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POSSIBLE AMPLIFICATION OF THE ANTINEOPLASTIC ACTION OF A FOLIC ACID ANTAGONIST BY METHYLCOBALAMINE ANALOGS

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The stimulant action of cyanocobalamine on the growth of transplanted tumors of various animal species (Rous sarcoma of chickens, PW-2 fibrosarcoma, sarcoma 45, and SSR [spontaneous sarcoma of rats] of rats, and Guerin's carcinoma, sarcoma 180, and lymphosarcoma of mice) and the attenuation of the curative effect of certain antineoplastic drugs in combined application with vitamin B_{12}, noted in early studies, are caused by the active biosynthesis of its coenzymes in the animals' bodies. Assessment of the functional role of methylcobalamine, one of the cobalamine coenzymes in the growth processes of normal and tumor cells, has drawn the greatest attention.

Methylcobalamine is a coenzyme of the methionine synthetase reaction, a key link defining the synergy of the action of cobalamines and folic acid compounds in cell proliferation processes. The special importance of methylcobalamine for activation of this enzyme system has been noted by a study of the disrupted metabolism of cobalamines in human leukoses. The poor effectiveness of combined cytostatic therapy in certain forms of acute leukosis involving high methylcobalamine concentrations in the blood has confirmed the specificity of its action in the body (Myasishcheva *et al.*, 1969). The active role of methylcobalamine in cell proliferation processes of hematopoietic tissue in healthy animals has now been established. Methylcobalamine increases the number of cells synthesizing DNA, their mitotic activity, and the size of the proliferative pool in the spleen of mice (Golenko *et al.*). A significant increase in the frequency of hemoblastosis development in mice has been found upon combined administration of methylcobalamine with endogenous blastomogens. An important point in the mechanism of the stimulant action of cobalamines is their inductive effect on methionine synthetase activity. In cultures of normal mammalian cells and human tumor cells, methionine synthetase activity rises noticeably with an increase in cobalamine concentration in the culture medium (Mangum *et al.*; Kamely *et al.*). However, various types of tumor cells differ from normal cells in their ability, on exposure to cobalamines, to increase the biosynthesis of methionine needed for rapid growth (Halpern *et al.*; Chello and Bertino). The salvage pathway with the aid of cobalamine-dependent methionine synthetase, which increases the intracellular pool of tetrahydrofolic acid independently of the folate reductase system, is evidently the principal mechanism of development of methotrexate (MTX) resistance in leukosis cells (Myasishcheva; Sauer and Jaenicke).

In this connection, there is a real possibility of amplifying the antineoplastic effect of this metabolite by combined application

with cobalamine coenzyme antagonists. An understanding of the mechanism of cobalamines' action formed the basis for directed synthesis of methylcobalamine analogs and their testing as potential antineoplastic compounds.

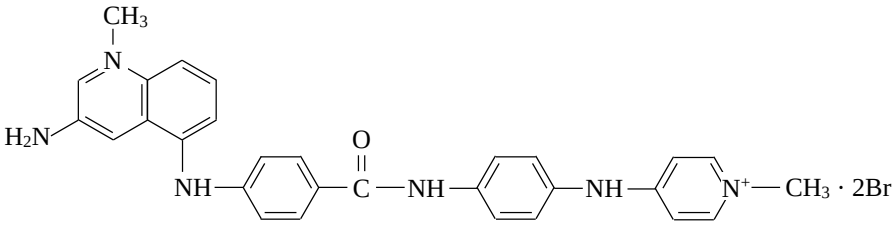
In chemotherapeutic experiments, we studied difluorochloromethylcobalamine and methylcobalamine chloropalladate, which had exhibited activity *in vitro* in suppressing bacterial cell growth and inhibiting DNA synthesis in human embryonic fibroblast culture (Myasishcheva *et al.*, 1977).

In developing a scheme of their combined action, we considered the basic aspects of the physiologic action of cobalamines in the body: monitoring of folic acid compounds' entry into cells and the formation of folate coenzymes, as well as the rate of cobalamine absorption by tumor cells (Burke *et al.*; Tisman and Herbert; Floodh and Ullberg). Accordingly, we could count on the selective action of the studied compounds and the possible reduction of cobalamine-dependent enzyme activity in the body. However, it was difficult to expect a significant effect from their isolated application. Therefore, we thought it important to assess the antineoplastic action of these compounds in the context of inhibition of dihydrofolate reductase activity using MTX.

Materials and Methods. The studies were conducted on mice of the $C_{57}BL$, CBA, BALB/c lines and BDF_1/$C_{57}BL$ × DBA(2) hybrids weighing 20–25 g, obtained from the USSR Academy of Medicine nursery. The antineoplastic activity of methylcobalamine analogs was studied on transplanted leukoses L-1210 and La and on solid tumors: mammary adenocarcinoma (Ca-755), cervical uterine cancer (CUC-5), and intestinal adenocarcinoma (ACATOL). As the principal object of study, we selected solid tumors, on which it is easier to detect the stimulant effect of methylcobalamine than on the L-1210 and La murine leukosis model with its high proliferative pool and very short animal lifespans.

Methylcobalamine (CH_3Cbl) and difluorochloromethylcobalamine (CF_2ClCbl) were prepared by the known method (Wood *et al.*, 1968), modified in the extraction section (Tachkova *et al.*). Methylcobalamine chloropalladate ($MetCbl{\cdot}PdCl_3$) was synthesized by Ye. G. Chauser's method. Methylcobalamine was administered intramuscularly at 10 mg/kg daily twice during the course of treatment 96 hours apart, and CF2ClCbl was administered daily subcutaneously in one 500 mg/kg dose or two 250 mg/kg doses daily for five days. The poorly soluble methylcobalamine chloropalladate was administered orally in a 2% starch suspension in a daily dose of 500 mg/kg over five days or twice 96 hours apart. The daily dose was administered all at once or at 250 mg twice daily. MTX from Lederle was used at 10 mg/kg intraabdominally at 96-hour intervals.

Our research studied the activity of cobalamine derivatives both in combined application with MTX and with a quinoline derivative (NSC-170319):



We obtained the drug from the U.S. National Cancer Institute in accordance with a U.S.–USSR agreement on cooperation in the area of tumor chemotherapy. According to the description provided by the American scientists, the drug is a methionine synthetase inhibitor (Carter *et al.*). The quinoline derivative was administered intraabdominally at 5 mg/kg daily or at 96-hour intervals, which corresponds to half the maximum tolerable dosage for the conditions. Treatment was begun 48 h after transplantation of the tumor. The results of the exposure were assessed 24 h after the end of the course of treatment and at various times throughout the animals' lives. Efficacy was measured by the percentage retardation of tumor growth, calculated by the conventional volume, and by the increase in the animals' lifespan. In each test, control and experimental groups were created so that their numbers would afford statistically significant minimum calculated percentage retardations

of tumor growth (60%) and increased mouse lifespans (25%). In accordance with these requirements, the test groups consisted of 6–10 mice, and the control groups consisted of 6–10 animals, depending on the tumor strain used.

Results and Discussion. Our research has revealed for the first time the stimulant effect of methylcobalamine on the growth of the transplanted Ca-755 and ACATOL tumors, and to a lesser extent on the growth of CUC-5 (Table 1). The greatest tumor growth rate under exposure to methylcobalamine was observed when Ca-755 was transplanted to BDF_1 hybrid mice, compared to the growth of the same tumor in purebred $C_{57}BL$ mice. The reproduction of tumor cells was stimulated during the period of methylcobalamine administration; the greatest difference in tumor size in the experimental and control groups was found immediately after discontinuation of the drug. At later times, tumor growth in mice that had received methylcobalamine slowed. When ACATOL was transplanted to mice of different genders, the tumor growth rate on exposure to methylcobalamine differed. The drug's stimulant action was considerably more marked in males (see Table 1).

As should be expected, the isolated action of methylcobalamine analogs retarded the growth of transplanted Ca-755 and CUC-5 tumors to a lesser extent, and only immediately after drug administration (Table 2).

Our comparative assessment revealed the greatest inhibitive activity with the use of methylcobalamine chloropalladate. The efficacy of Ca-755 growth retardation was more marked in BDF_1 hybrid mice compared to $C_{57}BL$ mice. As we have stated, it was also in BDF_1 mice that the stimulant action of methylcobalamine was significantly more marked. In this series of tests, the lifespans of BDF_1 mice with mammary adenocarcinoma increased 50% when they were exposed to CF_2ClCbl and methylcobalamine chloropalladate (see Table 2). At the same time, the administration of methylcobalamine derivatives produced no ACATOL growth retardation effect. We noted a large difference in the action of cobalamine derivatives on tumors depending on the application regime (see Table 2). Evidently, when a single large dose (500 mg/kg) is administered, the drugs can dissociate, with subsequent formation of an active form that stimulates tumor growth.

In accordance with our conjecture, when methylcobalamine analogs were combined with MTX, we observed an amplification of their action on the tumor (Ca-755, CUC-5; Table 3). The increased antineoplastic effect resulting from combined exposure was manifested immediately after the drug course and especially in the subsequent period: while the effect of MTX alone was absent, a fairly high percentage retardation of tumor growth remained.

Table 1. Effect of Methylcobalamine on Growth of Some Transplanted Tumors

Tumor	Drug Dosage, μg/kg	Days Drug Given after Tumor Transplant	Tumor Growth after Drug Administration, % of Control		
			1 day	7 days	14 days
Ca-755 $C_{57}BL$	10	2nd and 6th	+74	+21	+23
BDF_1	10	2nd and 6th	+180	+65	+10
ACATOL:					
females	10	2nd and 6th	+20	+23	+31
males	10	2nd and 6th	+126	+37	+33

Note: Here and in Tables 2–6, the plus sign denotes stimulation of tumor growth.

Table 2. Antineoplastic Action of Methylcobalamine Analogs

Tumor	Drug	Drug Dosage, μg/kg	Days Drug Given after Tumor Transplant	Tumor Growth Retardation, % of Control			Increase in Mouse Lifespan, % of Control
				1 day	7 days	16 days	
Ca-755	Chlorodifluoro-	250+250	2nd to 6th	30	+8		54
	methylcobalamine	250+250	2nd to 6th	43	38	0	16
CUC-5	(CF_2ClCbl)	250+250	2nd to 6th	0	0	0	0
ACATOL							
Ca-755	Combination of	250+250	2nd to 6th	90	59		50
(BDF_1)	trichloromethyl-	500	2nd to 6th	13	16	20	
	cobalamine with	250+250	2nd to 6th	80	23	0	10
CUC-5	$MetCbl \cdot PdCl_3$	500	2nd to 6th	+130	+15	+18	0
ACATOL		250+250	2nd to 6th	0	0	0	0

To understand the possible mechanisms of action of methylcobalamine analogs in the animals' bodies, we performed a comparative analysis of the growth of the same tumor strains under isolated exposure to a methionine synthetase inhibitor (quinoline derivative) and its combined action with MTX. The retardation of Ca-755, CUC-5, and ACATOL growth increased depending on the concentration of the drug. The drug was most effective against Ca-755. When the dosage was increased from 5 to 15 mg/kg, retardation of tumor growth increased to 40 and 96%, respectively. However, with increasing dosage, the drug's toxicity also increased noticeably. For example, with the L-1210 and La leukosis strains, the most optimal dosage according to our data was 10 mg/kg, at which the animals' lifespans increased three- to four-fold. At lower dosages, the drug's effect on mice with leukoses was substantially lower. For solid tumors, our studies revealed no significant increase in mouse lifespans. With combined administration of the drug with MTX, even at low dosages (5 mg/kg), we observed an additive effect, which was confirmed by the increased retardation of tumor growth (Table 4). With delayed treatment (on the eighth day after the tumor transplant) and daily administration of the drugs over five days (5 mg/kg of the quinoline derivative; 2 mg/kg of MTX), the results were even more demonstrative (Ca-755), but with the additive effect came general toxicity (Table 5).

An increase in tumor growth retardation and the animals' lifespan was noted with combined exposure to methylcobalamine chloropalladate and the quinoline derivative (NSC-176319, Table 6). Given the amplified action of MTX when used in combination with methylcobalamine analogs and a methionine synthetase inhibitor, we performed combination treatment of mice with Ca-755 using all three inhibitors: MTX, the quinoline derivative, and the most active cobalamine coenzyme analog, methylcobalamine chloropalladate (see Table 6).

The combined use of methionine synthetase inhibitors and dihydrofolate reductase resulted in a significant amplification of antineoplastic action, especially long after the end of treatment. Under these conditions, the retardation of tumor growth was 85% two weeks after discontinuation of the drugs, while at these times in the mouse groups that received each of the studied compounds in isolation or in two-drug combinations, the suppression of

Table 3. Antineoplastic Action of MTX and Methylcobalamine Analogs

Tumor	Drug	Drug Dosage, mg/kg	Days Drug Given after Transplant	Tumor Growth Retardation, % of Control				
				1 day	5 days	7 days	10 days	1[illegible] da[illegible]
Ca-755 ($C_{57}BL$)	MTX	10	2nd and 6th	75		10	+32	
	MetCbl·$PdCl_3$	250+250	2nd and 6th	58		20	14	
	MTX+MetCbl·$PdCl_3$	10+250+250 (given simultaneously)	2nd to 6th	97		75	0	
CUC-5 (CBA)	MTX	10	2nd and 6th	90		48		4
	MetCbl·$PdCl_3$	500	2nd and 6th	+220		+100		+8
	MTX+MetCbl·$PdCl_3$	10+500 (given simultaneously)	2nd and 6th	97		65		4
Ca-755 (hybrids)	MTX	10	2nd and 6th	87	81	45	67	
	CF_3ClCbl	500	2nd and 6th	+67	+5	+21	5	
	MTX+CF_2ClCbl	10+500 (CF_2ClCbl, given 3 h before MTX)	2nd and 6th	97	99	74	67	

Table 4. Action of Combined MTX and NSC-176319 on Mouse Tumors

Tumor	Drug	Drug Dosage, mg/kg	Days Drug Given after Tumor Transplant	Tumor Growth Retardatio		
				1 day	5 days	7–8 days
Ca-755 (BDF_1)	MTX	5	2nd and 6th	46	9	19
	NSC-176319	5	2nd and 6th	18	8	41
	MTX+NSC-176319	5+5 (given simultaneously)	2nd and 6th	81	62	66
CUC-5 (CBA)	MTX	10	2nd and 6th	69		74
	NSC-176319	10	2nd and 6th	20		55
	MTX+NSC-176319	10+10 (given simultaneously)	2nd and 6th	88		84
	MTX	10	2nd and 6th	45	53	44
ACATOL (BALB/c)	NSC-176319	5	2nd and 6th	12	27	30
	MTX+NSC-176319	10+5 (MTX given 20 min after NSC-176319)	2nd and 6th	65	43	40

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