nature* 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (e.g. $HRG\beta_{177-244}$).

An "ErbB hetero-oligomer" herein is a noncovalently associated oligomer comprising at least two different ErbB receptors. Such complexes may form when a cell expressing two or more ErbB receptors is exposed to an ErbB ligand and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al., *J. Biol. Chem.,* 269(20):14661-14665 (1994), for example. Examples of such ErbB hetero-oligomers include EGFR-HER2, HER2-HER3 and HER3-HER4 complexes. Moreover, the ErbB hetero-oligomer may comprise two or more HER2 receptors combined with a different ErbB receptor, such as HER3, HER4 or EGFR. Other proteins, such as a cytokine receptor subunit (e.g. gp130), may be included in the hetero-oligomer.

In the context of HER2 variants, such as HER2 fragments, the phrase "having the biological activity of a native human HER2" is used to refer to the qualitative ability of such fragments to induce tumor growth when overexpressed in an animal model (transgenic or non-transgenic) of the present invention.

"Tumor", as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

A cancer which "overexpresses" an ErbB receptor is one which has significantly higher levels of an ErbB receptor, such as HER2, at the cell surface thereof, compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. ErbB receptor overexpression may be determined in a diagnostic or prognostic assay by evaluating increased levels of the ErbB protein present on the surface of a cell (e.g. via an immunohistochemistry assay; IHC). Alternatively, or additionally, one may measure levels of ErbB-encoding nucleic acid in the cell, e.g. via fluorescent in situ hybridization (FISH; see WO98/45479 published October, 1998), Southern blotting, or polymerase chain reac-

tion (PCR) techniques, such as real time quantitative PCR (RT-PCR). One may also study ErbB receptor overexpression by measuring shed antigen (e.g., ErbB extracellular domain) in a biological fluid such as serum (see, e.g., U.S. Pat. No. 4,933,294 issued Jun. 12, 1990; WO91/05264 published Apr. 18, 1991; U.S. Pat. No. 5,401,638 issued Mar. 28, 1995; and Sias et al. *J. Immunol. Methods* 132: 73-80 (1990)). Aside from the above assays, various in vivo assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, e.g. a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, e.g. by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

The tumors overexpressing HER2 are rated by immunohistochemical scores corresponding to the number of copies of HER2 molecules expressed per cell, and can been determined biochemically: 0=0-10,000 copies/cell, 1+=at least about 200,000 copies/cell, 2+=at least about 500,000 copies/cell, 3+=at least about 2,000,000 copies/cell. Overexpression of HER2 at the 3+ level, which leads to ligand-independent activation of the tyrosine kinase (Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84: 7159-7163 [1987]), occurs in approximately 30% of breast cancers, and in these patients, relapse-free survival and overall survival are diminished (Slamon et al., *Science* 244: 707-712 [1989]; Slamon et al., *Science* 235: 177-182 [1987]).

Conversely, a cancer which is "not characterized by overexpression of an ErbB receptor" is one which, in a diagnostic assay, does not express higher than normal levels of ErbB receptor compared to a noncancerous cell of the same tissue type.

A "hormone independent" cancer is one in which proliferation thereof is not dependent on the presence of a hormone which binds to a receptor expressed by cells in the cancer. Such cancers do not undergo clinical regression upon administration of pharmacological or surgical strategies that reduce the hormone concentration in or near the tumor. Examples of hormone independent cancers include androgen independent prostate cancer, estrogen independent breast cancer, endometrial cancer and ovarian cancer. Such cancers may begin as hormone dependent tumors and progress from a hormone-sensitive stage to a hormone-refractory tumor following antihormonal therapy.

The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments, so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 (1984)). Chimeric antibodies of interest herein include primatized antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, Ape etc) and human constant region sequences.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragment(s).

An "intact" antibody is one which comprises an antigen-binding variable region as well as a light chain constant domain (C_L) and heavy chain constant domains, C_H1, C_H2 and C_H3. The constant domains may be native sequence constant domains (e.g. human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

"Humanized" forms of non-human (e.g., rodent) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

Humanized anti-ErbB2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 (HER-

CEPTIN®) as described in Table 3 of U.S. Pat. No. 5,821,337 expressly incorporated herein by reference; humanized 520C9 (WO93/21319) and humanized 2C4 antibodies. The heavy chain and light chain of humanized antibody 2C4 are shown in FIGS. **1** and **2**, respectively.

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc.

Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes". There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells in summarized is Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. Nos. 5,500, 362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS (USA)* 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, e.g. from blood or PBMCs as described herein.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)).

"Complement dependent cytotoxicity" or "CDC" refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (e.g. an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable", as used in connection with antibodies, refers to the fact that certain portions of the antibody variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues

26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework Region" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

An antibody "which binds" an antigen of interest, e.g. ErbB2 antigen, is one capable of binding that antigen with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting a cell expressing the antigen and/or for targeted delivery of a cytotoxic or other chemotherapeutic agent, such as a maytansinoid. Where the antibody is one which binds ErbB2, it will usually preferentially bind ErbB2 as opposed to other ErbB receptors, and may be one which does not significantly cross-react with other proteins such as EGFR, ErbB3 or ErbB4. In such embodiments, the extent of binding of the antibody to these non-ErbB2 proteins (e.g., cell surface binding to endogenous receptor) will be less than 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA). Sometimes, the anti-ErbB2 antibody will not significantly cross-react with the rat neu protein, e.g., as described in Schecter et al. *Nature* 312:513 (1984) and Drebin et al., *Nature* 312:545-548 (1984).

Unless indicated otherwise, the expressions "monoclonal antibody 4D5", and "4D5 monoclonal antibody" refer to an antibody that has antigen binding residues of, or derived from, the murine 4D5 antibody. For example, the monoclonal antibody 4D5 may be murine monoclonal antibody 4D5 (ATCC CRL 10463) or a variant thereof, such as humanized antibody 4D5, possessing antigen binding amino acid residues of murine monoclonal antibody 4D5. Exemplary humanized 4D5 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 (HERCEPTIN®) as in U.S. Pat. No. 5,821,337, with huMAb4D5-8 (HERCEPTIN®) being a preferred humanized 4D5 antibody.

An antibody having a "biological characteristic" of a designated antibody, such as the monoclonal antibody designated 4D5, is one which possesses one or more of the biological characteristics of that antibody which distinguish it from other antibodies that bind to the same antigen (e.g. ErbB2). For example, an antibody with a biological characteristic of 4D5 may show growth inhibitory effect on ErbB2 overexpressing cells in a manner that is dependent on the ErbB2 expression level and/or bind the same epitope in the extracellular domain of ErbB2 as that bound by 4D5 (e.g. which blocks binding of monoclonal antibody 4D5 to ErbB2).

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB expressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of ErbB expressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13.

Examples of "growth inhibitory" antibodies are those which bind to ErbB2 and inhibit the growth of cancer cells overexpressing ErbB2. Preferred growth inhibitory anti-ErbB2 antibodies inhibit growth of SK-BR-3 breast tumor cells in cell culture by greater than 20%, and preferably greater than 50% (e.g. from about 50% to about 100%) at an antibody concentration of about 0.5 to 30 μg/ml, where the growth inhibition is determined six days after exposure of the SK-BR-3 cells to the antibody (see U.S. Pat. No. 5,677,171 issued Oct. 14, 1997). The SK-BR-3 cell growth inhibition assay is described in more detail in that patent and hereinbelow. The preferred growth inhibitory antibody is monoclonal antibody 4D5, e.g., humanized 4D5.

A molecule (e.g. antibody) which "induces cell death" is one which causes a viable cell to become nonviable. The cell is generally one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. Preferably, the cell is a cancer cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic, prostate or bladder cancer cell. In vitro, the cell may be a SK-BR-3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Cell death in vitro may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e. in the absence of complement) and in the absence of immune effector cells. To determine whether the molecule is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. *Cytotechnology* 17:1-11 (1995)) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the PI uptake assay in BT474 cells. Examples of antibodies which induce cell death include anti-ErbB2 antibodies 7C2 and 7F3 (WO 98/17797, expressly incorporated herein by reference), including humanized and/or affinity matured variants thereof.

A molecule (e.g. antibody) which "induces apoptosis" is one which induces programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is usually one which overexpresses the ErbB2 receptor. Preferably the cell is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic, prostate or bladder cancer cell. In vitro, the cell may be a SK-BR-3, BT474, Calu 3 cell, MDA-MB-

453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the molecule which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an annexin binding assay using BT474 cells. Sometimes the pro-apoptotic molecule will be one which further blocks ErbB ligand activation of an ErbB receptor. In other situations, the molecule is one which does not significantly block ErbB ligand activation of an ErbB receptor. Further, the molecule may induce apoptosis, without inducing a large reduction in the percent of cells in S phase (e.g. one which only induces about 0-10% reduction in the percent of these cells relative to control). Examples of antibodies which induce apoptosis include anti-ErbB2 antibodies 7C2 and 7F3 (WO 98/17797, expressly incorporated herein by reference), including humanized and/or affinity matured variants thereof.

An antibody which "blocks" ligand activation of an ErbB receptor is one which reduces or prevents such activation as hereinabove defined, wherein the antibody is able to block ligand activation of the ErbB receptor substantially more effectively than monoclonal antibody 4D5, e.g. about as effectively as monoclonal antibodies 7F3 or 2C4 or Fab fragments thereof and preferably about as effectively as monoclonal antibody 2C4 or a Fab fragment thereof. For example, the antibody that blocks ligand activation of an ErbB receptor may be one which is about 50-100% more effective than 4D5 at blocking formation of an ErbB hetero-oligomer. Blocking of ligand activation of an ErbB receptor can occur by any means, e.g. by interfering with: ligand binding to an ErbB receptor, ErbB complex formation, tyrosine kinase activity of an ErbB receptor in an ErbB complex and/or phosphorylation of tyrosine kinase residue(s) in or by an ErbB receptor. Examples of antibodies which block ligand activation of an ErbB receptor include monoclonal antibodies 2C4 and 7F3 (which block HRG activation of ErbB2/ErbB3 and ErbB2/ErbB4 hetero-oligomers; and EGF, TGF-α, amphiregulin, HB-EGF and/or epiregulin activation of an EGFR/ErbB2 hetero-oligomer); and L26, L96 and L288 antibodies (Klapper et al. *Oncogene* 14:2099-2109 (1997)), which block EGF and NDF binding to T47D cells which express EGFR, ErbB2, ErbB3 and ErbB4. Humanized and/or affinity matured variants these and other antibodies within the definition are specifically included.

The term "epitope" is used to refer to binding sites for (monoclonal or polyclonal) antibodies on protein antigens.

Antibodies that bind to a certain epitope are identified by "epitope mapping." There are many methods known in the art for mapping and characterizing the location of epitopes on proteins, including solving the crystal structure of an antibody-antigen complex, competition assays, gene fragment expression assays, and synthetic peptide-based assays, as described, for example, in Chapter 11 of Harlow and Lane, Using Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1999. Competition assays are discussed below. According to the gene fragment expression assays, the open reading frame encoding the protein is fragmented either randomly or by specific genetic constructions and the reactivity of the expressed fragments of the protein with the antibody to be tested is determined. The gene fragments may, for example, be produced by PCR and then transcribed and translated into protein in vitro, in the presence of radioactive amino acids. The binding of the antibody to the radioactively labeled protein fragments is then determined by immunoprecipitation and gel electrophoresis. Certain epitopes can also be identified by using large libraries of random peptide sequences displayed on the surface of phage particles (phage libraries). Alternatively, a defined library of overlapping peptide fragments can be tested for binding to the test antibody in simple binding assays. The latter approach is suitable to define linear epitopes of about 5 to 15 amino acids.

An antibody binds "essentially the same epitope" as a reference antibody, when the two antibodies recognize identical or sterically overlapping epitopes. The most widely used and rapid methods for determining whether two epitopes bind to identical or sterically overlapping epitopes are competition assays, which can be configured in all number of different formats, using either labeled antigen or labeled antibody. Usually, the antigen is immobilized on a 96-well plate, and the ability of unlabeled antibodies to block the binding of labeled antibodies is measured using radioactive or enzyme labels.

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane domain of ErbB2, and extends from about residue 519 to about residue 625, inclusive within the ErbB2 extracellular domain sequence included in SEQ ID NO: 3, FIG. **4**. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in Harlow and Lane, supra, can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of ErbB2 (e.g. any one or more residues in the region from about residue 529 to about residue 625, inclusive in SEQ ID NO: 3).

The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain (see FIG. **4** and SEQ ID NO: 3).

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (e.g. any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2; see FIG. **4**, and SEQ ID NO: 3).

A tumor which "does not respond, or responds poorly, to treatment with a monoclonal anti-ErbB antibody" does not show statistically significant improvement in response to anti-ErbB antibody treatment when compared to no treatment or treatment with placebo in a recognized animal model or a human clinical trial, or which responds to initial treatment with anti-ErbB antibodies but grows as treatment is continued. A particularly suitable animal model for testing the efficacy of anti-ErbB antibodies is the transgenic animal model disclosed herein, and illustrated in Example 3.

The terms "treat" or "treatment" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as the development or spread of cancer. For purposes of this invention, beneficial or

desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment of the present invention. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors; leukemias and lymphoid malignancies, in particular breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic, prostate or bladder cancer. A preferred disorder to be treated in accordance with the present invention is malignant tumor, such as breast cancer, that overexpresses an ErbB receptor (e.g. ErbB2 and/or EGFR), and does not respond or responds poorly to treatment with antibody to the receptor(s) that is/are overexpressed. A particularly preferred disorder is an ErbB2-overexpressing breast cancer that does not respond or responds poorly to HERCEPTIN® therapy.

The term "therapeutically effective amount" refers to an amount of a drug effective to treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP) and/or determining the response rate (RR).

The term "objective response rate" refers to the number of treated individuals that respond to treatment as determined by a quantitative measure.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlomaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfomithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK7; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2'=-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions,* 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery*, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into

the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

The term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term also includes, as equivalents, analogs of either DNA or RNA made from nucleotide analogs, and as applicable, single (sense or antisense) and double-stranded polynucleotides. An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. The term "expression vector" includes plasmids, cosmids or phages capable of synthesizing the subject HER2 protein encoded by the respective recombinant gene carried by the vector. Preferred vectors are those capable of autonomous replication and/expression of nucleic acids to which they are linked. In the present specification, "plasmid" and "vector" are used interchangeably, as the plasmid is the most commonly used form of vector.

As used herein, the terms "transcriptional regulatory elements" and "transcriptional regulatory sequences" are used interchangeably and refer to nucleic acid, e.g. DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, enhancers, splicing signals and polyadenylation signals. These terms are intended to encompass all elements that promote or regulate transcription, including promoters, core elements required for basic interaction of RNA polymerase and transcription factors, upstream elements, enhancers, and response elements (Lewin, "Genes V" (Oxford University Press, Oxford) pages 847-873). Reference herein to the transcriptional regulatory elements of a gene or class of gene includes both all or an intact region of the naturally occurring transcriptional regulatory elements and modified forms of the transcriptional regulatory elements of the gene or group of genes. Such modified forms include rearrangements of the elements, deletions of some elements or extraneous sequences, and insertion of heterologous elements. The modular nature of transcriptional regulatory elements and the absence of position-dependence of the function of some regulatory elements such as enhancers make such modifications possible. Numerous techniques are available for dissecting the regulatory elements of genes to determine their location and function. Such information can be used to direct modification of the elements, if desired. It is preferred, however, that an intact region of the transcriptional regulatory elements of a gene be used.

The term "tissue-specific promoter" means a nucleotide sequence that serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells of a tissue, such as cells of a mammary gland. In an illustrative embodiment, gene constructs utilizing mammary gland-specific promoters can be used to preferentially direct expression of a HER2 protein or protein fragment in the mammary gland tissue.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "transfection" refers to the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of HER2.

As used herein, the term "transgene" refers to a nucleic acid sequence which is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can be operably linked to one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of a selected nucleic acid.

Accordingly, the term "transgene construct" refers to a nucleic acid which includes a transgene, and (optionally) such other nucleic acid sequences as transcriptionally regulatory sequence, polyadenylation sites, replication origins, marker genes, etc., which may be useful in the general manipulation of the transgene for insertion in the genome of a host organism.

The term "transgenic" is used herein as an adjective to describe the property, for example, of an animal or a construct, of harboring a transgene. For instance, as used herein, a "transgenic organism" is any animal, preferably a non-human mammal, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by trangenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extra-chromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express or overexpress a recombinant form of the subject HER2 proteins. The terms "founder line" and "founder ani-

mal" refer to those animals that are the mature product of the embryos to which the transgene was added, i.e., those animals that grew from the embryos into which DNA was inserted, and that were implanted into one or more surrogate hosts.

The terms "progeny" and "progeny of the transgenic animal" refer to any and all offspring of every generation subsequent to the originally transformed mammals. The term "non-human mammal" refers to all members of the class Mammalia except humans. "Mammal" refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as mouse, rat, rabbit, pig, sheep, goat, cattle and higher primates.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-ErbB2 antibodies disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

A "cardioprotectant" is a compound or composition which prevents or reduces myocardial dysfunction (i.e. cardiomyopathy and/or congestive heart failure) associated with administration of a drug, such as an anti-ErbB antibody or its maytansinoid conjugate, to a patient. The cardioprotectant may, for example, block or reduce a free-radical-mediated cardiotoxic effect and/or prevent or reduce oxidative-stress injury. Examples of cardioprotectants encompassed by the present definition include the iron-chelating agent dexrazoxane (ICRF-187) (Seifert et al. *The Annals of Pharmacotherapy* 28:1063-1072 (1994)); a lipid-lowering agent and/or anti-oxidant such as probucol (Singal et al. *J. Mol. Cell Cardiol.* 27:1055-1063 (1995)); amifostine (aminothiol 2-[(3-aminopropyl)amino]ethanethiol-dihydrogen phosphate ester, also called WR-2721, and the dephosphorylated cellular uptake form thereof called WR-1065) and S-3-(3-methylaminopropylamino)propylphosphorothioic acid (WR-151327), see Green et al. *Cancer Research* 54:738-741 (1994); digoxin (Bristow, M. R. In: Bristow M R, ed. *Drug-Induced Heart Disease*. New York: Elsevier 191-215 (1980)); beta-blockers such as metoprolol (Hjalmarson et al. *Drugs* 47:Suppl 4:31-9 (1994); and Shaddy et al. *Am. Heart J.* 129: 197-9 (1995)); vitamin E; ascorbic acid (vitamin C); free radical scavengers such as oleanolic acid, ursolic acid and N-acetylcysteine (NAC); spin trapping compounds such as alpha-phenyl-tert-butyl nitrone (PBN); (Paracchini et al., *Anticancer Res.* 13:1607-1612 (1993)); selenoorganic compounds such as P251 (Elbesen); and the like.

2. Detailed Description

The present invention is based on results obtained in a novel murine HER2-transgenic tumor model in which HERCEPTIN® or the murine antibody 4D5 from which HERCEPTIN® was derived, had little effect on tumor growth. Using this model to test the efficacy of HERCEPTIN® and HERCEPTIN®-maytansinoid conjugates, it was surprisingly found that while the transplanted tumor obtained from such transgenic mice responded poorly to HERCEPTIN® treatment, the HERCEPTIN®-maytansinoid conjugates were highly efficacious.

Accordingly, the present invention is based on the use of anti-ErbB antibody-maytansinoid conjugates in the treatment of ErbB overexpressing tumors that do not respond well to anti-ErbB antibody and/or maytansinoid treatment.

A. Production of anti-ErbB Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The production of antibodies will be illustrated with reference to anti-ErbB2 antibodies but it will be apparent for those skilled in the art that antibodies to other members of the ErbB receptor family can be produced and modified in a similar manner.

The ErbB2 antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of ErbB2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing ErbB2 at their cell surface (e.g. NIH-3T3 cells transformed to overexpress ErbB2; or a carcinoma cell line such as SK-BR-3 cells, see Stancovski et al. *PNAS* (*USA*) 88:8691-8695 (1991)) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with **3** volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al.,

Nature, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); and Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.,* 5:256-262 (1993) and Plückthun, *Immunol. Revs.,* 130:151-188 (1992).

In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy chain and light chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; and Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized Antibodies

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et

al., *J. Mol. Biol.,* 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

Example 1 below describes production of an exemplary humanized anti-ErbB2 antibody. The humanized antibody herein may, for example, comprise nonhuman hypervariable region residues incorporated into a human variable heavy domain and may further comprise a framework region (FR) substitution at a position selected from the group consisting of 69H, 71H and 73H utilizing the variable domain numbering system set forth in Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). In one embodiment, the humanized antibody comprises FR substitutions at two or all of positions 69H, 71H and 73H.

Various forms of the humanized antibody are contemplated. For example, the humanized antibody may be an antibody fragment, such as a Fab. Alternatively, the humanized antibody may be an intact antibody, such as an intact IgG1 antibody.

(iv) Human Antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germline mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and U.S. Pat. Nos. 5,591,669, 5,589,369 and 5,545,807.

Alternatively, phage display technology (McCafferty et al., *Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., *Nature,* 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., *J. Mol. Biol.* 222:581-597 (1991), or Griffith et al., *EMBO J.* 12:725-734 (1993). See, also, U.S. Pat. Nos. 5,565,332 and 5,573,905.

As discussed above, human antibodies may also be generated by in vitro activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

Human anti-ErbB2 antibodies are described in U.S. Pat. No. 5,772,997 issued Jun. 30, 1998 and WO 97/00271 published Jan. 3, 1997.

(v) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. WO 96/16673 describes a bispecific anti-ErbB2/anti-FcγRIII antibody and U.S. Pat. No. 5,837,234 discloses a bispecific anti-ErbB2/anti-FcγRI antibody. A

bispecific anti-ErbB2/Fcα antibody is shown in WO98/02463. U.S. Pat. No. 5,821,337 teaches a bispecific anti-ErbB2/anti-CD3 antibody.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in U.S. Pat. No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

(vii) Other Amino Acid Sequence Modifications

Amino acid sequence modification(s) of the anti-ErbB2 antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the anti-ErbB2 antibody are prepared by introducing appropriate nucleotide changes into the anti-ErbB2 antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the anti-ErbB2 antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may

alter post-translational processes of the anti-ErbB2 antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the anti-ErbB2 antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells *Science,* 244: 1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with ErbB2 antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed anti-ErbB2 antibody variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-ErbB2 antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the anti-ErbB2 antibody molecule include the fusion to the N- or C-terminus of the anti-ErbB2 antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the anti-ErbB2 antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;
(2) neutral hydrophilic: cys, ser, thr;
(3) acidic: asp, glu;
(4) basic: asn, gln, his, lys, arg;
(5) residues that influence chain orientation: gly, pro; and
(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the anti-ErbB2 antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human ErbB2. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

It may be desirable to modify the antibody of the invention with respect to effector function, e.g. so as to enhance antigen-dependent cell-mediated cyotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation

in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. *Anti-Cancer Drug Design* 3:219-230 (1989).

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Pat. No. 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

(viii) Glycosylation Variants

Antibodies are glycosylated at conserved positions in their constant regions (Jefferis and Lund, *Chem. Immunol.* 65:111-128 [1997]; Wright and Morrison, *TibTECH* 15:26-32 [1997]). The oligosaccharide side chains of the immunoglobulins affect the protein's function (Boyd et al., *Mol. Immunol.* 32:1311-1318 [1996]; Wittwe and Howard, *Biochem.* 29:4175-4180 [1990]), and the intramolecular interaction between portions of the glycoprotein which can affect the conformation and presented three-dimensional surface of the glycoprotein (Hefferis and Lund, supra; Wyss and Wagner, *Current Opin. Biotech.* 7:409-416 [1996]). Oligosaccharides may also serve to target a given glycoprotein to certain molecules based upon specific recognition structures. For example, it has been reported that in agalactosylated IgG, the oligosaccharide moiety 'flips' out of the inter-CH2 space and terminal N-acetylglucosamine residues become available to bind mannose binding protein (Malhotra et al., *Nature Med.* 1:237-243 [1995]). Removal by glycopeptidase of the oligosaccharides from CAMPATH-1H (a recombinant humanized murine monoclonal IgG1 antibody which recognizes the CDw52 antigen of human lymphocytes) produced in Chinese Hamster Ovary (CHO) cells resulted in a complete reduction in complement mediated lysis (CMCL) (Boyd et al., *Mol. Immunol.* 32:1311-1318 [1996]), while selective removal of sialic acid residues using neuraminidase resulted in no loss of DMCL. Glycosylation of antibodies has also been reported to affect antibody-dependent cellular cytotoxicity (ADCC). In particular, CHO cells with tetracycline-regulated expression of β(1,4)-N-acetylglucosaminyltransferase III (GnTIII), a glycosyltransferase catalyzing formation of bisecting GlcNAc, was reported to have improved ADCC activity (Umana et al., *Mature Biotech.* 17:176-180 [1999]).

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Glycosylation variants of antibodies are variants in which the glycosylation pattern of an antibody is altered. By altering is meant deleting one or more carbohydrate moieties found in the antibody, adding one or more carbohydrate moieties to the antibody, changing the composition of glycosylation (glycosylation pattern), the extent of glycosylation, etc.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites). Similarly, removal of glycosylation sites can be accomplished by amino acid alteration within the native glycosylation sites of the antibody.

The amino acid sequence is usually altered by altering the underlying nucleic acid sequence. Nucleic acid molecules encoding amino acid sequence variants of the anti-ErbB2 antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-ErbB2 antibody.

The glycosylation (including glycosylation pattern) of antibodies may also be altered without altering the amino acid sequence or the underlying nucleotide sequence. Glycosylation largely depends on the host cell used to express the antibody. Since the cell type used for expression of recombinant glycoproteins, e.g. antibodies, as potential therapeutics is rarely the native cell, significant variations in the glycosylation pattern of the antibodies can be expected (see, e.g. Hse et al., *J. Biol. Chem.* 272:9062-9070 [1997]). In addition to the choice of host cells, factors which affect glycosylation during recombinant production of antibodies include growth mode, media formulation, culture density, oxygenation, pH, purification schemes and the like. Various methods have been proposed to alter the glycosylation pattern achieved in a particular host organism including introducing or overexpressing certain enzymes involved in oligosaccharide production (U.S. Pat. Nos. 5,047,335; 5,510,261 and 5,278,299). Glycosylation, or certain types of glycosylation, can be enzymatically removed from the glycoprotein, for example using endoglycosidase H (Endo H). In addition, the recombinant host cell can be genetically engineered, e.g. make defective in processing certain types of polysaccharides. These and similar techniques are well known in the art.

The glycosylation structure of antibodies can be readily analyzed by conventional techniques of carbohydrate analysis, including lectin chromatography, NMR, Mass spectrometry, HPLC, GPC, monosaccharide compositional analysis, sequential enzymatic digestion, and HPAEC-PAD, which uses high pH anion exchange chromatography to separate oligosaccharides based on charge. Methods for releasing oligosaccharides for analytical purposes are also known, and include, without limitation, enzymatic treatment (commonly performed using peptide-N-glycosidase F/endo-β-galactosidase), elimination using harsh alkaline environment to release mainly O-linked structures, and chemical methods using anhydrous hydrazine to release both N- and O-linked oligosaccharides.

(viii) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. One may further select antibodies with certain biological characteristics, as desired.

For example, to identify growth inhibitory anti-ErbB2 antibodies, one may screen for antibodies which inhibit the growth of cancer cells which overexpress ErbB2. In one embodiment, the growth inhibitory antibody of choice is able to inhibit growth of SK-BR-3 cells in cell culture by about 20-100% and preferably by about 50-100% at an antibody concentration of about 0.5 to 30 μg/ml. To identify such antibodies, the SK-BR-3 assay described in U.S. Pat. No. 5,677,171 can be performed. According to this assay, SK-BR-3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin streptomycin. The SK-BR-3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 mls/35 mm dish). 0.5 to 30 μg/ml of the anti-ErbB2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SK-BR-3 cells by about 20-100% or about 50-100% may be selected as growth inhibitory antibodies.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, e.g., PI, trypan blue or 7AAD uptake may be assessed relative to control. The preferred assay is the PI uptake assay using BT474 cells. According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection (Rockville, Md.)) are cultured in Dulbecco's Modified Eagle Medium (D-MEM): Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclhone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3×10^6 per dish in 100×20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the appropriate monoclonal antibody. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200 rpm for 5 minutes at 4° C., the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12×75 tubes (1 ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake may be selected as cell death-inducing antibodies.

In order to select for antibodies which induce apoptosis, an annexin binding assay using BT474 cells is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 μg/ml of the monoclonal antibody. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a DNA staining assay using BT474 cells is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the preceding two paragraphs are incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37° C., then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

To identify an antibody which blocks ligand activation of an ErbB receptor, the ability of the antibody to block ErbB ligand binding to cells expressing the ErbB receptor (e.g. in conjugation with another ErbB receptor with which the ErbB receptor of interest forms an ErbB hetero-oligomer) may be determined. For example, cells naturally expressing, or transfected to express, ErbB receptors of the ErbB hetero-oligomer may be incubated with the antibody and then exposed to labeled ErbB ligand. The ability of the anti-ErbB2 antibody to block ligand binding to the ErbB receptor in the ErbB hetero-oligomer may then be evaluated.

For example, inhibition of HRG binding to MCF7 breast tumor cell lines by anti-ErbB2 antibodies may be performed using monolayer MCF7 cultures on ice in a 24-well-plate format essentially as described in Example 1 below. Anti-ErbB2 monoclonal antibodies may be added to each well and incubated for 30 minutes. ^{125}I-labeled rHRGβ$1_{177-224}$ (25 pm) may then be added, and the incubation may be continued for 4 to 16 hours. Dose response curves may be prepared and an IC_{50} value may be calculated for the antibody of interest. In one embodiment, the antibody which blocks ligand activation of an ErbB receptor will have an IC_{50} for inhibiting HRG binding to MCF7 cells in this assay of about 50 nM or less, more preferably 10 nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC_{50} for inhibiting HRG binding to MCF7 cells in this assay may, for example, be about 100 nM or less, more preferably 50 nM or less.

Alternatively, or additionally, the ability of the anti-ErbB2 antibody to block ErbB ligand-stimulated tyrosine phosphorylation of an ErbB receptor present in an ErbB hetero-oligomer may be assessed. For example, cells endogenously expressing the ErbB receptors or transfected to expressed them may be incubated with the antibody and then assayed for ErbB ligand-dependent tyrosine phosphorylation activity using an anti-phosphotyrosine monoclonal (which is optionally conjugated with a detectable label). The kinase receptor activation assay described in U.S. Pat. No. 5,766,863 is also available for determining ErbB receptor activation and blocking of that activity by an antibody.

In one embodiment, one may screen for an antibody which inhibits HRG stimulation of p180 tyrosine phosphorylation in MCF7 cells. For example, the MCF7 cells may be plated in 24-well plates and monoclonal antibodies to ErbB2 may be added to each well and incubated for 30 minutes at room temperature; then rHRGβ$1_{177-244}$ may be added to each well to a final concentration of 0.2 nM, and the incubation may be continued for 8 minutes. Media may be aspirated from each well, and reactions may be stopped by the addition of 100 μl of SDS sample buffer (5% SDS, 25 mM DTT, and 25 mM Tris-HCl, pH 6.8). Each sample (25 μl) may be electrophoresed on a 4-12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (at 1 μg/ml) immunoblots may be developed, and the intensity of the predominant reactive band at M_r~180,000 may be quantified by reflectance densitometry. The antibody selected will preferably significantly inhibit HRG stimulation of p180 tyrosine phosphorylation to about 0-35% of control in this assay. A dose-response curve

for inhibition of HRG stimulation of p180 tyrosine phosphorylation as determined by reflectance densitometry may be prepared and an IC_{50} for the antibody of interest may be calculated. In one embodiment, the antibody which blocks ligand activation of an ErbB receptor will have an IC_{50} for inhibiting HRG stimulation of p180 tyrosine phosphorylation in this assay of about 50 nM or less, more preferably 10 nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC_{50} for inhibiting HRG stimulation of p180 tyrosine phosphorylation in this assay may, for example, be about 100 nM or less, more preferably 50 nM or less.

One may also assess the growth inhibitory effects of the antibody on MDA-MB-175 cells, e.g, essentially as described in Schaefer et al. *Oncogene* 15:1385-1394 (1997). According to this assay, MDA-MB-175 cells may treated with an anti-ErbB2 monoclonal antibody (10 μg/mL) for 4 days and stained with crystal violet. Incubation with an anti-ErbB2 antibody may show a growth inhibitory effect on this cell line similar to that displayed by monoclonal antibody 2C4. In a further embodiment, exogenous HRG will not significantly reverse this inhibition. Preferably, the antibody will be able to inhibit cell proliferation of MDA-MB-175 cells to a greater extent than monoclonal antibody 4D5 (and optionally to a greater extent than monoclonal antibody 7F3), both in the presence and absence of exogenous HRG.

In one embodiment, the anti-ErbB2 antibody of interest may block heregulin dependent association of ErbB2 with ErbB3 in both MCF7 and SK-BR-3 cells as determined in a co-immunoprecipitation experiment substantially more effectively than monoclonal antibody 4D5, and preferably substantially more effectively than monoclonal antibody 7F3.

To screen for antibodies which bind to an epitope on ErbB2 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, or additionally, epitope mapping can be performed by methods known in the art (see, e.g. FIGS. **1**A and **1**B herein).

The results obtained in the cell-based assays described above can then be followed by testing in animal, e.g. murine, models, and human clinical trials. In particular, the inability or limited ability of an antibody to treat ErbB2 overexpressing tumors can be demonstrated in the transgenic mouse model disclosed in the present application as described in the Examples below.

B. Anti-ErbB Antibody-Maytansinoid Conjugates (Immunoconjugates)

Anti-ErbB antibody-maytansinoid conjugates are prepared by chemically linking an anti-ErbB antibody to a maytansinoid molecule without significantly diminishing the biological activity of either the antibody or the maytansinoid molecule. Maytansinoids are well known in the art and can be synthesized by known techniques or isolated from natural sources. Suitable maytansinoids are disclosed, for example, in U.S. Pat. No. 5,208,020 and in the other patents and non-patent publications referred to hereinabove. Preferred maytansinoids are maytansinol and maytansinol analogues modified in the aromatic ring or at other positions of the maytansinol molecule, such as various maytansinol esters.

There are many linking groups known in the art for making antibody-maytansinoid conjugates, including, for example, those disclosed in U.S. Pat. No. 5,208,020 (e.g., at column 7, lines 55-67 and at column 8, lines 1-14), or EP Patent 0 425 235 B1, and Chari et al. *Cancer Research* 52: 127-131 (1992). The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred. For example, for a compound as illustrated in FIG. **3**, "R" may be SH or may be SSR_1, where R_1 represents methyl, linear alkyl, branched alkyl, cyclic alkyl, simple or substituted aryl or heterocyclic.

In addition, there are many possible sites within the antibody molecule for linking maytansinoid to the antibody. For example, in one embodiment HERCEPTIN® can be linked to the maytansinoid at lysine 13 in the light chain, at lysine 32 in the heavy chain, at lysine 26 in both Fab fragments and at lysine 38 in the Fc fragment.

Conjugates of the antibody and maytansinoid may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl)hexanediamine), bis-diazoniumderivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Particularly preferred coupling agents include N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Carlsson et al., *Biochem. J.* 173:723-737 [1978]) and N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) to provide for a disulfide linkage.

The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with a hydroxyl group, and the C-20 position having a hydroxyl group. In a preferred embodiment, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

C. Pharmaceutical Formulations

Therapeutic formulations of the antibody-maytansinoid conjugates used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Preferred lyophilized anti-ErbB2

antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies or antibody-maytansinoid conjugates which bind to EGFR, ErbB2 (e.g. an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or additionally, the composition may further comprise a chemotherapeutic agent, cytotoxic agent, cytokine, growth inhibitory agent, anti-hormonal agent, and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

In one embodiment, the formulation comprises 5 mg/ml HERCEPTIN®-DM1, 100 mg/ml sucrose, 0.1% polysorbate 20 and 10 mM sodium succinate at pH 5.0.

D. Treatment with the Anti-ErbB2 Antibody-Maytansinoid Conjugates

It is contemplated that, according to the present invention, the anti-ErbB2 antibody-maytansinoid conjugates may be used to treat various diseases or disorders. Exemplary conditions or disorders include benign or malignant tumors; leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, stromal, blastocoelic, inflammatory, angiogenic and immunologic disorders.

Generally, the disease or disorder to be treated is cancer. Examples of cancer to be treated herein include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

The cancer will comprise ErbB-expressing cells, such that an anti-ErbB antibody herein is able to bind to the cancer, and will be typically characterized by overexpression of the ErbB receptor. In a preferred embodiment, the cancer comprises ErbB2-expressing cells, even more preferably, cells which are characterized by overexpression of the ErbB2 receptor. To determine ErbB, e.g. ErbB2 expression in the cancer, various diagnostic/prognostic assays are available. In one embodiment, ErbB2 overexpression may be analyzed by IHC, e.g. using the HERCEPTEST® (Dako). Parrafin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded a ErbB2 protein staining intensity criteria as follows:

Score 0

no staining is observed or membrane staining is observed in less than 10% of tumor cells.

Score 1+

a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells.

The cells are only stained in part of their membrane.

Score 2+

a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score 3+

a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells.

Those tumors with 0 or 1+ scores for ErbB2 overexpression assessment may be characterized as not overexpressing ErbB2, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing ErbB2.

Alternatively, or additionally, fluorescence in situ hybridization (FISH) assays such as the INFORM™ (sold by Ventana, Ariz.) or PATHVISION™ (Vysis, Ill.) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of ErbB2 overexpression in the tumor. In comparison with IHC assay, the FISH assay, which measures her2 gene amplification, seems to correlate better with response of patients to treatment with HERCEPTIN®, and is currently considered to be the preferred assay to identify patients likely to benefit from HERCEPTIN® treatment or treatment with the immunoconjugates of the present invention.

In one embodiment, the cancer will be one which expresses (and may overexpress) EGFR. Examples of cancers which may express/overexpress EGFR include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

Preferably, the immunoconjugates of the present invention and/or ErbB, e.g. ErbB2 or EGFR protein to which they are bound are internalized by the cell, resulting in increased therapeutic efficacy of the immunoconjugate in killing the cancer cell to which they bind. In a preferred embodiment, the

cytotoxic agent (maytansinoid) targets or interferes with nucleic acid in the cancer cell.

The treatment of the present invention targets ErbB overexpressing tumors that do not respond, or respond poorly, to treatment with an unconjugated anti-ErbB antibody. Such patients might have received prior treatment with an anti-ErbB antibody not conjugated to a maytansinoid moiety, where the prior treatment either did not result in significant improvement, or resulted in transient response. Prior treatment of any particular patient with an unconjugated anti-ErbB antibody is, however, not a prerequisite of identifying patients who are candidates for treatment in accordance with the present invention. An ordinary skilled physician can readily identify patients who are expected to benefit from treatment with the immunoconjugates of the present invention based on publicly available clinical data and his or her own experience. Treatment of mammals, and in particular human patients, with or without prior treatment with an (unconjugated) anti-ErbB antibody is specifically within the scope of the present invention.

The anti-ErbB antibody-maytansinoid conjugates are administered to a mammal, preferably to a human patient in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred.

Other therapeutic regimens may be combined with the administration of the anti-ErbB antibody-maytansinoid conjugates. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities.

In one preferred embodiment, the patient is treated with two or more different anti-ErbB antibodies, at least one of which is in the form of a maytansinoid conjugate. For example, the patient may be treated with a first anti-ErbB2 antibody-maytansinoid conjugate in which the antibody is growth inhibitory (e.g. HERCEPTIN®), and a second anti-ErbB2 antibody or antibody-immunoconjugate, e.g. an antibody-maytansinoid conjugate which blocks ligand activation of an ErbB receptor (e.g. 2C4 or a humanized and/or affinity matured variant thereof) or induces apoptosis of an ErbB2-overexpressing cell (e.g. 7C2, 7F3 or humanized variants thereof). In another embodiment, the treatment involves the administration of antibodies that specifically bind two or more different ErbB receptors, such as, for example, ErbB2 and EGFR receptors, where at least one of the anti-ErbB antibodies is administered as a maytansinoid conjugate. Preferably such combined therapy results in a synergistic therapeutic effect.

It may also be desirable to combine administration of the anti-ErbB antibody-maytansinoid conjugates, with administration of an antibody directed against another tumor-associated antigen, which is not member of the ErbB family of receptors. The other antibody in this case may, for example, bind to vascular endothelial growth factor (VEGF), and may be in the form of a maytansinoid conjugate, or another immunoconjugate.

In one embodiment, the treatment of the present invention involves the combined administration of an anti-ErbB2 antibody-maytansinoid conjugate (or conjugates) and one or more chemotherapeutic agents or growth inhibitory agents, including coadministration of cocktails of different chemotherapeutic agents. Preferred chemotherapeutic agents include taxanes (such as paclitaxel and doxetaxel) and/or anthracycline antibiotics. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992).

In a preferred embodiment, the treatment is initiated with an anti-ErbB antibody-maytansinoid conjugate, followed by maintenance treatment with an unconjugated or 'naked' anti-ErbB antibody. This strategy may eliminate or reduce tumor cells resistant to the naked antibody in the initial round because of the ability of the antibody-DM1 conjugate to effectively kill such tumor cells.

The antibody-maytansinoid conjugates may be combined with an anti-hormonal compound; e.g., an anti-estrogen compound such as tamoxifen; an anti-progesterone such as onapristone (see, EP 616 812); or an anti-androgen such as flutamide, in dosages known for such molecules. Where the cancer to be treated is hormone independent cancer, the patient may previously have been subjected to anti-hormonal therapy and, after the cancer becomes hormone independent, the anti-ErbB2 antibody (and optionally other agents as described herein) may be administered to the patient.

Sometimes, it may be beneficial to also coadminister a cardioprotectant (to prevent or reduce myocardial dysfunction associated with the therapy) or one or more cytokines to the patient. In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

Suitable dosages for any of the above coadministered agents are those presently used and may be lowered due to the combined action (synergy) of the agent and anti-ErbB2 antibody.

For the prevention or treatment of disease, the appropriate dosage of antibody-maytansinoid conjugates will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody-maytansinoid conjugate is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of antibody-maytansinoid conjugate is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. A preferred dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the anti-ErbB2 antibody-maytansinoid conjugate. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Based on the data disclosed herein, it is anticipated that one useful dosing protocol may entail approximately weekly (or less frequent) administration of the anti-ErbB antibody-maytansinoid conjugate where each dose of the conjugate is about 0.2-10 mg/kg, preferably about 1-3 mg/kg of the conjugate (e.g. where there are 1 to about 10, preferably about 3-4, maytansinoid molecules conjugated to each antibody mol-

ecule). From about 2-10, preferably about 4-6, dosages of the conjugate may be administered to the patient approximately every week.

In a preferred embodiment, the patients are treated initially with anti-ErbB-maytansinoid conjugate followed by therapy with unconjugated anti-ErbB antibody. Preferably, the anti-ErbB antibody in the conjugate and the unconjugated antibody are the same antibody. For example, treatment could be initiated with weekly injections of HERCEPTIN®-DM1 at about 0.5-5 mg/kg, preferably at about 1-3 mg/kg for 4-6 weeks, with the option of repeating this treatment. Patients can then be rolled over to conventional HERCEPTIN® therapy, which typically consists of treatment with a 4 mg/kg initial dose of HERCEPTIN®, followed by weekly treatment with a maintenance dose of 2 mg/kg. However, the 4 mg/kg initial dose may be omitted, with therapy going straight to the 2 mg/kg maintenance dose.

E. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-ErbB2 antibody-maytansinoid conjugate. In one embodiment the container is a 10 cc vial containing 10 mL of a solution comprising HERCEPTIN®-DM1.

The label or package insert indicates that the composition is used for treating the condition of choice, such as cancer. In one embodiment, the label or package inserts indicates that the composition comprising the antibody which binds ErbB2 can be used to treat cancer which expresses an ErbB receptor selected from the group consisting of epidermal growth factor receptor (EGFR), ErbB2, ErbB3 and ErbB4, preferably EGFR. In addition, the label or package insert may indicate that the patient to be treated is one having cancer characterized by excessive activation of an ErbB receptor selected from EGFR, ErbB2, ErbB3 or ErbB4. For example, the cancer may be one which overexpresses one of these receptors and/or which overexpresses an ErbB ligand (such as TGF-α). The label or package insert may also indicate that the composition can be used to treat cancer, wherein the cancer is not characterized by overexpression of the ErbB2 receptor. For example, whereas the present package insert for HERCEPTIN® indicates that the antibody is used to treat patients with metastatic breast cancer whose tumors overexpress the ErbB2 protein, the package insert herein may indicate that the antibody or composition is used to treat cancer that does not respond, or respond poorly, to treatment with HERCEPTIN®. In other embodiments, the package insert may indicate that the antibody-maytansinoid conjugate or composition can be used also to treat hormone independent cancer, prostate cancer, colon cancer or colorectal cancer.

Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises a maytansinoid conjugate of a first antibody which binds ErbB2 and inhibits growth of cancer cells which overexpress ErbB2; and (b) a second container with a composition contained therein, wherein the composition comprises a second antibody which binds ErbB2 and blocks ligand activation of an ErbB receptor, or a conjugate of this second antibody with a maytansinoid. The article of manufacture in this embodiment of the invention may further comprises a package insert indicating that the first and second compositions can be used to treat cancer. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

Further details of the invention are illustrated in the following non-limiting examples.

EXAMPLE 1

Production, Characterization and Humanization of Anti-ErbB2 Monoclonal Antibody 4D5

The murine monoclonal antibody 4D5 which specifically binds the extracellular domain of ErbB2 was produced as described in Fendly et al., *Cancer Research* 50:1550-1558 (1990). Briefly, NIH 3T3/HER2-3_{400} cells (expressing approximately 1×10^5 ErbB2 molecules/cell) produced as described in Hudziak et al *Proc. Natl. Acad. Sci.* (*USA*) 84:7158-7163 (1987) were harvested with phosphate buffered saline (PBS) containing 25 mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled ErbB2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified ErbB2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the ErbB2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653. Hybridoma supernatants were screened for ErbB2-binding by ELISA and radioimmunoprecipitation.

Epitope Mapping and Characterization

The ErbB2 epitope bound by monoclonal antibody 4D5 was determined by competitive binding analysis (Fendly et al. *Cancer Research* 50:1550-1558 (1990)). Cross-blocking studies were done by direct fluorescence on intact cells using the PANDEX™ Screen Machine to quantitate fluorescence. The monoclonal antibody was conjugated with fluorescein isothiocyanate (FITC), using established procedures (Wofsy et al. *Selected Methods in Cellular Immunology*, p. 287, Mishel and Schiigi (eds.) San Francisco: W.J. Freeman Co. (1980)). Confluent monolayers of NIH 3T3/HER2-3_{400} cells were trypsinized, washed once, and resuspended at 1.75×10^6 cell/ml in cold PBS containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3. A final concentration of 1% latex particles (IDC, Portland, Oreg.) was added to reduce clogging of the PANDEX™ plate membranes. Cells in suspension, 20 μl, and 20 μl of purified monoclonal antibodies (100 μg/ml to 0.1 μg/ml) were added to the PANDEX™ plate wells and incubated on ice for 30 minutes. A predetermined dilution of the FITC-labeled monoclonal antibody in 20 μl was added to each well, incubated for 30 minutes, washed, and the fluorescence was quantitated by the PANDEX™. Monoclonal antibodies were considered to share an epitope if each blocked binding of the other by 50% or greater in comparison to an irrelevant monoclonal antibody control. In this experiment, monoclonal antibody 4D5 was assigned epitope I (amino acid residues from about 529 to about 625, inclusive within the ErbB2 extracellular domain (see SEQ ID NO: 3).

The growth inhibitory characteristics of monoclonal antibody 4D5 were evaluated using the breast tumor cell line,

SK-BR-3 (see Hudziak et al. *Molec. Cell. Biol.* 9(3):1165-1172 (1989)). Briefly, SK-BR-3 cells were detached by using 0.25% (vol/vol) trypsin and suspended in complete medium at a density of 4×10^5 cells per ml. Aliquots of 100 μl (4×10^4 cells) were plated into 96-well microdilution plates, the cells were allowed to adhere, and 100 μl of media alone or media containing monoclonal antibody (final concentration 5 μg/ml) was then added. After 72 hours, plates were washed twice with PBS (pH 7.5), stained with crystal violet (0.5% in methanol), and analyzed for relative cell proliferation as described in Sugarman et al. *Science* 230:943-945 (1985). Monoclonal antibody 4D5 inhibited SK-BR-3 relative cell proliferation by about 56%.

Monoclonal antibody 4D5 was also evaluated for its ability to inhibit HRG-stimulated tyrosine phosphorylation of proteins in the M_r 180,000 range from whole-cell lysates of MCF7 cells (Lewis et al. *Cancer Research* 56:1457-1465 (1996)). MCF7 cells are reported to express all known ErbB receptors, but at relatively low levels. Since ErbB2, ErbB3, and ErbB4 have nearly identical molecular sizes, it is not possible to discern which protein is becoming tyrosine phosphorylated when whole-cell lysates are evaluated by Western blot analysis. However, these cells are ideal for HRG tyrosine phosphorylation assays because under the assay conditions used, in the absence of exogenously added HRG, they exhibit low to undetectable levels of tyrosine phosphorylation proteins in the M_r 180,000 range.

MCF7 cells were plated in 24-well plates and monoclonal antibodies to ErbB2 were added to each well and incubated for 30 minutes at room temperature; then $rHRG\beta1_{177-244}$ was added to each well to a final concentration of 0.2 nM, and the incubation was continued for 8 minutes. Media was carefully aspirated from each well, and reactions were stopped by the addition of 100 μl of SDS sample buffer (5% SDS, 25 mM DTT, and 25 mM Tris-HCl, pH 6.8). Each sample (25 μl) was electrophoresed on a 4-12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (4G10, from UBI, used at 1 μg/ml) immunoblots were developed, and the intensity of the predominant reactive band at M_r-180,000 was quantified by reflectance densitometry, as described previously (Holmes et al. *Science* 256:1205-1210 (1992); Sliwkowski et al. *J. Biol. Chem.* 269:14661-14665 (1994))

Monoclonal antibody 4D5 significantly inhibited the generation of a HRG-induced tyrosine phosphorylation signal at M_r 180,000. In the absence of HRG, but was unable to stimulate tyrosine phosphorylation of proteins in the M_r 180,000 range. Also, this antibody does not cross-react with EGFR (Fendly et al. *Cancer Research* 50:1550-1558 (1990)), ErbB3, or ErbB4. Monoclonal antibody 4D5 was able to block HRG stimulation of tyrosine phosphorylation by ~50%.

The growth inhibitory effect of monoclonal antibody 4D5 on MDA-MB-175 and SK-BR-3 cells in the presence or absence of exogenous rHRGβ1 was assessed (Schaefer et al. *Oncogene* 15:1385-1394 (1997)). ErbB2 levels in MDA-MB-175 cells are 4-6 times higher than the level found in normal breast epithelial cells and the ErbB2-ErbB4 receptor is constitutively tyrosine phosphorylated in MDA-MB-175 cells. Monoclonal antibody 4D5 was able to inhibit cell proliferation of MDA-MB-175 cells, both in the presence and absence of exogenous HRG. Inhibition of cell proliferation by 4D5 is dependent on the ErbB2 expression level (Lewis et al. *Cancer Immunol. Immunother.* 37:255-263 (1993)). A maximum inhibition of 66% in SK-BR-3 cells could be detected. However this effect could be overcome by exogenous HRG.

Humanization

The murine monoclonal antibody 4D5 was humanized, using a novel "gene conversion mutagenesis" strategy, as described in U.S. Pat. No. 5,821,337, the entire disclosure of which is hereby expressly incorporated by reference. The humanized monoclonal antibody 4D5 used in the following experiments is designated huMAb4D5-8. This antibody is of IgG1 isotype.

EXAMPLE 2

HERCEPTIN®-DM1 Conjugates

1. Purification of HERCEPTIN®

HERCEPTIN® (huMAb4D5-8, rhuMAb HER2, U.S. Pat. No. 5,821,337) (1 vial containing 440 mg antibody) was dissolved in 50 mL MES buffer (25 mM MES, 50 mM NaCl, pH 5.6). The sample was loaded on a cation exchange column (Sepharose S, 15 cm×1.7 cm) that had been equilibrated in the same buffer. The column was then washed with the same buffer (5 column volumes). HERCEPTIN® was eluted by raising the NaCl concentration of the buffer to 200 mM. Fractions containing the antibody were pooled, diluted to 10 mg/mL, and dialyzed into a buffer containing 50 mM potassium phosphate, 50 mM NaCl, 2 mM EDTA, pH 6.5.

2. Modification of HERCEPTIN® with SPP

The purified HERCEPTIN® antibody was modified with N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) to introduce dithiopyridyl groups. The antibody (376.0 mg, 8 mg/mL) in 44.7 mL of 50 mM potassium phosphate buffer (pH 6.5) containing NaCl (50 mM) and EDTA (1 mM) was treated with SPP (5.3 molar equivalents in 2.3 mL ethanol). After incubation for 90 minutes under argon at ambient temperature, the reaction mixture was gel filtered through a Sephadex G25 column equilibrated with 35 mM sodium citrate, 154 mM NaCl, 2 mM EDTA. Antibody containing fractions were pooled and assayed. The degree of modification of the antibody was determined as described above. Recovery of the modified antibody (HERCEPTIN®-SPP-Py) was 337 mg (89.7%) with 4.5 releasable 2-thiopyridine groups linked per antibody.

3. Conjugation of HERCEPTIN®-SPP-Py with DM1

The modified antibody (337.0 mg, 9.5 μmols of releasable 2-thiopyridine groups) was diluted with the above 35 mM sodium citrate buffer, pH 6.5, to a final concentration of 2.5 mg/mL. DM1 (1.7 equivalents, 16.1 μmols) in 3.0 mM dimethylacetamide (DMA, 3% v/v in the final reaction mixture) was then added to the antibody solution. The structure of DM1 is shown in FIG. **3**, where the nature of the "R" group is not critical and can be occupied, for example, by a variety of groups capable of forming a chemical bond with a linker. DM1 used in the present reaction was stored as an S—S form, which is more stable, and was reduced to the SH form for conjugation with the HERCEPTIN® antibody. The reaction proceeded at ambient temperature under argon for 20 hours. The structure of HERCEPTIN®-DM1 conjugates is illustrated in FIG. **4**.

The reaction was loaded on a Sephacryl S300 gel filtration column (5.0 cm×90.0 cm, 1.77 L) equilibrated with 35 mM sodium citrate, 154 mM NaCl, pH 6.5. The flow rate was 5.0 mL/min and 65 fractions (20.0 mL each) were collected. A major peak centered around fraction No. 47 (FIG. **5**). The major peak comprises monomeric HERCEPTIN®-DM1. Fractions 44-51 were pooled and assayed. The number of DM1 drug molecules linked per antibody molecule was determined by measuring the absorbance at 252 nm and 280 nm, and found to be 3.7 drug molecules per antibody molecule.

4. Anti-Proliferative Effect of HERCEPTIN®-DM1 Conjugate in vitro

SK-BR3 cells, which express 3+ level of HER2 on cell surface, were treated with HERCEPTIN®, HERCEPTIN®-DM1 conjugate, control mAb RITUXAN® or RITUXAN®-DM1 conjugates, and the effect of these treatments on cell proliferation was monitored. As shown in FIG. **6**, the extent of cell growth inhibition by treatment with HERCEPTIN®-DM1 was dramatically more pronounced than that with HERCEPTIN®, while the control RITUXAN® antibody did not inhibit cell growth. Although the RITUXAN®-DM1 did inhibit cell growth, it did so only at high concentrations. For example, the RITUXAN®-DM1 conjugate did not inhibit growth at concentration up to 1 μg/ml. In contrast, the HERCEPTIN®-DM1 conjugate was highly potent and significantly inhibited cell growth starting from 0.01 μg/ml and reaching a plateau at 0.1 μg/ml. The RITUXAN®-DM1 conjugate required 100 times higher concentration to achieve the same level of cell growth inhibition as HERCEPTIN®-DM1 conjugate. This is also reflected in a 100-fold difference in IC_{50} value, concentration required to inhibit cell growth by 50%, of the respective conjugates.

EXAMPLE 3

Transgenic Animals

In order to improve the clinical activity of HERCEPTIN®, a transgenic HER2 mouse model was developed in which novel HER2-directed therapies could be tested preclinically. Tumors arise readily in transgenic mice that express a mutationally activated form of neu, the rat homolog of HER2, but the HER2 that is overexpressed in breast cancers is not mutated and tumor formation is much less robust in transgenic mice that overexpress nonmutated HER2 (Webster et al., *Semin. Cancer Biol.* 5: 69-76 [1994]). To improve tumor formation with nonmutated HER2, a strategy was used to further enhance overexpression of nonmutated HER2 in a transgenic mouse.

Any promoter that promotes expression of HER2 in epithelial cells in the mouse mammary gland can be used in the disclosed constructs. Many of the milk protein genes are transcribed by promoter/enhancer elements that are specifically active in mammary glands. Milk protein genes include those genes encoding caseins (α-S_1 and β), β-lactoglobulin, α-lactalbumin, and whey acidic protein. The ovine β-lactoglobulin promoter is well characterized and widely used in the art (Whitelaw et al., *Biochem J.* 286: 31-39, [1992]). However, similar fragments of promoter DNA from other species are also suitable. A preferred promoter is the promoter derived from the Long Terminal Repeat (LTR) of the Mouse Mammary Tumor Virus (MMTV). A HER2 transgene construct of the present invention was generated using the MMTV LTR promoter.

To improve tumor formation with nonmutated HER2, we have made transgenic mice using a HER2 cDNA plasmid in which an upstream ATG was deleted in order to prevent initiation of translation at such upstream ATG codons, which would otherwise reduce the frequency of translation initiation from the downstream authentic initiation codon of HER2 (for example, see Child et al., *J. Biol. Chem.* 274: 24335-24341 [1999]). Additionally, a chimeric intron was added to the 5' end, which should also enhance the level of expression as reported earlier (Neuberger and Williams, *Nucleic Acids Res.* 16: 6713 [1988]; Buchman and Berg, *Mol. Cell. Biol.* 8: 4395 [1988]; Brinster et al., *Proc. Natl. Acad. Sci. USA* 85: 836 [1988]). The chimeric intron was derived from a Promega vector, pCI-neo mammalian expression vector (bp 890-1022). The cDNA 3'-end is flanked by human growth hormone exons 4 and 5, and polyadenylation sequences. Moreover, FVB mice were used because this strain is more susceptible to tumor development. The promoter from MMTV-LTR was used to ensure tissue-specific HER2 expression in the mammary gland. Animals were fed the AIN 76A diet in order to increase susceptibility to tumor formation (Rao et al., *Breast Cancer Res. and Treatment* 45: 149-158 [1997]). The nucleotide sequence of this transgene plasmid construct (SEQ ID NO: 1) is shown in FIG. **7**.

Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Taconic (Germantown, N.Y.). Many strains are suitable, but FVB female mice are preferred because of their higher susceptibility to tumor formation. FVB males were used for mating and vasectomized CD.1 studs were used to stimulate pseudopregnancy. Vasectomized mice can be obtained from any commercial supplier. Founders were bred with either FVB mice or with 129/BL6×FVB p53 heterozygous mice. The mice with heterozygosity at p53 allele were used to potentially increase tumor formation. However, this has proven unnecessary. Therefore, some F1 tumors are of mixed strain. Founder tumors are FVB only. Six founders were obtained with some developing tumors without having litters.

EXAMPLE 4

HER2 Transgenic Mouse as a Tumor Model to Evaluate HER2-Directed Therapies

Mammary gland biopsies of one founder transgenic mouse made as described in Example 3, showed 3+ expression of HER2, as determined by immunohistochemical staining, at about 2 months of age. The amount of HER2 extracellular domain (ECD) shed into serum was measured and found to be about 1.2 ng/ml (Huang et al., supra). This mouse subsequently developed a mammary tumor at 5 months of age, after bearing 4 litters. The tumor was surgically resected under aseptic conditions and minced into small pieces, 2 mm^3, which were then transplanted into the mammary fat pad of wild-type FVB female mice. As can be seen in FIG. **8**A, the amount of HER2 ECD shed into serum increased over time following transplant and was found to be directly proportional to the weight of the tumor that developed (FIG. **8**B). Tumors developed in 22 of 31 recipient mice, with a latency of 5 weeks. With subsequent passage, tumors developed with shorter latency and grew more rapidly, and tumor incidence increased to >95% of recipients. HER2 expression, as determined by immunohistochemical staining, was 3+ but heterogeneous in the primary tumor, but became uniformly 3+ after the first passage.

Treatment of tumor-bearing mice with HERCEPTIN® or 4D5, the murine antibody from which humanized HERCEPTIN® was derived, had only a modest effect on the growth of the transplanted tumors (FIG. **9**). HER2 expression was 3+ in tumors that grew during HERCEPTIN® or 4D5 therapy, indicating that there was no selection of HER2-negative tumors. Moreover, as can be seen in FIG. **10**, cy3-HERCEPTIN® was detected decorating tumor cells after injection into tumor-bearing mice, indicating that the lack of efficacy was not due to failure of the antibody to access the tumor. In addition, HER2 appears to be activated in the tumor cells, as evidenced by the binding of an anti-tyrosine phosphorylated HER2 antibody (FIG. **10**).

Based on the persistent expression of HER2 and the failure of this tumor model to respond to HERCEPTIN®, a novel

approach was tested, using HERCEPTIN® conjugated to maytansinoid DM1 as described in Example 3. FIG. **9** shows that the HERCEPTIN®-DM1 conjugate has dramatic anti-tumor activity in this model. RITUXAN®, an unrelated anti-CD20 monoclonal antibody, was used as a negative control for these studies. There was little response to HERCEPTIN® compared to the control antibody, RITUXAN®, but there was striking anti-tumor activity of the maytansinoid conjugate of HERCEPTIN®. As shown in FIG. **9**, all of the mice treated with HERCEPTIN®-maytansinoid showed striking shrinkage of their tumors, though none of the tumors disappeared. After approximately 4 weeks, tumors began to regrow. Five animals were sacrificed at this time. Their tumors were found to express HER2 at 3+ levels. Thus, there was no selection for HER2-negative tumors. Based on this observation, the remaining 3 mice were treated with HERCEPTIN®-maytansinoid for 5 consecutive days. The tumors again regressed in response to the treatment.

Despite its effectiveness at shrinking tumors and suppressing tumor growth, HERCEPTIN®-DM1 does not kill normal human cells, indicating a selective activity. The effect of various concentrations of HERCEPTIN®-DM1 on human mammary epithelial cells, human hepatocytes and human small airway epithelial cells was investigated. At antibody concentrations of up to 10 μg/ml, the conjugate had no significant effect on cell number.

The pharmacokinetics of HERCEPTIN®-DM1 was evaluated in mice and cynomolgous monkeys. It was determined that the HERCEPTIN®-DM1 pharmacokinetics was linear with respect to dose in both mouse and cynomolgous monkeys following i.v. administration. Dose response analysis in mice indicated that tumor suppression increased with increasing exposure to HERCEPTIN®-DM1 and reached maximum suppression following a dose of at least 18 mg/kg given once a week. The concentration-effect relationship will be further characterized in future studies.

FIG. **11** shows the results of treating mice with HER-2 over-expressing tumors with HERCEPTIN®-DM1 once a week for 5 weeks. Each dose contained 300 μg/kg of DM1 and 18 mg/kg of HERCEPTIN®. Another group of animals received HERCEPTIN® twice a week at a dose of 18 mg/kg body weight. Animals that did not receive any treatment were used as control. As shown in FIG. **11**, in animals that received HERCEPTIN®-DM1 tumor size was dramatically controlled and, perhaps more importantly, the tumor size was kept under control even after the therapy was stopped.

In yet another experiment, the amount of HERCEPTIN®-DM1 conjugate as well as the frequency of administration were varied while keeping the total number of doses at five. The evaluation was carried out in nude mice containing HER2 tumor transplants in mammary pads. As shown in FIG. **12**, some animals received HERCEPTIN®-DM1 at 300, 100, 30 or 10 μg DM1/kg twice a week for the total of 5 doses. Another group of animals received HERCEPTIN®-DM1 at 300 or 100 μg DM1/kg once a week for the total of 5 doses. For comparison, HERCEPTIN® alone was administered at 18 mg/kg twice a week or a control monoclonal antibody (E25 directed against CD20, also known as RITUXAN®) was administered twice a week. Consistent with earlier results in this HERCEPTIN® insensitive model, HERCEPTIN® failed to control the growth of mammary tumors. However, HERCEPTIN®-DM1 conjugate showed dramatic anti-tumor activity in a dose-dependent manner. For example, higher doses showed more potent anti-tumor activity than lower doses in both of the groups, i.e. those receiving treatments twice a week and once a week. Furthermore, twice a week treatment was more effective in keeping the tumor size smaller than once a week treatment schedule. Interestingly, however, there was no significant difference in the profile of tumor growth inhibition at 300 μg DM1/kg dose of HERCEPTIN®-DM1 conjugate whether administered twice a week or once a week. This suggests that at 300 μg DM1/kg dose, once a week schedule of treatment is effective in controlling tumor growth and that a higher frequency of administration is not necessary.

The results of a similar experiment are depicted in FIG. **13**. The results of three different dosing regimens of HERCEPTIN®-DM1 conjugate on tumor size are shown compared to matching dosing regimens of RITUXAN®-DM1. Tumor size was reduced and tumor growth was suppressed for at least about 50 days by treatment with 5 doses of HERCEPTIN®-DM1 at a concentration of 300 μg DM1/kg. This was true both when the HERCEPTIN®-DM1 was administered once a week and when it was administered twice a week. By contrast, administration of 5 doses of HERCEPTIN®-DM1 twice a week at a concentration of 100 μg DM1/kg did not shrink tumor size and suppressed tumor growth for somewhat less time. Matched RITUXAN®-DM1 treatment showed little effect on tumor size, indicating that the observed effect is specific to HERCEPTIN®-DM1. Similarly, unconjugated RITUXAN® (control MAb E25) showed no efficacy.

As can be seen clearly in FIG. **14**, a dose of HERCEPTIN®-DM1 (300 μg DM1/kg) once a week for five weeks caused tumors to shrink and prevented regrowth for more than 60 days. FIG. **14** also shows that a second round of treatment with HERCEPTIN®-DM1 after tumor regrowth begins is capable of shrinking tumors a second time. Neither unconjugated RITUXAN® (control MAb E25) nor RITUXAN®-DM1 had any obvious effect on tumor growth.

As shown here, the mammary tumor transplanted from HER2 transgenic mouse serves as a very useful model in evaluating various anti-tumor compounds as well as in establishing efficacy of different treatment regimens in preclinical studies. The model is particularly unique as it shares an important attribute of a fraction of human mammary tumors which are either completely or partially refractory to the treatment of HERCEPTIN® inspite of overexpression of HER2 at 2+ or 3+ levels. Thus, HER2 transgenic model described herein provides a valuable tool not only to study the mechanism of resistance to the anti-tumor activity of HERCEPTIN®, but also for screening compounds or modified HERCEPTIN®, including conjugates, for anti-tumor activity. The insight gained from such studies is likely to help in developing effective breast cancer therapies with broad coverage including HERCEPTIN®-resistant metastatic HER2 overexpressing breast carcinomas. The present HER2 transgenic model is particularly suited for preclinical research as well as drug development, and is a better alternative to in vitro studies carried out using breast carcinoma cell lines. It is an in vivo system with normal stroma and microenvironment of breast epithelium with cell-cell and cell-matrix interactions that are typical of a tissue. It also takes into account local factors and cytokines produced in the normal course of mammary gland development and attendant regulatory networks. It is also suitable to carry out pharmacokinetics studies of drug candidates at a smaller scale, which can then be scaled up for studies in non-human primate models. The results provide a sound basis for actual clinical trials in human subjects. As per the strategy described herein, the development of a HER2 transgenic model does not need to involve any in vitro selection, and requires minimal in vivo selection, the latter being limited to the extent of serial passage of the mammary tissue in order to reduce the duration of time needed to develop tumors and obtaining homogenous overexpression of

HER2 in mammary cells. Moreover, breeding of these mice provides a continuous source of tissues for various follow-up or supplemental studies. This is particularly significant since the availability of clinical samples of tissues from breast cancer patients is highly limited.

The HERCEPTIN®-DM1 conjugate as described herein was found to have superior activity over HERCEPTIN® in this HER2 transgenic model that mimics HERCEPTIN®-resistant metastatic HER2 overexpressing breast carcinomas. Approximately, 85% of breast cancer patients either do not respond to HERCEPTIN® therapy or repond poorly. The molecular basis of the resistance is not clearly understood. However, it is not due to a lower level of HER2 expression since these tumors also overexpress HER2 at 2+ or 3+ levels. Nevertheless, this significant proportion of breast cancer patients is not able to avail themselves of the powerful potential of HERECEPTIN® therapy. Preclinical studies carried out using the HERCEPTIN®-insensitive HER2 transgenic mouse model as outlined in this application shows a dramatic response of these tumors to HERCEPTIN®-DM1 conjugate as compared to HERCEPTIN®.

The HERCEPTIN®-DM1 conjugate was found to effectively control the growth of HERCEPTIN®-resistant tumors in a dose-dependent manner at a dosage of 100 μg DM1/kg and above. Administration of the tested conjugate at 300 μg DM1/kg once a week brought about a very impressive inhibition of tumor growth. Five such doses completely prevented the emergence of tumor for more than 60 days, and when the tumor did begin to reemerge, a second round of HERCEPTIN®-DM1 was able to control the growth. This is in contrast with a rapid growth of tumors in animals treated with control monoclonal antibody (RITUXAN®), maytansinoid conjugated control monoclonal antibody (RITUXAN®-DM1) or unconjugated HERCEPTIN®. Thus, the preclinical studies presented herein clearly show that the HERCEPTIN®-DM1 conjugate is able to elicit a dramatic anti-tumor response even in HERCEPTIN®-resistant breast tumors. The better objective response rate obtained with the HERCEPTIN®-DM1 conjugate will allow a higher fraction of breast cancer patients to benefit from this powerful therapy. The fact that the effect of HERCEPTIN®-DM1 is dose-dependent suggests that in an actual clinical setting, the strategy is likely to provide a considerable maneuver of doses to achieve the best anti-tumor activity. Moreover, the duration of anti-tumor response is significantly longer, permitting less frequent administration of the conjugate without compromising the therapeutic efficacy. The resultant cost-effectiveness and convenience is quite significant. Furthermore, the conjugate is likely to improve survival rate among the responders. For example, the median time to disease progression in HERCEPTIN® treated patients was only 3.1 months. With the superior therapeutic efficacy of HERCEPTIN®-DM1 as compared to HERCEPTIN®, survival rate is likely to be increased.

All references cited throught the specification, and the references cited therein, are hereby expressly incorporated by reference.

Deposit of Biological Material

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209, USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	Oct. 17, 1996
7F3	ATCC HB-12216	Oct. 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	Apr. 8, 1999

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of the deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the cultures to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.12 with particular reference to 886 OG 638).

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the constructs deposited, since the deposited embodiments are intended to illustrate only certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below, but should not be construed to limit the invention.

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Lys Gly Lys Ala Ser Leu Thr Val Asp Arg Ser Ser Arg Ile Val Tyr
65   70   75   80

Met Glu Leu Arg Ser Leu Thr Phe Glu Asp Thr Ala Val Tyr Tyr Cys
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Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
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Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr Asn Gln Arg Phe
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Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser Lys Asn Thr Leu Tyr
65   70   75   80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
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Thr Leu Val Thr Val Ser Ser
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Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
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Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
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Thr Leu Val Thr Val Ser Ser
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Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Val Gln Ala
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Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Thr
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Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser Arg Phe Ser Gly
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Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro

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Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly
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Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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<210> SEQ ID NO 9
<211> LENGTH: 1255
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

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Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His
        35                  40                  45

Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr
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Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
65                  70                  75                  80

Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu
                85                  90                  95

Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
            100                 105                 110

Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro
        115                 120                 125

Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser
    130                 135                 140

Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
145                 150                 155                 160

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
                165                 170                 175

Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys
            180                 185                 190

His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser
        195                 200                 205

Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys
    210                 215                 220

Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys
225                 230                 235                 240

Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu
                245                 250                 255

His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val
            260                 265                 270

Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg
        275                 280                 285

Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu
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Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln
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Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys
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Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp
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Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe
385                 390                 395                 400

Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
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Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg
            420                 425                 430

Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu
        435                 440                 445

Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly
    450                 455                 460

Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val
465                 470                 475                 480

Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu His Thr
                485                 490                 495

Ala Asn Arg Pro Glu Asp Glu Cys Val Gly Glu Gly Leu Ala Cys His
            500                 505                 510

Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr Gln Cys
        515                 520                 525

Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu Glu Cys
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Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg His Cys
545                 550                 555                 560

Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr Cys
                565                 570                 575

Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr Lys Asp
            580                 585                 590

Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro Asp Leu
        595                 600                 605

Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln
    610                 615                 620

Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp Asp Lys
625                 630                 635                 640

Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Val Ser
                645                 650                 655

Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val Phe Gly
            660                 665                 670

Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg
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Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly
    690                 695                 700

Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu
705                 710                 715                 720

Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys
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Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile
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Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu
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Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg
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Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu
785                 790                 795                 800

Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg
                805                 810                 815

Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly
            820                 825                 830

Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala
        835                 840                 845

Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe
    850                 855                 860

Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp
865                 870                 875                 880

Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg
                885                 890                 895

Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val
            900                 905                 910

Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala
        915                 920                 925

Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro
    930                 935                 940

Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met
945                 950                 955                 960

Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe
                965                 970                 975

Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu
            980                 985                 990

Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu
        995                 1000                1005

Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu
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Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly Ala Gly
1025                1030                1035                1040

Gly Met Val His His Arg His Arg Ser Ser Ser Thr Arg Ser Gly Gly
                1045                1050                1055

Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Ala Pro Arg
            1060                1065                1070

Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe Asp Gly
        1075                1080                1085

Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His
    1090                1095                1100

Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val Pro Leu
1105                1110                1115                1120

Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser Pro Gln
                1125                1130                1135

Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro
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Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu
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Arg Ala Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val
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Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln
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Gly Gly Ala Ala Pro Gln Pro His Pro Pro Pro Ala Phe Ser Pro Ala
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Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asp Pro Pro Glu Arg Gly Ala
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Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro Glu Tyr
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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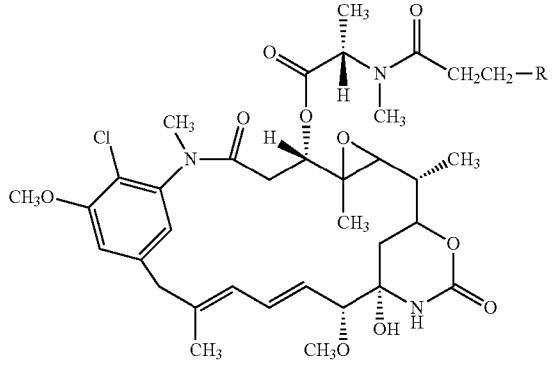
What is claimed is:

1. An immunoconjugate comprising an anti-ErbB2 antibody conjugated to a maytansinoid, wherein the antibody is huMAb4D5-8.

2. The immunoconjugate of claim **1**, wherein the maytansinoid is DM1 having the structure:



and wherein the antibody is chemically linked to the maytansinoid via a disulfide or thioether group at "R" shown in the structure.

3. The immunoconjugate of claim **1**, wherein the immunoconjugate comprises from 3 to 5 maytansinoid molecules per antibody molecule.

4. The immunoconjugate of claim **1**, wherein the antibody and the maytansinoid are conjugated by a chemical linker selected from N-succinimidyl-3-(2-pyridyldithio) propionate, N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) and succinimidyl-4-(N-maleimidomethyl)cyclohexanel-1-carboxylate.

5. A pharmaceutical composition comprising an immunoconjugate of any of claims **1** to **4**, and a pharmaceutically acceptable carrier.

6. The immunoconjugate of claim **4**, wherein the antibody and the maytansinoid are conjugated by succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate.

7. The immunoconjugate of claim **2**, wherein the antibody and the maytansinoid are conjugated by a chemical linker selected from N-succinimidyl-3-(2-pyridyldithio)propionate, N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) and succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate.

8. The immunoconjugate of claim **7**, wherein the antibody and the maytansinoid are conjugated by succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,337,856 B2
APPLICATION NO. : 11/949351
DATED : December 25, 2012
INVENTOR(S) : Walter Blättler and Ravi V. J. Chari

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Cover Page, Section (54) and at Column 1, lines 1-3, Title "METHODS OF TREATMENT USING ANTI-ERBB ANTIBODY-MAYTANSINOID CONJUGATES" should be -- huMab4D5 ANTI-ErbB2 ANTIBODY-MAYTANSINOID CONJUGATES --.

On the Cover Page, Section (73), "Assignee: Immunogen, Inc., Waltham, MA (US)", should be -- Assignee: ImmunoGen, Inc., Waltham, MA (US) --.

On Page 2, First Column, under FOREIGN PATENT DOCUMENTS, "JP 2003203395 1/2003", should be -- JP 2003503395 1/2003 --.

On Page 3, First Column, "Hudziak et al., "p185^{HER2} Monoclonal Antibody Has Antiproliferation Effects In Vitro and Sensitizes Human Brest Tumor Cells to Tumor Necrosis Factor", *Molecular and Cellular Biology*, vol. 9, No. 3, pp. 1165-1172 (1989).", should be -- Hudziak et al., "p185^{HER2} Monoclonal Antibody Has Antiproliferation Effects In Vitro and Sensitizes Human Breast Tumor Cells to Tumor Necrosis Factor", *Molecular and Cellular Biology*, vol. 9, No. 3, pp. 1165-1172 (1989). --.

Signed and Sealed this
Second Day of April, 2013

Teresa Stanek Rea
Acting Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

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On the Title Page, Item (73), "Assignee: Immunogen, Inc., Waltham, MA (US)", should be -- Assignee: ImmunoGen, Inc., Waltham, MA (US) --.

On Title Page 2, First Column, under FOREIGN PATENT DOCUMENTS, "JP 2003203395 1/2003", should be -- JP 2003503395 1/2003 --.

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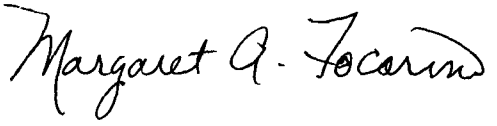
This certificate supersedes the Certificate of Correction issued April 2, 2013.

Signed and Sealed this
Tenth Day of December, 2013



Margaret A. Focarino
Commissioner for Patents of the United States Patent and Trademark Office

On Title Page 3, First Column, "Kraus et al., "Isolation and characterization of *ERBB3*, a third member of the *ERBB*/epidermal grwoth factor receptor family: Evidence for overexpression in a subset of human mammary tumors," *Proc. Natl. Acad. Sci. USA*, vol. 86, pp. 9193-9197 (1989).", should be -- Kraus et al., "Isolation and characterization of *ERBB3*, a third member of the *ERBB*/epidermal growth factor receptor family: Evidence for overexpression in a subset of human mammary tumors," *Proc. Natl. Acad. Sci. USA*, vol. 86, pp. 9193-9197 (1989). --.

On Title Page 3, First Column, "Lewis et al., "Differential responses of human tumor cell lines to anti-p185^{HER2} monoclonal antibodies", *Cancer Immunol. Immunother.*, vol. 37, pp. 225-263.", should be -- Lewis et al., "Differential responses of human tumor cell lines to anti-p185^{HER2} monoclonal antibodies", *Cancer Immunol. Immunother.*, vol. 37, pp. 225-263 (1993). --.