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Combining Combinatorial Chemistry and Affinity Chromatography: Highly Selective Inhibitors of Human Betaine: Homocysteine *S*-Methyltransferase

Michaela Collinsová,[1] Carmen Castro,[2] Timothy A. Garrow,[2] Athanasios Yiotakis,[3] Vincent Dive,[4] and Jiří Jiráček[1,*]
[1]Institute of Organic Chemistry and Biochemistry
Academy of Sciences of the Czech Republic
Flemingovo nám. 2
16610 Praha 6
Czech Republic
[2]Department of Food Science and Human Nutrition
University of Illinois
Urbana, Illinois 61801
[3]Department of Chemistry
Laboratory of Organic Chemistry
University of Athens
Panepistimiopolis Zografou 15771
Athens
Greece
[4]CEA
CE-Saclay
Département d'Ingénierie et d'Etudes des Protéines
91191 Gif/Yvette
France

Summary

A new method to find novel protein targets for ligands of interest is proposed. The principle of this approach is based on affinity chromatography and combinatorial chemistry. The proteins within a crude rat liver homogenate were allowed to interact with a combinatorial library of phosphinic pseudopeptides immobilized on affinity columns. Betaine: homocysteine S-methyltransferase (BHMT) was one of the proteins that was retained and subsequently eluted from these supports. The phosphinic pseudopeptides, which served as immobilized ligands for the isolation of rat BHMT, were then tested for their ability to inhibit human recombinant BHMT in solution. The most potent inhibitor also behaved as a selective ligand for the affinity purification of BHMT from a complex media. Further optimization uncovered Val-Phe-ψ[PO_2^--CH_2]-Leu-His-NH_2 as a potent BHMT inhibitor that has an IC_{50} of about 1 μM.

Introduction

The challenge of functional genomics and proteomics is to translate sequencing data into a precise understanding of how proteins function in cells, tissues, or the whole organism [1–3].

Small ligands that are able to specifically interact with proteins can be very effective tools in the search for proteome function [4, 5]. The classical approaches for the identification of new protein ligands include structure activity studies, molecular computer modeling, or combinatorial techniques. The problem with these approaches is that they generally use only one protein target. Given the high number of proteins in mammalian organisms, high-throughput screening procedures have been developed to handle this complicated task [6, 7]. However, the potential drawback of such methods is that the full range of proteins that the chosen ligands may interact with are not discovered if the screening is performed with only one or a few proteins. Lack of information regarding how many proteins a given ligand can interact with precludes our ability to completely understand the full spectrum of effects that ligand may have in a complex media such as a living cell. In this respect, approaches that study the affects of ligands in whole cells are becoming increasingly important. New compounds are routinely tested for biological activity using cultured cells and many new potential drugs have been studied this way [8]. However, in most of these cases, the full spectrum of protein targets a new drug may interact with will likely remain unknown and further investigation is needed to identify these other protein targets [9, 10]. Screening ligand libraries using biosensor chips or arrays grafted with proteins has been discussed as methods of choice to identify new protein-ligand interactions [7, 11–13] (and the references herein). These methods allow real time recording of ligand-protein interactions and subsequent elution of these complexes can be used for protein identification by mass spectrometry.

However, rather than employ one of these sophisticated approaches to discover novel protein-ligand interactions, we designed a simpler method based on affinity capture principles coupled to combinatorial chemistry. This approach is not intended to replace various chip-based technologies, but the advantage of our method is that it is simple and that a diverse range of chemistry can be applied on the appropriate resin supports. Although the immobilization of ligands on chips has made significant progress, the flexibility of the chemistry that can be applied to chips is still far behind that which is possible to apply on standard solid support resins. We anticipated that the recently developed solid supports that are compatible with both organic synthesis and affinity chromatography would be useful for our methodology [14–17]. These new supports make it possible to prepare affinity columns harboring libraries of diverse ligands rather than a single affinity ligand. This feature considerably extends the capacity of such supports to selectively isolate interacting proteins from crude preparations. These aspects, in addition to our observation that few investigators have applied standard combinatorial chemistry in the field of affinity chromatography, led us to develop this project.

For this study, phosphinic pseudopeptides (pseudopeptides phosphinates) were selected as potential ligands. We previously reported a convenient method to prepare libraries of such compounds [18–20]. Phosphinic pseudopeptides have been shown to function as transition state analogs of zinc metalloproteases and

*Correspondence: jiracek@uochb.cas.cz

these compounds are highly potent inhibitors of this protein family [21]. Given the ability of the phosphoryl group to interact with zinc atoms [22], we anticipated that phosphinic peptides might also interact with other zinc metalloenzymes that do not belong to this family of zinc proteases [23]. To test this idea, we prepared affinity columns harboring 361 different phosphinic peptides and used them to isolate all interacting proteins from crude rat liver homogenates. Then, by applying a deconvolution process, we identified the most specific ligand within the phosphinate peptide library that had the highest affinity toward one newly discovered protein target, betaine: homocysteine *S*-methyltransferase (BHMT). This ligand was then used to prepare highly selective affinity column for BHMT. Furthermore, the ligand was also used as the parent structure to rationally develop new and more potent inhibitors of this enzyme.

Results

Affinity Chromatography of Proteins from Soluble Rat Liver Homogenate on Columns Grafted with Phosphinic Pseudopeptide Mixtures of the General Formula Ac-X_{aa}-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-X_{aa}'-(βAla)$_2$-PL-PEGA

All 19 different affinity supports having the general formula Ac-X_{aa}-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-X_{aa}'-(βAla)$_2$-PL-PEGA and Ac-(βAla)$_2$-PL-PEGA (blank resin, BL) were prepared as described in Experimental Procedures. Each support had an equimolar mixture of 19 proteinogenic amino acids in the X_{aa}' position and a unique and different amino acid in the X_{aa} position (Cys was omitted). Soluble homogenate from rat livers was prepared and applied to each support and BL resin to perform affinity chromatography. After interaction with proteins, washing steps and elutions were performed on each support with either the corresponding mixture of soluble phosphinic pseudopeptides or guanidine hydrochloride (GnCl). BL resin was eluted only with GnCl. Eluted proteins were analyzed by electrophoresis.

Figure 1 shows electrophoretic analysis of fractions obtained from supports with amino acids Trp, Tyr, Phe, Met (Figure 1A), and Pro, Ser, Thr, Lys, Arg (Figure 1B) in position X_{aa}. Proteins eluted from BL resin and proteins present in RLH are also shown in Figure 1A. Highly selective and discreet protein elution was observed when affinity columns were eluted with the different mixtures of phosphinic peptides (e.g., lines W-i, F-i, P-i, S-i, Figures 1A and 1B). However, other proteins still remained immobilized on columns after elution with soluble phosphinic pseudopeptide mixtures as demonstrated by the electrophoretic profiles obtained when columns were washed with GnCl (e.g., lines W-g, Y-g, P-g, S-g). The electrophoretic analysis of samples from the remaining affinity supports resulted in similar patterns (data not shown). The density of protein bands and the occurrence relative to BL fraction were taken into account when selecting bands for N-terminal sequencing. The proteins labeled P1 to P20 (Figure 1), some of them as very weak bands, were blotted onto PVDF membranes and submitted for *N*-terminal sequencing.

N-Terminal Sequencing of Proteins P1–P20

The results of the *N*-terminal sequencing of proteins P1–P20 are shown in Table 1. Protein bands P1–P7, P11, P12, and P14–P16 revealed no peptide sequence, probably due to a blocked *N*-terminus or simply too little protein for detection.

Protein P8 was identified as 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) [24], protein P13 as Δ3,Δ2-enoyl-CoA isomerase (EC 5.3.3.8), protein P17 as L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), and protein band P18 was identified as enoyl-CoA hydratase 1 (ECH, EC 4.2.1.17) [25]. These proteins are enzymes involved in the mitochondrial/peroxisomal fatty acid metabolism and some studies indicate that they could be components of a liver multifunctional enzyme/complex [26].

Band P9 was identified as rat elongation factor-1 α (lacking residues 1–32), a protein that fulfills an essential cellular function in protein synthesis in that it binds charged tRNA molecules and transports them to the acceptor site on the ribosome [27].

The *N*-terminal sequencing of protein P10 resulted in a peptide sequence APIAGKKAKRGI. This sequence matched with the *N*-terminal sequence (*N*-terminal Met is missing) of rat liver betaine: homocysteine *S*-methyltransferase (BHMT, EC 2.1.1.5). BHMT is a cytoplasmic Zn-metalloenzyme responsible for the transfer of methyl group from betaine to L-homocysteine giving dimethylglycine and L-methionine [28, 29].

Protein bands P19 and P20 probably belong to different isoforms of rat glutathione *S*-transferase (GST, Table 1) [30, 31]. GSTs are widely distributed and abundant enzymes responsible for the detoxification of endo- and exogenous electrophiles via their conjugation to the reduced glutathione.

Inhibition Assays of ECH and GST with Soluble Phosphinic Pseudopeptide Libraries

Specific elution of ECH (P18) and GST (P19) with soluble inhibitors (Figures 1A and 1B, e.g., lines W-i or S-i) led us to determine if ECH and GST interactions with the affinity columns involved the active sites of these enzymes. To do this, inhibition experiments were performed with the different soluble pseudopeptide mixtures. We used bovine liver ECH (crotonase) and crotonoylcoenzyme-A as a substrate, following the protocols described by He and coworkers [32]. The activity of rat liver GST was determined using glutathione and 1-chloro-2,4-dinitrobenzene as substrates [33]. Some experiments were preformed using the relatively pure and enzymatically active preparations of both enzymes obtained from affinity chromatography (e.g., inhibitor-eluted fractions W-i or S-i shown in Figure 1, band P18/ECH, and band P19/theta-GST). All 19 soluble phosphinic pseudopeptide sublibraries of general formula Ac-X_{aa}-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-X_{aa}'-NH_2 were tested in inhibition assays. These pseudopeptide mixtures failed to inhibit ECH and GST in concentrations up to 400 μM.

Inhibition of Human Recombinant BHMT with Phosphinic Pseudopeptides

The fact that BHMT (P10) is a metalloenzyme led to investigate its interaction with our phosphinic pseudo-

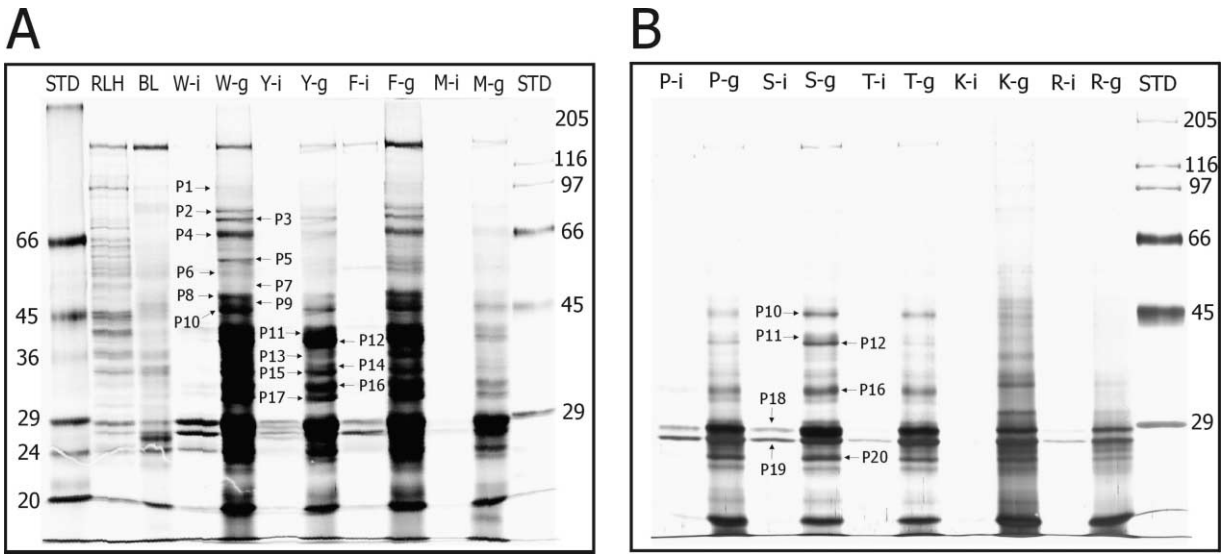

Figure 1. Electrophoretic Analysis of Fractions Obtained from Affinity Supports Ac-X_{aa}-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-X_{aa}'-$(\beta Ala)_2$-PL-PEGA

(A) Electrophoretic analysis of fractions obtained from affinity supports of general formula Ac-X_{aa}-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-X_{aa}'-$(\beta Ala)_2$-PL-PEGA having Trp (W), Tyr (Y), Phe (F), or Met (M) residues in the X_{aa} position. RLH means rat liver homogenate (5 μg of proteins was applied) and line BL shows proteins eluted from blank resin.

(B) Electrophoretic analysis of fractions obtained from affinity supports with Pro (P), Ser (S), Thr (T), Lys (K), or Arg (R) residues in the X_{aa} position. Fractions eluted with soluble inhibitor mixtures are marked as e.g., P-i (where mixture Ac-Pro-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-X_{aa}'-NH_2 was used), fractions eluted with GnCl are marked as e.g., P-g. Fifteen μl from each fraction (400 μl) were applied on the gel. Molecular weights of standards (STD) are shown in kDa. Proteins marked P1–P20 were submitted to *N*-terminal sequencing. For details, see Experimental Procedures.

peptides. The results of the inhibition assays of human recombinant BHMT with soluble phosphinic pseudopeptide mixtures are shown in Figure 2. The effective inhibition of BHMT activity by these mixtures strongly supports the hypothesis that BHMT was immobilized onto the affinity supports through the direct interaction

Table 1. *N*-Terminal Sequencing of Isolated Proteins

Protein band	*N*-terminal sequence found	Protein identified
P1–P7	nd	nd
P8	IPPAPLAKTD	3-Hydroxy-3-methylglutaryl-CoA synthase mitochondrial precursor (lacking residues 1–38), EC 4.1.3.5
P9	GIDKRTIEKFEK	Elongation factor-1 alpha (lacking residues 1-32)
P10	APIAGKKAKRGI	Betaine: homocysteine *S*-methyltransferase EC 2.1.1.5
P11, P12	nd	nd
P13	ATQQDFENAMN	Rat homologue of mouse peroxisomal Δ3,Δ2-Enoyl-CoA isomerase, EC 5.3.3.8
P14–P16	nd	nd
P17	KKILIKHVTV	L-3-Hydroxyacyl-CoA dehydrogenase Mitochondrial precursor (lacking residues 1–22), EC 1.1.1.35
P18	GANFQYIITEKKG	Enoyl-CoA hydratase 1, mitochondrial precursor (lacking residues 1–29), EC 4.2.1.17
P19	GLELYLDLLSQP	Glutathione *S*-transferase subunit 12, class-theta, EC 2.5.1.18
P20	PMTLGYWDIRG	Glutathione *S*-transferase chain 4, class-mu, EC 2.5.1.18
P21	APIAGKKAKRGI	Betaine: homocysteine *S*-methyltransferase EC 2.1.1.5
P22	APAGGPRVKKGILESLD	Rat homologue of mouse betaine: homocysteine *S*-methyltransferase 2
P23	YVAEKISGQKVNEAAXD VAEKISGQKVNEAAXDI	Betaine: homocysteine *S*-methyltransferase (lacking residues 1–88 or 1–89), EC 2.1.1.5

Results of *N*-terminal sequencing of proteins P1–P20 shown in Figure 1 and of proteins P21–P23 shown in Figure 4B. nd means not determined (due to the *N*-terminal blocking).

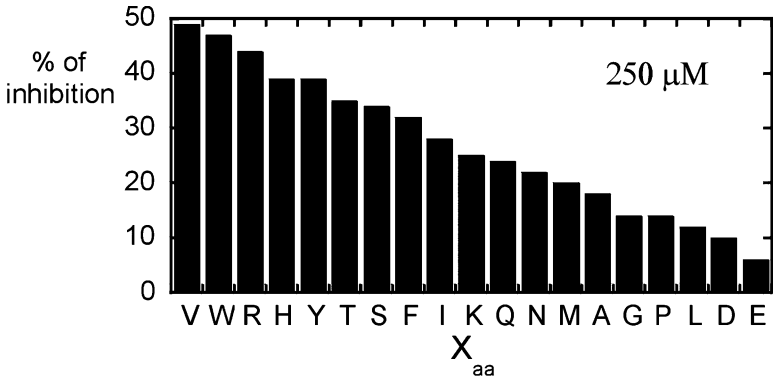

Figure 2. Inhibition of Human Recombinant BHMT by Soluble Phosphinic Pseudopeptide Mixtures Ac-X_{aa}-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-X_{aa}'-NH_2

Amino acids in the X_{aa} position are shown in a single letter code. The mixtures were tested at 250 μM concentration and the values are means of three independent measurements. For details, see Experimental Procedures.

between phosphinic ligands and its active site. The level of inhibition of individual inhibitor mixtures at 250 μM concentration varied from 50% to 5%. The presence of negatively charged residues like Asp or Glu in the X_{aa} position resulted in a very poor inhibition. The highest inhibition was observed with the mixture having Val residue in the X_{aa} position. This mixture was selected for further study.

Subsequently, 19 different phosphinic pseudopeptides of general formula Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-X_{aa}'-NH_2 were prepared. Each pseudopeptide differed by having a single and different amino acid in the X_{aa}' position. The ability of these pseudopeptides to inhibit human recombinant BHMT at 250 μM concentration is displayed in Figure 3. These data revealed a clear preference of the enzyme for His residue in the X_{aa}' position suggesting that Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-His-NH_2 should behave as a rather selective inhibitor of BHMT.

Affinity Purification of Rat Liver BHMT on Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-His-AOA-βAla-Dap-Sepharose

The Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-His-NH_2 pseudopeptide was synthesized stepwise via suitable spacer on NHS-activated Sepharose (Figure 4A) and used for affinity purification of BHMT from crude rat liver homogenate as described in Experimental Procedures. Figure 4B shows electrophoretic analysis of fractions obtained from this affinity chromatography. Three major protein bands labeled P21–P23 were submitted to the *N*-terminal sequencing (Table 1). Protein P21 was identified as rat BHMT, yielding the same *N*-terminal sequence as obtained from protein band P10 of the gel displayed in Figure 1B. This identity was confirmed by specific recognition of protein P21 by polyclonal antibodies raised against human recombinant BHMT (Figure 4C, lines B1, H1, and I1). A protein migrating at the same level was also recognized by the antibodies in the line loaded with a crude homogenate from rat liver (Figure 4C, RLH). Sequencing of band P22 revealed the sequence APAGGPRVKKGILESLD, which is very similar to the *N*-terminal sequence MAPAGSTRAKKGVLERLDS of the so-called mouse BHMT 2 [34]. We believe that protein P22 is probably the rat homolog of mouse BHMT 2. Sequencing protein P23 gave two sequences (Table 1), apparently shifted by one amino acid. By searching in the protein databases, we found that protein P23 is the degradation product of the rat BHMT lacking *N*-terminal residues 1–88 or 1–89. Such a degraded form of BHMT has already been described by other authors [35]. Protein P21 was present as a faint band in fractions eluted with betaine (lines B1–B2) or with compound Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-His-NH_2 (lines I1–I2). On the other hand, three DL-homocysteine elutions (lines H1–H3) resulted in a thick band P21 (with decreasing intensity from line H1 to H3) and weaker bands P22 and P23, consistent with the idea that these inhibitors have high affinity for the homocysteine binding site. Washing the column with sodium dodecylsulfate (SDS) and GnCl solutions (lines S1, S2 and G1, G2, respectively) revealed that almost no other protein remained on the affinity column after previous elutions. Overall, these results demonstrate that Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-

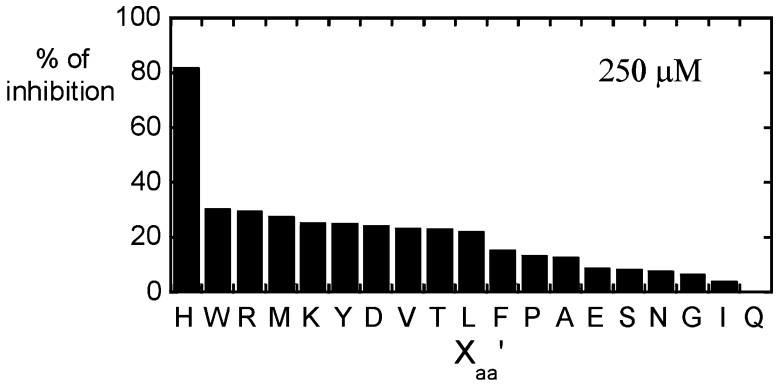

Figure 3. Inhibition of Human Recombinant BHMT by Single Soluble Phosphinic Pseudopeptides Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-X_{aa}'-NH_2

Amino acids in the X_{aa}' position are shown by single letter code. The compounds were tested at 250 μM concentration and the values are means of three independent measurements. For details, see Experimental Procedures.

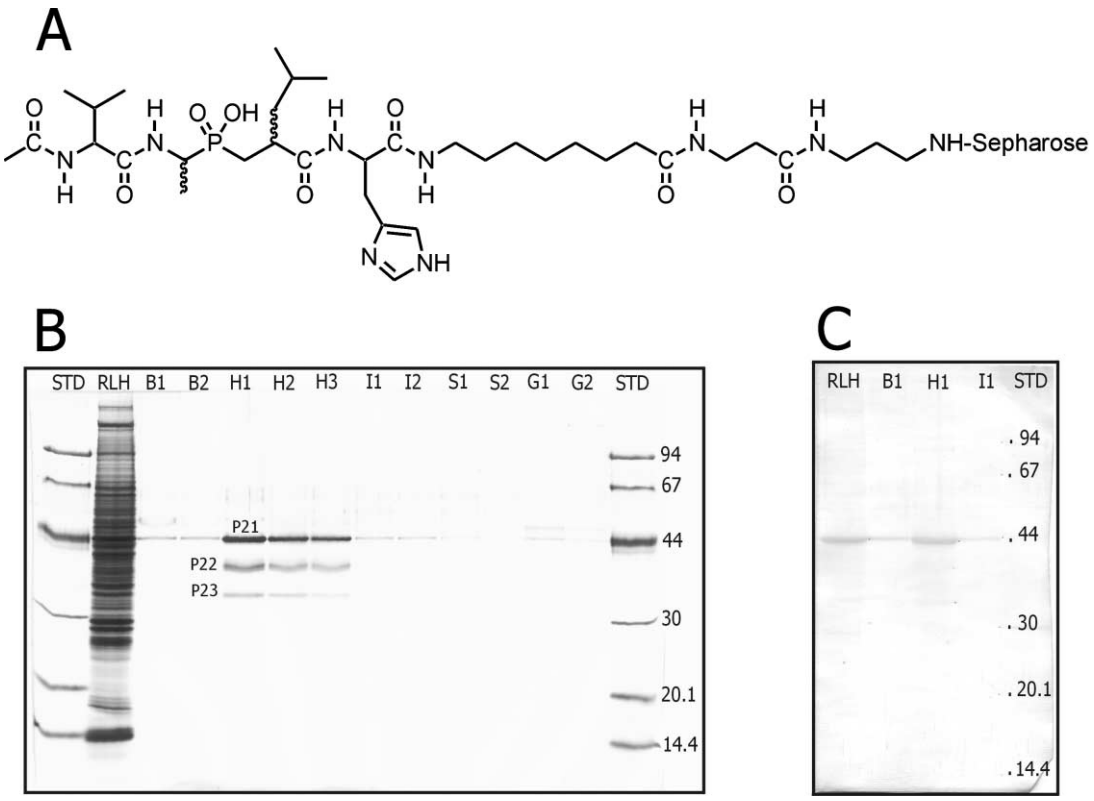

Figure 4. Affinity Purification of Rat Liver BHMT

(A) Structure of affinity matrix used for the isolation of rat liver BHMT.

(B) Electrophoretic analysis of fractions obtained from the affinity support shown above. RLH means rat liver homogenate (20 μg of proteins was applied). Fractions B1–B2 were eluted with betaine, fractions H1–H3 with DL-homocysteine, fractions I1–I2 with a solution of compound **1** (Table 2), fractions S1–S2 with 1% SDS, and fractions G1–G2 with 6 M GnCl. Fifteen μl from each fraction (700 μl) were applied on the gel. Molecular weights of standards (STD) are shown in kDa. Proteins marked as P21–P23 were submitted to *N*-terminal sequencing.

(C) Western blot analysis of selected fractions from affinity purification of rat liver BHMT with polyclonal antibodies against human recombinant BHMT. For details, see Experimental Procedures.

His-NH_2 has high specificity toward BHMT since it was able to selectively retain the enzyme on the affinity column while the other proteins in the crude extract were easily washed away.

Estimate of the Yield of BHMT Purification

We performed densitometric analysis of the immunoblot shown in Figure 4C to calculate the yield of BHMT. The data revealed that all BHMT positive fractions obtained from Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-His-AOA-βAla-Dap-Sepharose affinity column (7 μmoles was used) should contain about 0.15% of total 44 kDa BHMT present in 45 ml of RLH applied. The comparison with the immunoblot of pure recombinant human BHMT (not shown) revealed that all BHMT positive fractions eluted from this column should contain about 130 μg (3 nmoles) of 44 kDa BHMT. Additionally, we performed another immunoblotting analysis (not shown) of fractions S-g, T-g, RLH, and BL that are shown in Figures 1A and 1B. BHMT specific polyclonal antibodies did not detect any positive band in the BL line. However, 44 kDa BHMT positive bands were detected in fractions RLH, S-g, and T-g, which indicates that the interaction was specific to pseudopeptides. The densitometric analysis of the immunoblot revealed that S-g fraction from Ac-Ser-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-X_{aa}'-(βAla)$_2$-PL-PEGA affinity column (5 μmoles) contained about 4 μg of 44 kDa BHMT.

Optimization of Phosphinic Pseudopeptide Inhibitors of BHMT

Several analogs of pseudopeptide Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-His-NH_2 (compound **1**, Table 2) were synthesized in order to reveal the role of different structural elements have in insuring the affinity and potency of this compound toward BHMT. Given the significant role of His in position X_{aa}' of compound **1** for the inhibition of BHMT, we hypothesized that His or Cys residues in this position might interact directly with the catalytic Zn^{2+} ion of BHMT. Both His and Cys side chains are known as powerful ligands of zinc [36]. However, compound **2**, having Cys in the X_{aa}' position, did not inhibit BHMT at 100 μM concentration. Compounds **3** and **4** are analogs of pseudopeptide **1** with different amino acid side chains adjacent to the phosphinic moiety and were prepared using available phosphinic pseudopeptide precursors [20]. Inhibition potency of compound **3**, in respect to compound **1**, shows that the substitution of methyl by phenyl residue is very well tolerated by

Table 2. Inhibition of Human BHMT by Phosphinic Pseudopeptide Analogs

	Compound, 100 μM	% of Inhibition
1	Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-His-NH_2	57.4
2	Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-Cys-NH_2	0.0
3	Ac-Val-DL-Phe-ψ[PO_2^--CH_2]-DL-Leu-His-NH_2	53.7
4	Ac-Val-DL-Phe-ψ[PO_2^--CH_2]-DL-Ala-His-NH_2	8.7
5	Ac-Val-Ala-Leu-His-NH_2	25.9
6	Ac-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-NH_2	0.0
7	Ac-Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-His	0.0
8	Val-DL-Ala-ψ[PO_2^--CH_2]-DL-Leu-His-NH_2	70.6
9	Val-DL-Phe-ψ[PO_2^--CH_2]-DL-Leu-His-NH_2	80.3

Inhibition potencies of phosphinic pseudopeptide analogs at 100 μM concentration toward human recombinant BHMT. All compounds (with the exception of peptide **5**) are mixtures of four diastereoisomers.

BHMT. In contrast, the substitution of isobutyl side chain (compound **1**) by methyl (compound **4**) resulted in a poor inhibitor of BHMT. Compound **5** is a natural tetrapeptide, without phosphinic moiety, with the sequence mimicking that of compound **1**. This peptide is a very weak inhibitor of BHMT when compared to compound **1**, demonstrating the importance of the phosphinate moiety for the recognition of compound **1** by BHMT. However, the sole presence of a phosphinic pseudodipeptide moiety, as it is in the case of compound **6**, is not sufficient for potent inhibition. Thus, the amino acids in the X_{aa} and X_{aa}' positions are key elements for insuring tight interaction of the phosphinic pseudopeptide with BHMT. Probing the role of the *N*- or *C*-terminal protecting groups provided interesting observations. Indeed, compound **7**, which bears a free carboxylate group in the *C*-terminus instead of an amide group, did not inhibit BHMT at 100 μM concentration. This result is consistent with the ability of compound **1**, when linked to the solid support by its *C*-terminus (Figure 4A), to interact effectively with BHMT. Finally, the removal of the *N*-acetyl group improved significantly the inhibitory potency (compound **8**, Table 2). Compound **9**, containing a pseudo-phenylalanine residue, is the most potent inhibitor of this series.

The four diastereoisomers of compounds **8** and **9** were resolved by RP-HPLC and tested to evaluate their potency toward human BHMT. As shown in Table 3, among the four diastereoisomers for each compound, two exhibit higher affinity for BHMT (compounds **8a**, **8b** and **9a**, **9c**), pinpointing the role of the stereochemistry for the potency of these phosphinic pseudopeptides.

Determination of IC_{50} Values of Phosphinic Pseudopeptides Val-Phe-ψ[PO_2^--CH_2]-Leu-His-NH_2 toward Human Recombinant BHMT

We determined the IC_{50} values toward human recombinant BHMT for separated diastereoisomers of the most strongly inhibiting compound **9**. The results shown in Table 3 demonstrate that all diastereoisomers of compound **9** are relatively potent inhibitors of BHMT with IC_{50} values in low micromolar range. Two of the diastereoisomers, **9a** and **9c**, are significantly stronger than the other two. We observed a marked increase in inhibition when the pH of the buffer was changed from 7.5 to 6.9. The diastereosomers **9b**, **9c**, and **9d** had IC_{50} values about 20% lower at pH 6.9 than that observed at pH 7.5. Interestingly, the drop in IC_{50} value of diastereoisomer **9a** was even more dramatic, from 2.4 μM to 1.0 μM. Additionally, we found that L-methionine, one of the products of BHMT catalyzed reaction, is a rather weak inhibitor of human recombinant BHMT with IC_{50} value of about 250 μM. Compounds **9a** and **9c** are the most potent inhibitors of BHMT developed in this study and these compounds may be the most potent inhibitors of BHMT published to date [37, 38].

Discussion

Six of the proteins that were eluted from phosphinic pseudopeptide affinity columns were identified as enzymes. Two of these enzymes, ECH and GST, were eluted in a highly selective manner by phosphinic pseudopeptide mixtures in solution and very pure preparations of both enzymes were obtained (Figures 1A and 1B, e.g., lines W-i and S-i). This means that the library contained highly selective ligands for both enzymes. Interestingly, attempts to determine if these phosphinic peptides bind to the active site of GST and ECH failed. The simplest explanation for this observation is to consider that the binding sites for phosphinic pseudopeptides are outside of the active sites of these enzymes.

Table 3. Inhibition of Human BHMT by Individual Diastereoisomers of Compounds **8** and **9**

	Compound	% of Inhibition 50 μM	IC_{50} (μM) pH 6.9	IC_{50} (μM) pH 7.5
8a	Val-Ala-ψ[PO_2^--CH_2]-Leu-His-NH_2	54.5	nd	nd
8b	Val-Ala-ψ[PO_2^--CH_2]-Leu-His-NH_2	49.1	nd	nd
8c	Val-Ala-ψ[PO_2^--CH_2]-Leu-His-NH_2	22.2	nd	nd
8d	Val-Ala-ψ[PO_2^--CH_2]-Leu-His-NH_2	22.5	nd	nd
9a	Val-Phe-ψ[PO_2^--CH_2]-Leu-His-NH_2	82.9	1.0	2.4
9b	Val-Phe-ψ[PO_2^--CH_2]-Leu-His-NH_2	44.2	9.0	12.0
9c	Val-Phe-ψ[PO_2^--CH_2]-Leu-His-NH_2	87.2	1.7	2.0
9d	Val-Phe-ψ[PO_2^--CH_2]-Leu-His-NH_2	64.8	3.7	5.0
	L-Met	nd	nd	250

Inhibition potencies (at 50 μM concentration) of individual diastereoisomers of compounds **8** and **9** towards human recombinant BHMT. The IC_{50} values of individual diastereoisomers **a–d** of compound **9** and L-methionine towards human recombinant BHMT were determined in 50 mM K-phosphate buffer at two different pH. The diastereoisomers of pseudopeptides **8** and **9** are numbered **a–d** in the order they were eluted by RP-HPLC. nd means not determined. For details, see Experimental Procedures.

It would be of interest to confirm this hypothesis. As the 3D structures of both proteins are available [39, 40], localization of binding sites on these enzymes could also be possible by probing the protein surfaces with phosphinic pseudopeptide structures. The selective interaction of ECH and GST with our affinity columns could be exploited for the development of selective affinity purification procedures for these enzymes.

Protein P8 was identified as 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5), protein P13 as Δ3,Δ2-enoyl-CoA isomerase (EC 5.3.3.8), and protein P17 as L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35). These proteins, as well as ECH, are enzymes involved in the mitochondrial/peroxisomal fatty acid metabolism. We suppose that these enzymes could be retained on the affinity columns as components of previously reported multienzyme complex [26] through the interaction with ECH.

Protein P9 was identified as rat elongation factor-1 α. At present, we do not have a direct and rapid method to prove the specific interaction of our inhibitors with this protein involved in the proteosynthesis. However, further investigation of this interaction could be interesting because compounds interacting with elongation factor-1 α display growth inhibitory effects on human tumor cells [41].

Protein P10 was identified as BHMT. Soluble phosphinic pseudopeptide mixtures did not elute this protein but GnCl treatment was necessary to release the protein from the columns (Figure 1). Strikingly, in this case, several observations lead us to conclude that the phosphinic pseudopeptides interact with the active site of BHMT. First, soluble phosphinic pseudopeptides were shown to inhibit BHMT activity. Second, homocysteine and to a lesser extent betaine, the substrates of BHMT, were able to elute BHMT with high selectivity from the affinity column grafted with the phosphinic pseudopeptide inhibitor Ac-Val-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-His-NH_2 (Figure 4B). The more efficient elution of BHMT with homocysteine compared to betaine suggests that the phosphinic inhibitor occupies the homocysteine binding site of BHMT tighter than the betaine binding site, if it occupies the latter site at all. All major protein bands (P21–P23) eluted from the affinity column with homocysteine are BHMT or BHMT-related enzymes. A weak band of BHMT protein was observed in the inhibitor-eluted fractions following the betaine/homocysteine washings. Almost no protein was subsequently eluted from the column using the nonspecific and denaturing agents, SDS and GnCl. The experiment shown in Figure 4 reveals that Ac-Val-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-His-NH_2 displays excellent selectivity for BHMT since it functioned as an affinity ligand for the enzyme with such high specificity that it could be used to purify the enzyme to homogeneity from a crude liver extract in single chromatographic step.

The densitometry analysis of the immunoblots revealed that the capacity of Ac-Ser-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-X_{aa}'-$(\beta Ala)_2$-PL-PEGA affinity column (5 μmoles was used) to capture 44 kDa BHMT was about 30 times lower than the capacity of Ac-Val-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-His-AOA-βAla-Dap-Sepharose affinity column (7 μmoles was used). The reason for this difference in capacity is probably due to the high preference of BHMT for His amino acid in the X_{aa}' position of pseudopeptides (Figure 3). In fact, His was only one of 19 amino acids present in the X_{aa}' position of Ac-Ser-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-X_{aa}'-$(\beta Ala)_2$-PL-PEGA affinity column containing 19 different pseudopeptides. This fact underscores the remarkable selectivity of Ac-Val-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-His-AOA-βAla-Dap-Sepharose for BHMT.

Structure activity studies performed with Ac-Val-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-His-NH_2, which was obtained by combinatorial approach (Figures 2 and 3), indicate that the selectivity of the interaction between BHMT and this phosphinic pseudopeptide relies on several structural features which are (1) the presence of the phosphinate moiety, (2) the histidine residue, and (3) the C-terminal carboxamide group (Tables 2 and 3).

Recently, the mechanism for the binding of homocysteine to the active site of a BHMT-related enzyme, methionine synthase, was defined [42, 43]. This mechanism involves the formation of a zinc tetrathiolate complex formed by three cysteine ligands of the enzyme and the thiolate group of the homocysteine substrate. It is likely that similar mechanism occurs upon the binding of homocysteine to the active site of BHMT where the Zn^{2+} ion is also coordinated by 3 cysteine ligands [44]. This hypothesis has recently been supported by the resolution of a crystal structure of human BHMT in complex with a transition state inhibitor [45]. We suppose that the phosphinic inhibitors developed in this study interact with the zinc atom of BHMT via their phosphinate groups, that is, the phosphinates compete with homocysteine for the enzyme's catalytic Zn. This explanation is supported by our observation that homocysteine rather than betaine could be used to effectively elute enzyme from the phosphinate pseudopeptide column. The direct interaction of phosphinate group oxygen atoms with a catalytic zinc ion was recently proved by the resolution of the structure of the complex between the metalloproteinase astacine and a phosphinate inhibitor [22].

We demonstrated the successful application of a new and simple method for the discovery of new protein targets for artificial ligands of interest. Despite the very limited diversity of the pseudopeptide library (only 361 sequences) used, we isolated a large number of proteins from which the majority was specifically retained on affinity columns due to the presence of the pseudopeptides. *N*-terminal sequencing of twenty of these isolated proteins resulted in the identification of BHMT, which we subsequently showed to be selectively and relatively potently inhibited by one of the pseudopeptides from the library. Overall, we believe that this method, especially in conjunction with 2D electrophoresis and MALDI mass spectrometry, holds promise as an additional method for the discovery of new specific protein-ligand interactions.

The phosphinic pseudopeptide inhibitors of BHMT developed in this study may be a very promising tool for studying the physiological function of BHMT. For example, in the absence of a mouse strain devoid of BHMT activity, the design and use of selective inhibitors of this enzyme as drugs may help to elucidate whether BHMT has an important role in the regulation of plasma homocysteine levels. This would be an important finding

since hyperhomocysteinemia is believed to be a risk factor for vascular diseases and thrombosis [46].

Our future effort will be focused on the synthesis and characterization of phosphinate molecules of non-peptide nature mimicking the hypothetical transition state occurring upon binding of betaine and homocysteine to the active site of BHMT.

Significance

A new and simple approach for the discovery of novel protein targets for synthetic ligands is proposed. The principle of a new method is based on combination of affinity chromatography and combinatorial chemistry. Zn-metalloenzyme betaine: homocysteine *S*-methyltransferase (BHMT) was isolated from crude rat liver homogenate using affinity chromatography on supports with mixtures of immobilized phosphinic pseudopeptides. The respective soluble phosphinic pseudopeptides were then tested for their ability to inhibit human recombinant BHMT. We discovered potent and highly selective compounds, which can be effective tools in a study of BHMT functions and properties. The results reported in this study validate the concept that combining combinatorial chemistry to affinity chromatography protocols makes possible to identify, without any a priori hypothesis, novel protein targets for phosphinic peptides. And vice versa, selective inhibitors of BHMT were developed despite the rather limited diversity of the phosphinic pseudopeptide library used to fish out the proteins. We believe that our new approach can be a valuable tool in the search for the function of the proteome.

Experimental Procedures

Fmoc-protected amino acids, Rink Amide AM resin, 2-Chlorotrityl resin, Amino-PEGA resin, photolabile linker (PL, 4-[4-(1-aminoethyl)-2-methoxy-5-nitro-phenoxy]-butyric acid) were purchased from Novabiochem. NHS-activated Sepharose was purchased from Amersham Biosciences. Plastic columns for affinity chromatography were from MoBiTec GmbH (Göttingen, Germany). Anti-rabbit IgG peroxidase conjugated antibody, bovine liver ECH (crotonase), crotonoylcoenzyme-A, glutathione, and 1-chloro-2,4-dinitrobenzene were purchased from Sigma-Aldrich. *N*-methyl-C^{14}-betaine was prepared and supplied by Moravek Biochemicals (Brea, CA). Human recombinant BHMT and polyclonal antibodies against human recombinant BHMT were prepared as described previously [29, 47].

Inhibitor and Affinity Support Synthesis

The soluble pseudopeptide library of general formula Ac-X_{aa}-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-X_{aa}'-NH_2 and single soluble pseudopeptides were prepared and characterized as described previously [18–20, 48, 49]. The same methodology was applied for the synthesis of the immobilized library of general formula Ac-X_{aa}-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-X_{aa}'-$(\beta Ala)_2$-PL-PEGA and Ac-$(\beta Ala)_2$-PL-PEGA (blank resin) using Amino-PEGA resin [14, 15]. Photolabile linker (PL) [50, 51] was introduced to allow the cleavage and the subsequent control of the quality of synthesized structures. Ac-Val-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-His-AOA-βAla-Dap-Sepharose (AOA is 8-aminooctanoic acid, Dap is 1,3-diaminopropane) was prepared using NHS-activated Sepharose as described elsewhere [16, 17].

Preparation of Soluble Rat Liver Homogenate for Affinity Chromatography Procedures

Male Wistar rats weighing approximately 250 g were decapitated and their livers were perfused with cold saline, excised, and immediately frozen at –72°C. All following procedures were carried out at 4°C. When needed, rat livers (approximately 70 g) were thawed, minced with scissors, and homogenized in the same volume of 25 mM Hepes/NaOH buffer (pH 7.5) containing 0.2 mM 1,4-dithiothreitol (DTT) (buffer A) using a teflon glass homogenizer. The homogenate was centrifuged at 2400 g for 20 min. The supernatant was removed and temporarily stored while the pellet was resuspended in half volume of buffer A and centrifuged again as above. Then, both supernatants were combined and centrifuged at 100,000 g for 65 min. The pellet was discarded and the resulting supernatant was diluted with buffer A to the final volume of 200 ml, divided into 20 equal portions, and immediately used for affinity chromatography or frozen at –72°C. The protein concentration was measured by the procedure of Bradford [52] using bovine serum albumin as standard and was determined to be about 30 mg per ml.

Affinity Chromatography on Immobilized Pseudopeptide Libraries of General Formula Ac-X_{aa}-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-X_{aa}'-$(\beta Ala)_2$-PL-PEGA

All steps were performed at 4°C. Before use, the individual affinity resins (5 μmoles of each) were placed into 2.5 ml plastic columns with porous frits and connectors for tubings. The resins were thoroughly washed with buffer A. Then, 10 ml of soluble rat liver homogenate (RLH) were applied on each affinity column and allowed to circulate overnight at a flow rate of 0.5 ml per min. Thereafter, the affinity matrixes were washed with 20 ml of buffer A, 50 ml of 1.5 M NaCl in buffer A, 10 ml of buffer A, 50 ml of 0.1% Triton X-100 in buffer A, and 40 ml of buffer A. The bound proteins were at first eluted from the affinity resins with 0.4 ml of 0.2 mM solution of respective soluble inhibitor mixtures in buffer A by slowly rotating the columns for 6 hr and then with 0.4 ml of 6 M GnCl in water for 4 hr. Ac-$(\beta Ala)_2$-PL-PEGA resin was eluted only with 6 M GnCl. All fractions were analyzed by electrophoresis.

Affinity Chromatography on Ac-Val-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-His-AOA-βAla-Dap-Sepharose

For the most part, these procedures were the same as described above. The small differences are briefly described as follows. Rat liver homogenate (45 ml) was applied on affinity resin (7 μmol) and allowed to circulate for 6 hr. The bound proteins were eluted stepwise from the affinity gel by slowly rotating for 6 hr in 0.7 ml of each of following agents in buffer A: (1) 10 mM betaine (twice), (2) 1 mM DL-homocysteine (three times), (3) 250 μM Ac-Val-DL-Ala-$\psi[PO_2^-$-$CH_2]$-DL-Leu-His-NH_2 (twice), (4) 1% SDS (twice), and (5) with 6 M GnCl (twice). Resin was washed with buffer A between different elutions. All fractions were analyzed by electrophoresis.

Gel Electrophoresis

SDS-PAGE was performed according to Laemmli [53] on 0.7 mm thick gels (11% acrylamide in running gel, 5% acrylamide in stacking gel). Proteins were visualized by silver staining procedure as described by Blum et al. [54]. The volume of 15 μl was applied from each fraction (0.4 ml or 0.7 ml). Fraction containing GnCl were dialyzed prior to the electrophoresis.

Western Blot Analysis

Samples were resolved by electrophoresis and electroblotted onto PVDF membrane (Immobilon P, Millipore) at 2.5 mA/cm^2 for 1.5 hr using in 20 mM CAPS/NaOH buffer (pH 10.3) supplemented with 10% methanol and 0.01% SDS. All incubations and washes were performed at room temperature with 20 mM Tris/HCl buffer (pH 7.5) supplemented with 140 mM NaCl and 0.1% Tween-20 (T-TBS buffer). Blocking was accomplished by incubating the membrane in 3% bovine serum albumine in T-TBS buffer (for 1 hr). The membrane was then incubated for 30 min with a rabbit serum BHMT antibody, washed with T-TBS, and incubated for another 30 min with an anti-rabbit IgG peroxidase conjugated antibody in T-TBS. Before staining, the membrane was washed with T-TBS and then developed with diaminobenzidine.

For *N*-terminal sequencing, the blotted proteins were stained in PVDF membrane with Coomassie Brilliant Blue R-250. Bands of interest were excised from a membrane and submitted to sequencing on Perkin-Elmer Applied Biosystems 491 Protein Sequencer.

Peptide sequences were submitted to various protein databases for identification [55].

BHMT Assay

Compounds were tested for their ability to inhibit BHMT activity (Tables 2 and 3) using an assay procedure we have described previously in detail [56], with several modifications. Briefly, DL-homocysteine was freshly prepared by dissolving DL-homocysteine thiolactone hydrochloride (15.4 mg) in 400 μl of 2 M NaOH. The solution was allowed to stand for 5 min at room temperature. The solution was then neutralized by the addition of 600 μl of a saturated solution of KH_2PO_4 and immediately used in BHMT assay. The standard BHMT assay (500 μl) contained 0.7 μM BHMT, different concentrations of inhibitor, 100 μM DL-homocysteine, 250 μM betaine (0.1 μCi *N*-methyl-C^{14}-betaine was added into each reaction mixture and the desired betaine concentration was adjusted with cold betaine), 10 mM β-mercaptoethanol, and 50 mM Tris/HCl (pH 7.5). Human recombinant BHMT was first mixed with inhibitor(s) then the substrates were added and the mixture incubated at 37°C for 30 min. The reaction was stopped by transferring the reaction tubes into ice water and by adding 2.5 ml of ice-cold water. The samples were applied to a Dowex 1 × 4 (200–400 mesh) and the non-reacted betaine was washed from the column with water. Dimethylglycine and methionine were eluted into scintillation vials with 1.5 ml of 1.5 M HCl and then 8 ml of scintillation mixture were added to each vial and counted. Blanks contained all the reaction components except enzyme and their values were subtracted from the sample values. All samples were assayed in triplicates and results are expressed relative (%) to a sample containing no inhibitor.

Inhibition curves for the determination of IC_{50} of inhibitors (Table 3) were measured in 50 mM K-phosphate buffer at pH 7.5 or 6.9 using 0.2 μM BHMT, 10 μM DL-homocysteine, 50 mM betaine (0.5 μCi), and variable amounts of inhibitors. The inhibition at ten different inhibitor concentrations was determined for each curve. All samples were assayed in duplicates and each curve was measured twice.

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References

1. Crews, C.M. (1996). Deciphering isozyme function: exploring cell biology with chemistry in the post-genomic era. Chem. Biol. *3*, 961–965.
2. Eisenberg, D., Marcotte, E.M., Xenarios, I., and Yeates, T.O. (2000). Protein function in the post-genomic era. Nature *405*, 823–826.
3. Pandey, A., and Mann, M. (2000). Proteomics to study genes and genomes. Nature *405*, 837–846.
4. Schreiber, S.L. (1998). Chemical genetics resulting from a passion for synthetic organic chemistry. Bioorg. Med. Chem. *6*, 1127–1152.
5. Lowe, C.R. (2001). Combinatorial approaches to affinity chromatography. Curr. Opin. Chem. Biol. *5*, 248–256.
6. MacBeath, G., and Schreiber, S.L. (2000). Printing proteins as microarrays for high-throughput function determination. Science *289*, 1760–1763.
7. Kodadek, T. (2001). Protein microarrays: prospects and problems. Chem. Biol. *8*, 105–115.
8. Crews, C.M., and Splittgerber, U. (1999). Chemical genetics: exploring and controlling cellular processes with chemical probes. Trends Biochem. Sci. *24*, 317–320.
9. Taunton, J., Hassig, C.A., and Schreiber, S.L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science *272*, 408–411.
10. Chen, J.K., Lane, W.S., and Schreiber, S.L. (1999). The identification of myriocin-binding proteins. Chem. Biol. *6*, 221–235.
11. Nelson, R.W., Nedelkov, D., and Tubbs, K.A. (2000). Biosensor chip mass spectrometry: a chip-based proteomics approach. Electrophoresis *21*, 1155–1163.
12. Baird, C.L., and Myszka, D.G. (2001). Current and emerging commercial optical biosensors. J. Mol. Recognit. *14*, 261–268.
13. Stoll, D., Templin, M.F., Schrenk, M., Traub, P.C., Vohringer, C.F., and Joos, T.O. (2002). Protein microarray technology. Front. Biosci. *7*, C13–C32.
14. Meldal, M. (1992). PEGA: a flow stable polyethylene glycol dimethyl acrylamide copolymer for solid phase synthesis. Tetrahedron Lett. *33*, 3077–3080.
15. Meldal, M., Auzanneau, F.-I., Hindsgaul, O., Palcic, M.M. (1994). A PEGA resin for use in the solid-phase chemical-enzymatic synthesis of glycopeptides. J. Chem. Soc. Chem. Commun. 1849–1850.
16. Tegge, W., and Frank, R. (1997). Peptide synthesis on Sepharose™ beads. J. Pept. Res. *49*, 355–362.
17. Gast, R., Glökler, J., Höxter, M., Kiess, M., Frank, R., and Tegge, W. (1999). Method for determining protein kinase substrate specificities by the phosphorylation of peptide libraries on beads, phosphate-specific staining, automated sorting, and sequencing. Anal. Biochem. *276*, 227–241.
18. Jiracek, J., Yiotakis, A., Vincent, B., Lecoq, A., Nicolaou, A., Checler, F., and Dive, V. (1995). Development of highly potent and selective phosphinic peptide inhibitors of zinc endopeptidase 24–15 using combinatorial chemistry. J. Biol. Chem. *270*, 21701–21706.
19. Jiracek, J., Yiotakis, A., Vincent, B., Checler, F., and Dive, V. (1996). Development of the first potent and selective inhibitor of the zinc endopeptidase neurolysin using a systematic approach based on combinatorial chemistry of phosphinic peptides. J. Biol. Chem. *271*, 19606–19611.
20. Yiotakis, A., Vassiliou, S., Jiracek, J., and Dive, V. (1996). Protection of the hydroxyphosphinyl function of phosphinic dipeptides by adamantyl. Application to the solid-phase synthesis of phosphinic peptides. J. Org. Chem. *61*, 6601–6605.
21. Dive, V., Lucet-Levannier, K., Georgiadis, D., Cotton, J., Vassiliou, S., Cuniasse, P., and Yiotakis, A. (2000). Phosphinic peptide inhibitors as tools in the study of the function of zinc metallopeptidases. Biochem. Soc. Trans. *28*, 455–460.
22. Grams, F., Dive, V., Yiotakis, A., Yiallouros, I., Vassiliou, S., Zwilling, R., Bode, W., and Stocker, W. (1996). Structure of astacin with a transition-state analogue inhibitor. Nat. Struct. Biol. *3*, 671–675.
23. Collinsova, M., and Jiracek, J. (2000). Phosphinic acid compounds in biochemistry, biology and medicine. Curr. Med. Chem. *7*, 629–647.
24. Hegardt, F.G. (1999). Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis. Biochem. J. *338*, 569–582.
25. Hiltunen, J.K., and Qin, Y.-M. (2000). β-oxidation-strategies for the metabolism of a wide variety of acyl-CoA esters. Biochim. Biophys. Acta *1484*, 117–128.
26. Palosaari, P.M., and Hiltunen, J.K. (1990). Peroxisomal bifunctional protein from rat liver is a trifunctional enzyme possessing 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and delta *3*, delta 2-enoyl-CoA isomerase activities. J. Biol. Chem. *265*, 2446–2449.
27. Ann, D.K., Lin, H.H., Lee, S., Tu, Z.-J., and Wang, E. (1992). Characterization of the statin-like S1 and rat elongation factor 1 α as two distinctly expressed messages in rat. J. Biol. Chem. *267*, 699–712.
28. Finkelstein, J.D., Harris, B.J., and Kyle, W.E. (1972). Methionine metabolism in mammals: kinetic study of betaine-homocysteine methyltransferase. Arch. Biochem. Biophys. *153*, 320–324.
29. Millian, N.S., and Garrow, T.A. (1998). Human betaine-homocysteine methyltransferase is a zinc metalloenzyme. Arch. Biochem. Biophys. *356*, 93–98.
30. Ogura, K., Nishiyama, T., Okada, T., Kajita, J., Narihata, H., Watabe, T., Hiratsuka, A., and Watabe, T. (1991). Molecular

cloning and amino acid sequencing of rat liver class theta glutathione S-transferase Yrs-Yrs inactivating reactive sulfate esters of carcinogenic arylmethanols. Biochem. Biophys. Res. Commun. *181*, 1294–1300.

31. Alin, P., Mannervik, B., and Jornvall, H. (1986). Cytosolic rat liver glutathione transferase 4–4. Primary structure of the protein reveals extensive differences between homologous glutathione transferases of classes α and μ. Eur. J. Biochem. *156*, 343–350.
32. He, X.Y., Yang, S.Y., and Schulz, H. (1992). Inhibition of enoyl-CoA hydratase by long-chain L-3-hydroxyacyl-CoA and its possible effect on fatty acid oxidation. Arch. Biochem. Biophys. *298*, 527–531.
33. Habig, W.H., Pabst, M.J., and Jakoby, W.B. (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. *249*, 7130–7139.
34. Chadwick, L.H., McCandless, S.E., Silverman, G.L., Schwartz, S., Westaway, D., and Nadeau, J.H. (2000). Betaine-homocysteine methyltransferase-2: cDNA cloning, gene sequence, physical mapping, and expression of the human and mouse genes. Genomics *70*, 66–73.
35. Ueno, T., Ishidoh, K., Mineki, R., Tanida, I., Murayama, K., Kadowaki, M., and Kominami, E. (1999). Autolysosomal membrane-associated betaine homocysteine methyltransferase. Limited degradation fragment of a sequestered cytosolic enzyme monitoring autophagy. J. Biol. Chem. *274*, 15222–15229.
36. Vallee, B.L., and Auld, D.S. (1990). Zinc coordination, function, and structure of zinc enzymes and other proteins. Biochemistry *29*, 5647–5659.
37. Awad, W.M., Jr., Whitney, P.L., Skiba, W.E., Mangum, J.H., and Wells, M.S. (1983). Evidence for direct methyl transfer in betaine: homocysteine S-methyl-transferase. J. Biol. Chem. *258*, 12790–12792.
38. Lee, K.H., Cava, M., Amiri, P., Ottoboni, T., and Lindquist, R.N. (1992). Betaine:homocysteine methyltransferase from rat liver: purification and inhibition by a boronic acid substrate analog. Arch. Biochem. Biophys. *292*, 77–86.
39. Engel, C.K., Mathieu, M., Zeelen, J.P., Hiltunen, J.K., and Wierenga, R.K. (1996). Crystal structure of enoyl-coenzyme A (CoA) hydratase at 2.5 Å resolution: a spiral fold defines the CoA-binding pocket. EMBO J. *15*, 5135–5145.
40. Wilce, M.C.J., and Parker, M.W. (1994). Structure and function of glutathione S-transferases. Biochim. Biophys. Acta *1205*, 1–18.
41. Ahuja, D., Geiger, A., Ramanjulu, J.M., Vera, M.D., Sirdeshpande, B., Pfizenmayer, A., Abazeed, M., Krosky, D.J., Beidler, D., Joullié, M.M., et al. (2000). Inhibition of protein synthesis by didemnins: cell potency and SAR. J. Med. Chem. *43*, 4212–4218.
42. Peariso, K., Goulding, C.W., Huang, S., Matthews, R.G., and Penner-Hahn, J.E. (1998). Characterization of the zinc binding site in methionine synthase enzymes of *Escherichia coli*: the role of zinc in the methylation of homocysteine. J. Am. Chem. Soc. *120*, 8410–8416.
43. Peariso, K., Zhou, Z.S., Smith, A.E., Matthews, R.G., and Penner-Hahn, J.E. (2001). Characterization of the zinc sites in cobalamin-independent and cobalamin-dependent methionine synthase using zinc and selenium X-ray absorption spectroscopy. Biochemistry *40*, 987–993.
44. Breksa, A.P., III, and Garrow, T.A. (1999). Recombinant human liver betaine-homocysteine S-methyltransferase: identification of three cysteine residues critical for zinc binding. Biochemistry *38*, 13991–13998.
45. Evans, J.C., Huddler, D.P., Jiracek, J., Castro, C., Millian, N.S., Garrow, T.A., and Ludwig, M.L. (2002). Betaine-homocysteine methyltransferase. Zinc in a distorted barrel. Structure *10*, 1159–1171.
46. Refsum, H., Ueland, P.M., Nygard, O., and Vollset, S.E. (1998). Homocysteine and cardiovascular disease. Annu. Rev. Med. *49*, 31–62.
47. Rao, P.V., Garrow, T.A., John, F., Garland, D., Millian, N.S., and Zigler, J.S., Jr. (1998). Betaine-homocysteine methyltransferase is a developmentally regulated enzyme crystallin in rhesus monkey lens. J. Biol. Chem. *273*, 30669–30674.
48. Koval, D., Kasicka, V., Jiracek, J., Collinsova, M., and Garrow, T.A. (2002). Analysis and characterization of phosphinic pseudopeptides by capillary zone electrophoresis. Electrophoresis *23*, 215–222.
49. Koval, D., Kasicka, V., Jiracek, J., Collinsova, M., and Garrow, T.A. (2002). Determination of dissociation constant of phosphinate group in phosphinic pseudopeptides by capillary zone electrophoresis. J. Chromatography B Analyt. Technol. Biomed. Life Sci. *770*, 145–154.
50. Holmes, C.P. (1997). Model studies for new o-nitrobenzyl photolabile linkers: substituent effects on the rates of photochemical cleavage. J. Org. Chem. *62*, 2370–2380.
51. Rinnová, M., Novakova, M., Kasicka, V., and Jiracek, J. (2000). Side reactions during photochemical cleavage of an α-methyl-6-nitroveratryl-based photolabile linker. J. Pept. Sci. *6*, 355–365.
52. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.
53. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–681.
54. Blum, H., Beier, H., and Gross, H.J. (1987). Improved silver staining of plant-proteins, RNA and DNA in polyacrylamide gels. Electrophoresis *8*, 93–99.
55. Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.
56. Garrow, T.A. (1996). Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase. J. Biol. Chem. *271*, 22831–22838.