Discovery of JANUVIA™ (Sitagliptin), a Selective Dipeptidyl Peptidase IV Inhibitor for the Treatment of Type2 Diabetes

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Abstract: The emergence of glucagon-like peptide 1 (GLP-1) as a well validated approach to the treatment of type 2 diabetes and preclinical validation of dipeptidyl peptidase IV (DPP-4) inhibition as an alternate, oral approach to GLP-1 therapy prompted the initiation of a DPP-4 inhibitor program at Merck in 1999. DPP-4 inhibitors *threo*- and *allo*-isoleucyl thiazolidide were in-licensed to jump start the program; however, development was discontinued due to profound toxicity in rat and dog safety studies. The observation that both compounds inhibit the related proline peptidases DPP8 and DPP9 led to the hypothesis that inhibition of DPP8 and/or DPP9 could evoke severe toxicities in preclinical species. Indeed, the observed toxicities were recapitulated with a selective dual DPP8/9 inhibitor but not with an inhibitor selective for DPP-4. Thus, medicinal chemistry efforts focused on identifying a highly selective DPP-4 inibitor for clinical development. Initial work in an α-amino acid series related to isoleucyl thiazolidide was discontinued due to lack of selectivity; however, SAR studies on two screening leads led to the identification of a highly selective β-amino acid piperazine series. In an effort to stabilize the piperazine moiety, which was extensively metabolized *in vivo*, a series of bicyclic derivatives were prepared, culminating in the identification of a potent and selective triazolopiperazine series. Unlike their monocyclic counterparts, these analogs typically showed excellent pharmacokinetic properties in preclinical species. Optimization of this series led to the discovery of JANUVIA™ (sitagliptin), a highly selective DPP-4 inhibitor for the treatment of type 2 diabetes.

INTRODUCTION

The pathogenesis of type 2 diabetes (T2DM) involves a set of three primary defects: insulin resistance, β cell dysfunction, and hepatic glucose overproduction. These defects are the principal targets of both current and future therapy. Currently available classes of oral antihyperglycemic agents include PPARγ agonists, sulfonyureas/meglitinides, and biguanides. These agents are used either in monotherapy or, increasingly, in combinations to lower glucose levels. Despite the availability of a range of agents for T2DM, many diabetic patients fail to achieve or to maintain glycemic targets. In addition, current therapies have limited durability and/or are associated with significant side effects (GI intolerance, hypoglycemia, weight gain, lactic acidosis and edema). Thus, there remain critical unmet medical needs in the treatment of this disorder. With an increasing understanding of the molecular pathways involved in glucose control, a range of new potential targets have emerged for treatment of the key areas of pathogenesis. In particular, there has been increased emphasis on new therapies that increase the circulating concentrations of insulin in a glucose dependent manner, most notably, glucagon-like peptide 1 (GLP-1) analogs [1] and dipeptidyl peptidase 4 (DPP-4) inhibitors [2]. In this review we describe Merck's DPP-4 inhibitor program, which was initiated in 1999 and culminated with the discovery of JANUVIA™ (sitagliptin), a potent and highly selective inhibitor of DPP-4 that shows excellent promise for the treatment of T2DM.

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TARGET SELECTION

Over the last decade, GLP-1 receptor agonism has emerged as one of the best validated approaches for the treatment of T2DM. For example, in 1997 it was reported that continuous infusion of GLP-1 to diabetic humans resulted in normalization of both postprandial and fasting glucose [3]. More recently, sub-chronic (6 wk) continuous infusion of GLP-1 was shown to result in profound and significant decreases in fasting plasma glucose (14.1 to 10.1 mM) and HbA1c (9.2 to 7.9 %) [4]. It is generally accepted that the key mechanisms responsible for glucose lowering by GLP-1 receptor agonism are: (i) stimulation of glucose-dependent insulin biosynthesis and secretion, (ii) glucose-dependent inhibition of glucagon release, and (iii) delayed gastric emptying.

Also in the 1990s, it became increasingly clear that GLP1 was very tightly regulated *in vivo*, specifically via proteolysis at the *N*-terminus to produce an inactive peptide, and that the key enzyme responsible for this inactivation was DPP-4, a proline specific dipeptidyl aminopeptidase [5,6]. These findings resulted in the initiation of programs at several companies to identify DPP-4 resistant GLP-1 analogs, and also led to the testing of DPP-4 inhibitors in animal models of diabetes, where increased levels of GLP-1, enhanced insulin secretion, and improved glucose tolerance were observed [7-9].

The human validation of GLP-1, together with preclinical validation of DPP-4 inhibition as an alternate oral approach to GLP-1, prompted Merck to initiate a project on this enzyme in 1999. Our enthusiasm for this mechanism was based primarily on the view that this approach would have at least three potential advantages over currently available agents. First, because GLP-1 stimulates insulin release in a

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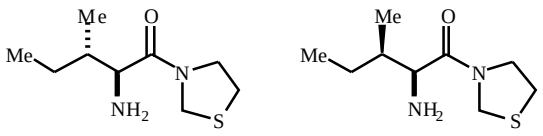
strictly glucose-dependent manner, little or no risk of hypoglycemia was anticipated. Second, no weight gain was expected with DPP-4 inhibitors. Finally, rodent studies with GLP-1 analogs had demonstrated a role for this peptide in the regulation of β-cell mass [10]; if these findings translated to the clinic, there was the potential that DPP-4 inhibitors could have long-term beneficial effects on β-cell function.

At the onset of this program, there were also several concerns regarding potential safety issues for this class. DPP-4 is a type II membrane bound cell surface protein that is ubiquitously expressed, and like many other cell surface molecules, DPP-4 had been implicated in a wide range of biological functions. Two potential issues were of most concern: first, DPP-4 is identical to the T cell activation marker CD26, and data in model systems suggested a potential co-stimulatory role for this enzyme in T cell activation [11]. Moreover, there were reports that some DPP-4 inhibitors (Lys [Z(NO_2)] pyrrolidide and related compounds) had several effects on immune cells, including inhibition of proliferation [12]. Second, DPP-4 had been shown to cleave a number of immunoregulatory, endocrine, and neurological peptides *in vitro* [13]. While some comfort was later provided by the report that DPP-4 deficient mice develop normally, and are healthy [14], a finding that we subsequently confirmed [15], we were acutely aware of the potential for mechanism-based toxicities, and, when possible, exploited opportunities to address these issues as the medicinal chemistry program progressed, as described below.

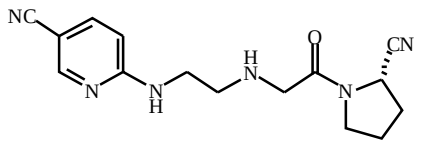
PROBIODRUG LICENSING EXPERIENCE

When we initiated our internal screening and medicinal chemistry program, two compounds were already advancing through human clinical trials, Probiodrug's isoleucyl thiazolidide (**1**) and NVP-DPP728 (**3**) from Novartis (Fig. **1**) [16,17]. Thus, in order to "jump start" our internal program, in late 2000 we elected to in-license L-*threo*-isoleucyl thiazolidide (P32/98) and its *allo* stereoisomer (L-*allo*-isoleucyl thiazolidide, **2**). In single dose pharmacodynamic studies, P32/98 had been shown to be well tolerated, increased active GLP-1, and reduced glycemic excursion following food or glucose intake in normal volunteers [18]. In addition, Probiodrug reported enhanced insulin secretion and improved glucose tolerance in single dose studies in a small number of diabetic patients [19].



P32/98 (**1**)

allo-Ile-thiazolidide (**2**)



NVP-DPP728 (**3**)

Fig (1). Early DPP-4 inhibitors.

Probiodrug had evaluated the safety of L-*threo*-isoleucyl thiazolidide in 4-week toxicity studies in rats and dogs [20]. In rats, toxicities were limited to the presence of lung histiocytosis and thrombocytopenia at relatively high doses (77.5 and 698 mg/kg, respectively). In dogs, acute central nervous system (CNS) toxicities, characterized by ataxia, seizures, convulsions, and tremor, were observed at 75 mg/kg, and bloody diarrhea was also observed at 225 mg/kg upon acute dosing. No additional toxicities were noted in these 4 week studies. However, in subsequent chronic toxicity studies at MRL, upon 5-6 weeks of treatment with this compound in dogs, mortality and profound toxicities occurred at doses ≥ 25 mg/kg/day. These toxicities included anemia, thrombocytopenia, splenomegaly, and multiple organ pathology mainly affecting the lymphoid system and gastrointestinal tract.

As noted above, we also in-licensed the *allo* isomer of isoleucyl thiazolidide, which, when compared to the *threo* isomer, has virtually identical affinity to DPP-4, similar pharmacokinetic and metabolic profiles, and similar *in vivo* efficacy in an oral glucose tolerance test in diet induced obese mice [20]. In parallel with the chronic toxicity studies with L-*threo*-isoleucyl thiazolidide, this compound was evaluated in an acute tolerability study in dogs and in 4-week toxicity studies in rats. As with the *threo* isomer, bloody diarrhea was observed in dogs, but the *allo* isomer was > 10-fold more toxic when compared on either a dose level or plasma exposure basis. In the rat studies, lung histiocytosis and thrombocytopenia were observed as had been seen with the *threo* compound, with the *allo* compound toxic at > 10-fold lower dosage. In addition, the other profound toxicities that were observed in dogs with the *threo* compound (e.g., anemia, splenomegaly, and mortality with multiple organ pathology) were observed with the *allo* compound in rats. As a result of these findings, development of both compounds was discontinued in early 2001.

DPP8/9 TOXICITY STUDIES

The toxicities observed with the *threo* and *allo* compounds deepened our concern about the potential safety of this mechanism. However, the finding that the *allo* isomer was approximately 10-fold more toxic in rats and dogs, despite having comparable pharmacodynamic activity and pharmacokinetics in both species, suggested that these toxicities were likely not due to DPP-4 inhibition, but instead were potentially due to off-target activity. In this regard, subsequent to the initiation of our program, it had become increasingly clear that DPP-4 was a member of larger family of 'DPP-4 activity- and/or structure-homologues' (DASH) proteins, enzymes that are unified by their common post-proline cleaving serine dipeptidyl peptidase mechanism [21]. Enzymes that had recently been described included quiescent cell proline dipeptidase (QPP) (aka DPP7) [22], DPP8 [23], DPP9 [24], and fibroblast activation protein (FAP) [25]. As the functions of these enzymes were unknown, determining the selectivity of our inhibitors was a key element of our medicinal chemistry program, and thus counterscreens for these enzymes were developed.

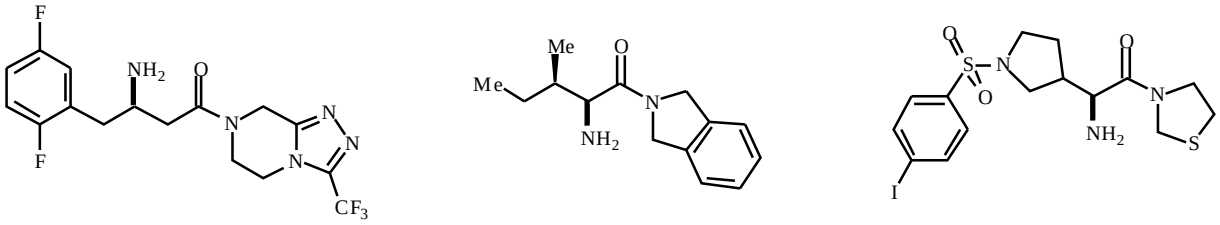
The selectivity of the *allo* and *threo* compounds was determined in the DASH family counterscreens, as well as in

an in-house panel of other proteases and by MDS Pharma Services (PanLabs) in a panel of 170 receptor and enzyme assays. No significant activity (IC_{50} < 100 μM) was observed in any of the in-house protease and PanLabs assays, with the exception of the sigma σ_1 receptor for the *allo* compound (K_i = 42 μM). However, for DPP-4 related dipeptidyl peptidases, inhibition was not only observed for DPP-4, but also for the closely related dipeptidyl peptidases, QPP, DPP8, and DPP9. Both the *allo* and *threo* isomers showed comparable QPP inhibition activity (IC_{50} = 18 μM and 14 μM, respectively); however, the potency for inhibition of DPP8 and DPP9 differed by 5- to 10-fold, with the *allo* isomer being more potent in each case (220 nM vs. 2200 nM for DPP8 and 320 nM vs. 1600 nM for DPP9) [20]. Since these differences in inhibition of DPP8/DPP9 were consistent with the observed differences in dose necessary to produce toxicity, we hypothesized that inhibition of DPP8 and/or DPP9 was responsible for the observed toxicities of both compounds in preclinical species.

To obtain evidence that DPP8/9 inhibition was responsible for the toxicities observed with the *allo* and *threo* isomers, DPP-4, QPP, and DPP8/9 selective compounds **4**, **5**, and **6** (Fig. **2**), respectively, were identified and evaluated in 2 week rat toxicity studies and in acute dog tolerability studies [20]. The results from these studies showed a remarkable similarity between the effects produced by the DPP8/DPP9 selective inhibitor and the *allo* compound (Table **1**). In rats, the DPP8/9 inhibitor produced alopecia, thrombocytopenia, reticulocytopenia, enlarged spleen, multi-organ histopathological changes, and mortality. In dogs, the DPP8/9 inhibitor produced gastrointestinal toxicity. The QPP inhibitor produced reticulocytopenia in rats only, and no toxicities were noted in either species for the selective DPP-4 inhibitor. These results provided compelling evidence that inhibition of DPP8/9, but not selective DPP-4 inhibition, is associated with multi-organ toxicities in preclinical species.

There were two major reasons that we had a high level of confidence in this conclusion. First, at least two structurally distinct compounds that inhibit DPP8/9 showed remarkably similar toxicities in rats and dogs. As noted above, the DPP8/9 inhibitor is highly selective over all other proline specific enzymes, and inhibition of the *allo* compound is limited to DPP-4, DPP8/9, and weak inhibition of QPP. We also showed that the DPP8/9 inhibitors produce similar toxicities in DPP-4-deficient mice and wild type mice, establishing that the observed toxicities were not due to inhibition of DPP-4 [20]. Second, the degree of toxicity observed with the *allo* and *threo* compounds correlated with their affinity for DPP8/9.

With the finding that DPP8/9 could produce a variety of toxicities *in vivo*, we hypothesized that some of immune effects that had been observed with Lys[Z(NO_2)]–pyrrolidide and related compounds [13] could instead be due to



4, DPP-4 selective
DPP-4 IC_{50} = 27 nM

5, DPP8/9 selective
DPP8 IC_{50} = 38 nM
DPP9 IC_{50} = 55 nM

6, QPP selective
QPP IC_{50} = 19 nM

Fig. (2). DPP-4, DPP8/9 and QPP selective inhibitors used in comparative toxicity studies.

Table 1. Comparative Toxicity Studies in Rats (2 Weeks of Treatment at Doses of 10, 30, 100 mg/kg/Day) and Dogs (Single Dose, 10 mg/kg PO) with Selective Inhibitors 4, 5, and 6. A Check (√) Indicates the Toxicity was Observed. Historical Data from *threo* and *allo* Isoleucyl Thiazolidide Safety Studies are Shown for Comparison

Species	Toxicity	DPP-4 Selective (4)	DPP8/9 Selective (5)	QPP Selective (6)	*threo*-Ile thia (1)	*allo*-Ile thia (2)
Rat	Alopecia		√			√
	Thrombocytopenia		√		√	√
	Anemia					√
	Reticulocytopenia		√	√	n.d.[a]	n.d.
	Splenomegaly		√			√
	Mortality		√			√
Dog	Bloody diarrhea		√		√	√

[a]not determined

inhibition of DPP-8/9, and we proceeded to assess the selectivity of these compounds. We found that they have greater intrinsic potency against DPP8 and DPP9 than DPP-4 [20]. Moreover, we discovered that the DPP8/9 selective inhibitor, but not the selective DPP-4 inhibitor, attenuated proliferation and IL-2 release in human *in vitro* models of T cell activation [20]. These results strongly suggest that proteolytic activity is not required for the putative co-stimulatory function of DPP-4/CD26, and that immunological effects previously observed with several DPP-4 inhibitors compounds in preclinical models were likely due to off-target inhibition of DPP8/9. This result provided a greater level of confidence that inhibition of DPP-4 would not result in compromised immune function.

While the significance to human safety is unknown, the finding that DPP8/9 inhibition produces profound toxicity in preclinical species, and is also likely responsible for effects on immune function that have been previously attributed to DPP-4, prompted us to refocus our program on the discovery of highly selective DPP-4 inhibitors.

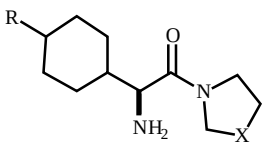
INITIAL MEDICINAL CHEMISTRY EFFORTS: CYCLOHEXYLGLYCINE LEAD

We initiated our medicinal chemistry program prior to the discovery of the preclinical toxicity associated with inhibition of DPP8/9. Thus, we focused initially on identifying a "Best in Class" compound by improving upon the potency of isoleucyl thiazolidide (IC_{50} = 420 nM in our hands) and the short half-life of NVP-DPP728, which we found to be only ~15 min in rats. We thought the latter issue might be due to chemical instability. This compound, like many of the most potent DPP-4 inhibitors, contained a reactive electrophile (a nitrile in this case) that was believed, and a close analog later shown, to form a covalent bond with the active site serine [26]. This electrophile is six atoms away from an amine, perfectly set up to cyclize. In order to improve upon the half-life, we chose from the onset to focus on structures which lacked this electrophile, even though we knew these structures had been generally shown to be less potent.

While we were waiting for results from our internal screening efforts, we initiated SAR studies based on the known α-amino acid derived inhibitors. The most potent inhibitor reported in the literature that did not contain an electrophile was cyclohexylglycyl thiazolidide (**7**, Table **1**), discovered by chemists at Ferring [27]. With an IC_{50} of 89 nM in our hands, this compound was already 4-fold more potent than Probiodrug's related clinical candidate. In order to further improve the potency and identify proprietary compounds, substitution on the cyclohexyl ring was explored [28,29]. In particular, amides, carbamates and sulfonamides at the 4-position on the cyclohexyl ring provided compounds such as **8**, **9** and **10** (Table **2**) with improved potency and oral bioavailabilities in rats of 81%, 76% and 46%, respectively [27].

Once the toxicity of the thiazolidide derivatives emerged, but before we had assays in hand for DPP8 and DPP9, we wondered whether the thiazolidine ring might be responsible for the observed toxicity. If the thiazolidine ring opened *in vivo*, a free thiol would be revealed, which could be reactive and lead to toxicity. To eliminate this possibility, we shifted our focus to non-sulfur containing heterocycles. The simple pyrrolidide derivatives were generally less potent (compare, for example, compounds **11** vs. **8**, **12** vs. **9**, and **13** vs. **10**, Table **2**); however, the addition of fluorine to the ring resulted compounds with increased potency. Indeed the (3*S*)-3-fluoropyrrolidine amides **14**, **15**, and **16** (Table **2**) are nearly equi-potent to the corresponding thiazolidide analogs [30].

Table 2. 4-Substituted Cyclohexylglycine Analogs



Compound	R	X	DPP-4 IC_{50} (nM)
7	H	S	89
8	(3,4-di-F-Ph)CONH-	S	54
9	$PhCH_2OCONH$-	S	25
10	(4-CF_3O-Ph)SO_2NH-	S	22
11	(3,4-di-F-Ph)CONH-	CH_2	190
12	$PhCH_2OCONH$-	CH_2	94
13	(4-CF_3O-Ph)SO_2NH-	CH_2	89
14	(3,4-di-F-Ph)CONH-	(*S*)-CHF	54
15	$PhCH_2OCONH$-	(*S*)-CHF	56
16	(4-CF_3O-Ph)SO_2NH-	(*S*)-CHF	36

The 4-trifluoromethoxybenzenesulfonamide derivative **16** has excellent pharmacokinetic properties across species, with a half-life of 4 h, 12 h and 5 h in rats, dogs and rhesus monkeys, respectively, and oral bioavailability of 37% to 89% [30]. It was profiled extensively as a potential preclinical development candidate. Only reticulocytopenia at 100 mg/kg/day was observed in a 2-week toxicity study in rats, and it was clean in acute tolerability studies in dogs [Lankas, G.; unpublished results]. Despite an attractive profile, further work on this compound was discontinued due to unacceptable levels of DPP8 and DPP9 inhibition (IC_{50} = 1400 nM and 1700 nM, respectively).

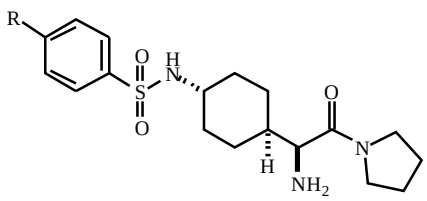
Once the potential toxicity associated with inhibition of the DPP8 and DPP9 enzymes was discovered, our goal was to identify an inhibitor with a >1000-fold window for DPP-4 inhibition over inhibition of these enzymes. A clue for how to achieve specificity came from a pair of positional scanning libraries that were developed for these aminopeptidases [31]. Each library consisted of dipeptidyl aminomethylcoumarin substrates. In the first "P_1" sublibrary, each well contained a spatially addressed amino acid at the P_1 position coupled to an isokinetic mixture of amino acids at P_2. In the

second "P_2" sublibrary, each well contained a spatially addressed amino acid at the P_2 position coupled to an isokinetic mixture of amino acids at P_1. Results for cleavage of the libraries by DPP-4 and DPP8 are summarized in Fig. (**3**). While both enzymes showed a strong preference for cleavage of substrates with a proline at the P_1 position, DPP-4 was much more promiscuous at P_2. In particular, dipeptides containing acidic amino acids such as glutamic acid were readily cleaved by DPP-4 whereas the rate of cleavage of these dipeptides by DPP8 was greatly reduced. We reasoned that incorporation of such acidic functionality at the P_2 position of our inhibitors could provide analogs with improved selectivity.

A variety of acidic derivatives were prepared. The two most selective compounds in this series, **17** [E. Parmee, unpublished results] and **18** [28], are illustrated in Fig. (**4**). Both are >100-fold selective for DPP-4 over DPP8; however, both suffer from poor oral bioavailability in rats (<1%). Further optimization did not yield selective compounds with improved pharmacokinetic properties and this series was put on hold.

α-AMINO ACID SERIES REVISITED

The α-amino acid series was ultimately re-examined following the discovery of sitagliptin. Because the *allo* isomer of isoleucyl thiazolidide was a more potent inhibitor of DPP8 and DPP9 than the *threo* isomer, we wondered whether we could improve selectivity by incorporating a "*threo*" bias into this series, an approach that was not possible with the symmetrical cyclohexylglycine derivatives. As shown in Fig. (**5**), when the ethyl sidechain of isoleucyl thiazolide was replaced with phenyl to provide β-methyl phenylalanine analog **19**, potency decreased by ~2-fold but selectivity was greatly improved [J. Xu, unpublished results].



17, R = COOH
DPP-4 IC_{50} = 15 nM
DPP8 IC_{50} = 2800 nM

18, R = $CF_3CH_2SO_2NH$-
DPP-4 IC_{50} = 2.6 nM
DPP8 IC_{50} = 15,000 nM

Fig. (4). DPP-4 inhibitors containing acidic functionality.

This compound was >100-fold selective over both DPP8 and DPP9. Potency could be enhanced by appending a second phenyl ring. The 4-fluorobiphenyl derivative **20** is a 64 nM DPP-4 inhibitor with excellent selectivity over both DPP8 and DPP9, though both **19** and **20** show decreased selectivity over QPP [32]. By incorporating polar functionality in place of the methyl group to give *N*,*N*-dimethylamide **21**, both potency and selectivity over QPP were improved [33].

While inhibitor **21** has excellent pharmacokinetic properties across species, its efficacy in an oral glucose tolerance test in mice was less than anticipated based on the exposures obtained and its inhibition of mouse DPP-4. This observation was attributed to its high plasma protein binding [33]. Replacement of the terminal phenyl ring with a heterocycle provided compounds with reduced serum shift, leading to triazolopyridine **22** Fig. (**5**) which was chosen for extensive preclinical evaluation [34]

FROM SCREENING HITS TO β-AMINO ACID LEAD

While medicinal chemistry efforts in the α-amino acid series were ongoing, screening of the Merck sample

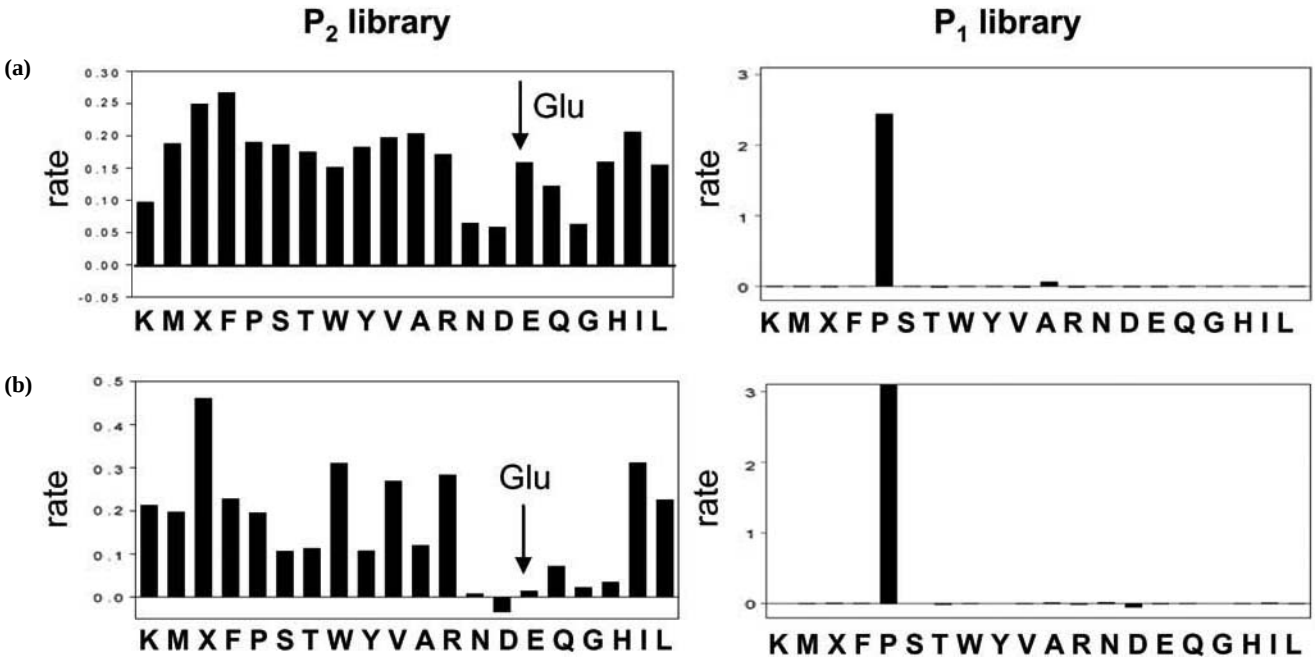


Fig. (3). Cleavage of peptide scanning libraries by (a) DPP-4 and (b) DPP8.

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