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Identification of methionine aminopeptidase 2 as a molecular target of the organoselenium drug ebselen and its derivatives/analogues: Synthesis, inhibitory activity and molecular modeling study

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ABSTRACT

A collection of twenty-six organoselenium compounds, ebselen and its structural analogues, provided a novel approach for inhibiting the activity of human methionine aminopeptidase 2 (MetAP2). This metalloprotease, being responsible for the removal of the amino-terminal methionine from newly synthesized proteins, plays a key role in angiogenesis, which is essential for the progression of diseases, including solid tumor cancers. In this work, we discovered that ebselen, a synthetic organoselenium drug molecule with anti-inflammatory, anti-oxidant and cytoprotective activity, inhibits one of the main enzymes in the tumor progression pathway. Using three-step synthesis, we obtained twenty-five ebselen derivatives/analogues, ten of which are new, and tested their inhibitory activity toward three neutral aminopeptidases (MetAP2, alanine and leucine aminopeptidases). All of the tested compounds proved to be selective, slow-binding inhibitors of MetAP2. Similarly to ebselen, most of its analogues exhibited a moderate potency (IC_{50} = 1–12 μM). Moreover, we identified three strong inhibitors that bind favorably to the enzyme with the half maximal inhibitory concentration in the submicromolar range.

Alanine (APNs), leucine (LAPs), and methionine aminopeptidases (MetAPs) are three major groups of neutral aminopeptidases containing metal ion(s) in their active site that catalyze the removal of amino acids from the N-terminus of a peptide or protein. Biomedical significance of these exopeptidases is related to therapeutic intervention against devastating human diseases. The leucine and alanine aminopeptidases represent promising targets for the development of a new generation of anti-inflammatory drugs. The alanine and methionine aminopeptidases possess a well-recognized potential for the design of anti-angiogenesis agents. Furthermore, neutral aminopeptidases are involved in the apoptosis of cancer cells, which makes them highly interesting objects of oncological research.[1–4]

Human MetAP type 2 (MetAP2) is one of the three known methionine aminopeptidases responsible for the removal of the N-terminal translation initiator methionine from newly synthesized proteins, which is a critical step in protein maturation.[3,5] Protein translation mainly begins with a methionine in eukaryotes and a formylated methionine in prokaryotes. Consequently, the removal of methionine is indispensable for post-translational amino group modifications, protein stability and proper localization.[6,7] MetAP2 possesses a characteristic bimetallic center activated by cobalt, manganese or iron, surrounded by residues that form a pita-bread-shaped fold.[8–10] MetAPs are expressed in many mammalian tissues and cell lines, but only type 2 is upregulated during cell proliferation.[11] Higher expression of MetAP2 has been observed in tumor cells compared with normal cells.[12,13] To date, increased expression of MetAP2 has been correlated with several types of cancer, for instance, mesothelioma,[14] neuroblastoma,[15] and colorectal carcinoma.[16] MetAP2 became a promising target molecule after the discovery that the potent anti-angiogenic agent fumagillin and its synthetic analogue ovalicin bind covalently and inhibit its aminopeptidase activity without affecting the ability of the enzyme to stabilize eukaryotic initiation factor-2.[17] TNP-470, one of the synthetic analogues of fumagillin that advanced to clinical trials,[18] as well as more recently published analogues with improved pharmacological profiles,[19] act similarly. Reversible inhibitors are generally considered as safer due to the decreased risk of toxicity or immunogenic responses. The non-covalent inhibitors of MetAP2 include compounds based on fumagillin,[20] bestatin,[21] 1,2,4-triazole,[22] and most recently pyrazolo[4,3-b]indole.[23] Other types of reversible ligands of MetAP2, such as anthranilic acid sulfonamides[24–26] and bengamides,[27] have also been reported.[28] In

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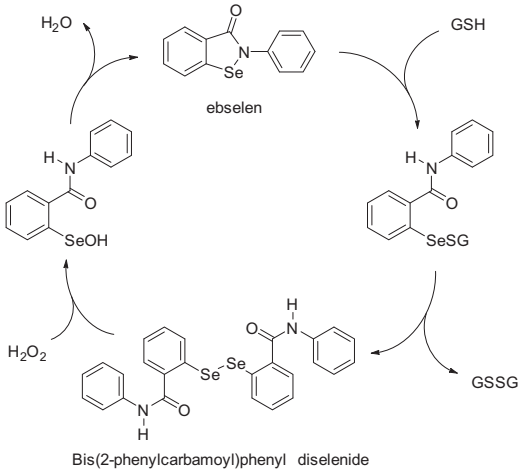
0960-894X/

this communication, we describe the discovery and optimization of the nontoxic synthetic selenium-containing drug ebselen as an inhibitor of MetAP2.

Ebselen, IUPAC name 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one, also called PZ51, is a low-molecular-weight drug with a pleiotropic mode of action and with very low toxicity in humans.[29] It was first identified as an anti-inflammatory agent with glutathione peroxidase-like activity in living cells.[30] One of the most likely modes of action of ebselen is as follows: the Se–N bond is readily cleaved by the thiol group of glutathione (GSH) to produce the corresponding selenenyl sulfides, which undergo disproportionation toward GSSG and 2,2′-dicarbamoyldiphenyl diselenide. The diselenide is oxidized to the corresponding selenenic acid in the presence of hydroperoxides, such as hydrogen peroxide (H_2O_2). After water elimination, ebselen is regenerated (Scheme 1).[31,32]

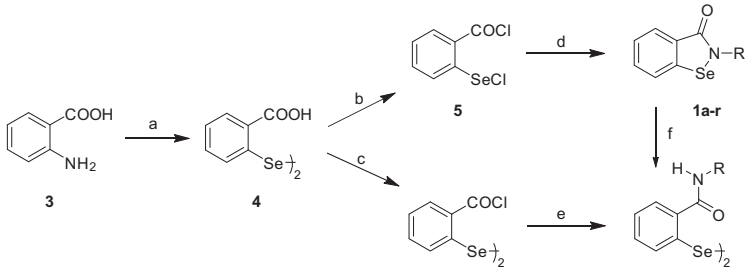
Ebselen is a well-known agent with therapeutic activity in neurological disorders, acute pancreatitis, and noise-induced hearing loss. It also exhibits antiatherosclerotic, antithrombotic, antioxidant and cytoprotective properties.[29,33–35] A recent study showed that hypoxia-induced cytotoxicity in human alveolar cells is reduced by ebselen owing to these properties.[36] An antiviral effect on hepatitis C virus nonstructural protein 3 was also recently demonstrated.[37] Ebselen and its derivatives act as antiproliferative compounds against pancreatic and renal,[38] liver, breast,[39] and lung cancer and cervical adenocarcinoma.[40] Organoselenium compounds exhibit strong electrophilic activity and are therefore capable of forming selenenyl-sulfide bonds with the cysteine residues in proteins.[32,41,42] The anticancer activity is usually linked to the inhibition of a selenocysteine-containing enzyme, thioredoxin reductase, that is overexpressed in many types of cancer.[43] All the above examples prove not only the broad spectrum of ebselen activity, but also convenience and safety of its use. However, no reports are available that show the relationship between ebselen and aminopeptidases.



Scheme 1. Plausible mechanism for the GSH activity of ebselen.[31,32]

Ebselen has previously been prepared by several methods,[44–49] first by Lesser and Weiss in 1924.[49] In this work, we synthesized ebselen and its derivatives/analogues using a four-step literature procedure starting from cheap and easily available reagents, anthranilic acid and elementary selenium.[47,48] The overall procedure for obtaining ebselen, its derivatives/analogues and their acyclic dimeric forms is outlined in Scheme 2. The protonation of anthranilic acid (**3**), diazotization, and dilithium diselenide selenenylation with nitrogen elimination gave 2,2′-dicarboxydiphenyl diselenide (**4**), a stable crystalline compound. The reaction of diselenide with thionyl chloride in the presence of dimethylformamide (DMF) produced 2-(chloroseleno)benzoyl chloride (**5**) or its acyclic form **6** depending on the quantity of thionyl chloride used, i.e., 7 equiv or 3.5 equiv, respectively. Acylation or tandem selenenylation/acylation reaction with appropriate amines alone or in the presence of Et_3N base in anhydrous acetonitrile (benzisoselenazolones **1a**–**r**) or appropriate amines in the presence of Na_2CO_3 in anhydrous dichloromethane (diselenides **2a**, **2c**–**e** and **2l**–**n**) completed the reaction sequence. We used ammonia or eighteen structurally diversified primary amines (methyl, aniline and its derivatives, including heteroatom- and halogen-substituted, benzyl, *p*-*tert*-butylbenzyl, and phenylethyl, Scheme 2). In particular, benzyl diselenide **2p** was prepared from corresponding benzisoselenazolone by hydrogenation with hydrazine monohydrate. The final compounds were purified by recrystallization or standard liquid column chromatography.

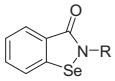
Twenty-six compounds, eighteen benzisoselenazol-3(2*H*)-ones (**1a**–**r**) and eight 2,2′-dicarbamoyldiaryl diselenides (**2a**, **2c**–**e**, **2l**–**n** and **2p**), ten of which are new (**1f**, **1i**–**m**, **1q**, **1r**, **2l** and **2m**), were obtained. The products were characterized by ^{1}H, ^{13}C, and ^{77}Se NMR spectroscopy, mass spectrometry and melting point (see Supplementary data).



R = Ph (**1a, 2a**), 4-methylphenyl (**1b**), 2-hydroxyphenyl (**1c, 2c**), 2-methoxyphenyl (**1d, 2d**), 4-methoxyphenyl (**1e, 2e**), 4-*n*-butoxyphenyl (**1f**), 3-fluorophenyl (**1g**), 4-fluorophenyl (**1h**), 2-methoxy-5-methylphenyl (**1i**), 4-methoxy-2-methylphenyl (**1j**), 5-*tert*-butyl-2-methoxyphenyl (**1k**), 2-chloro-4-methylphenyl (**1l, 2l**), 5-chloro-2-methoxyphenyl (**1m, 2m**), H (**1n, 2n**), Me (**1o**), Bz (**1p, 2p**), 4-*tert*-butylbenzyl (**1q**), 2-phenylethyl (**1r**)

Scheme 2. Synthesis of the benzisoselenazol-3(2*H*)-ones **1a**–**r** and bis(2-carbamoyl)phenyl diselenides **2a**, **2c**–**e**, **2l**–**n** and **2p**. Reagents and conditions: (a) (i) HCl, (ii) $NaNO_2$, 0 °C, (iii) Li_2Se_2, 0 °C; (b) 7 equiv $SOCl_2$, DMF, benzene, reflux; (c) 3.5 equiv $SOCl_2$, DMF, benzene, reflux; (d) RNH_2, Et_3N, MeCN, or RNH_2, MeCN, (e) RNH_2, Na_2CO_3, CH_2Cl_2, (f) $H_2NNH_2 \times H_2O$, MeOH, 25 °C.

Table 1
Inhibitory activity of ebselen and its derivatives obtained by substitution/functionalization of the phenyl ring toward MetAP2 aminopeptidase (measured after 30 min of incubation). The most significant inhibition is highlighted in bold. The activity of the reference compound (A832234), a transition state analogue inhibitor, is given



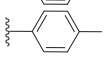
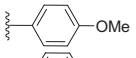
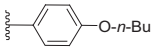
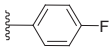
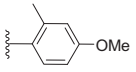
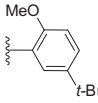
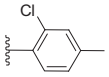
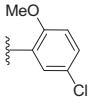
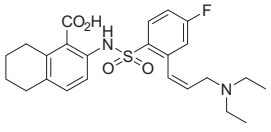
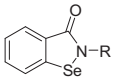
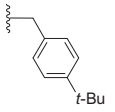
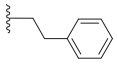
Entry	R	IC_{50} [μM]
1a, ebselen		2.43 ± 0.35
1b		24.3 ± 2.2
1c		10.2 ± 1.3
1d		9.98 ± 0.51
1e		2.07 ± 0.19
1f		6.27 ± 0.85
1g		0.935 ± 0.13
1h		10.9 ± 1.9
1i		0.92 ± 0.16
1j		1.35 ± 0.45
1k		2.67 ± 0.31
1l		**0.121 ± 0.066**
1m		2.35 ± 0.16
A832234, the reference compound		0.011[26]

Table 2
Inhibitory activity of ebselen analogues possessing substituents other than phenyl in position 2 toward human MetAP2 (measured after 30 min of incubation). The most significant inhibition is highlighted in bold



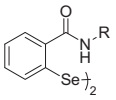
Entry	R	IC_{50} [μM]
1n	H	4.97 ± 0.25
1o	Me	10.4 ± 1.2
1p		**0.32 ± 0.09**
1q		8.85 ± 1.32
1r		7.57 ± 0.42

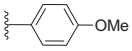
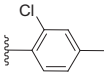
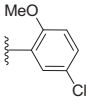
The compounds employed for inhibitory studies were formally divided into three groups. The first group included ebselen and 2-phenylbenzisoselenazol-3(2*H*)-ones with the phenyl rings mono- or disubstituted with different residues and functional groups, such as Me, *t*-Bu, OH, OMe, *n*-BuO, and halogens (**1a**–**m**). The second group (compounds **1n**–**r**) comprised analogues of ebselen based on the benzisoselenazol-3(2*H*)-one core modified in position 2 (the nitrogen atom). Instead of phenyl, the benzisoselenazolone system remained unsubstituted (**1n**) or it was substituted by methyl (**1o**), benzyl (**1p**), *p*-*tert*-butylbenzyl (**1q**) or phenylethyl (**1r**). The structure of the third group of the studied compounds (**2a**, **2c**–**e**, **2l**–**n**) was based on diselenide, the acyclic form of ebselen. Seven derivatives were chosen to investigate the mechanism by which this modification influences the activity. As mentioned in the introductory part the dimeric Se–Se form of ebselen has been assigned as the product of benzisoselenazolone interference with glutathione in physiological conditions. Thus, measuring the activity of 2,2′-dicarbamoyldiaryl diselenides is of fundamental value in the context of *in vivo* studies.

The collection of compounds was screened for the inhibition of three neutral metallo-aminopeptidases, APN, LAP and MetAP2 (see Supplementary data). Organoselenium products were found to be good, generally low-micromolar-range, slow-binding inhibitors of exclusively MetAP2. They were inactive toward APN and LAP below inhibitor concentration 2 mM, apparently due to the lack of specific interactions within their binding sites. Steady-state binding of the inhibitors to the active site of MetAP2 was achieved over time. The slow binding mechanism was not defined, the organoselenium compounds showed a mixed type A and B mechanism. The plot of the first-order rate constant (k_{app}) versus inhibitor concentration ([I]) was neither linear (type A) nor hyperbolic (type B, see Supplementary data). Therefore, it was not possible to distinguish between mechanism type A (reversible slow-binding) and type B (enzyme isomerization).[50] The non-covalent, competitive mode of inhibition clearly predominates. This was confirmed by regain of the enzymatic activity after dilution experiments and dialysis. However, we do not exclude conformational changes upon covalent modifications of solvent-exposed cysteine residues. To avoid uncertainties we decided to present the inhibitory potency as the half maximal inhibitory concentration (IC_{50}) determined after 30 min incubation of enzyme with an inhibitor (Tables 1–3).

Selenazolones of the first group appeared moderate to good inhibitors of methionine aminopeptidase 2, with nearly all of the IC_{50} constants in the low micromolar range, e.g. 2.43 μM for the lead ebselen (Table 1). The inhibitory potency varied within an order of magnitude up and down to ebselen depending on character and position of substitution. For example the affinity of compound **1b** with methyl substitution in the *para* position is diminished ten-fold compared to **1a**. In the case of substitution by hydroxy (**1c**) or methoxy group (**1d**) at the *ortho* position, the activity also decreased (four-fold). A methoxy group in the *para* position (**1e**) did not influence the inhibitory potency, whereas a butoxy group in the *para* position (**1f**) reduced the activity. Therefore, electron-donating functional groups were generally not well tolerated. The without strongly electron-withdrawing fluoride substitution exerted an ambiguous influence on the affinity of ligand. Fluoride in the *meta* position (**1g**) increased the inhibitory potency to the submicromolar range. However, the opposite effect was

Table 3
Inhibitory activity for diselenides, the acyclic forms of ebselen and analogues, toward human MetAP2 (measured after 30 min of incubation)



Entry	R	IC_{50} [μM]
2a		**0.23 ± 0.04**
2c		5.44 ± 0.71
2d		6.79 ± 0.23
2e		13.9 ± 0.23
2l		119.2 ± 4.8
2m		4.47 ± 0.38
2n	H	9.13 ± 0.96
2p		3.43 ± 0.63

measured for **1h** with F in the *para* position. The combination of a methyl in the *meta* position with a methoxy group in the non-adjacent *ortho* position in compound **1i** position resulted in a three-fold improvement in the IC_{50} value to 920 nM. The substitution of the compound **1d** with chlorine (**1m**) improved the inhibitory potency by four-fold. A similar modification in the case of compound **1b** resulted in the best inhibitor in this study (**1l**, IC_{50} = 121 nM).

Replacing phenyl in position 2 with less hydrophobic substituents, hydrogen (**1n**) or methyl (**1o**), was not beneficial (Table 2). The inhibition constants indicated two- and four-fold drop in activity, respectively. In contrast, benzyl substitution (**1p**) resulted in a very active inhibitor with IC_{50} = 320 nM, improved by almost one order of magnitude compared with ebselen. Interestingly, there is an increase in affinity only in case of the compound with benzyl substitution. Further extension of the hydrophobic fragment to *p*-*tert*-butylbenzyl (**1q**) or phenylethyl (**1r**) was no more profitable.

The impact of the opening of isoselenazolones on the inhibitory activity was also tested. Interestingly, the acyclic diselenide form of ebselen (**2a**) was found to be one order of magnitude more potent than **1a**. For other cases there is no essential difference in affinity between compounds **1c**–**e**, **1m**, **1n** and **1p** and corresponding diselenides. Only for the *o*-chloro-*p*-methylphenyl substituent, the acyclic counterpart (**2l**) proved to be three orders of magnitude less active than **1l**.

The SAR discussion was further illustrated by molecular modeling study on the binding mode of the most significant ligands with human MetAP2. This study confirmed the binding to the active site and thus, competitive mechanism of inhibition. The modeled interactions of ebselen (**1a**) shows that the molecule occupies an intersection between putative S1 and S1′ regions, with the *N*-phenyl ring buried deeper, and adopts a bent-shaped conformation which fits well to the hydrophobic environment (Fig. 2A and Graphical Abstract). Edge-to-face interactions between surrounding imidazoles and aromatic rings of the inhibitor, namely His231 with *N*-phenyl and both His231 and His331 with the ligand bicyclic system can be proposed as the principal hydrophobic contacts. The oxygen atom of the selenazole ring coordinates well with one of the active site metal ions. The close O···Co distance is 1.13 Å while the other contact is not tight (3.43 Å).

Indiscriminate docking results were obtained upon modeling interactions of the most potent inhibitor **1l** with the MetAP2 active site. In this case more than one conformation showed comparable thermodynamic stability. The binding mode corresponding to that described for ebselen was one of those possibilities. A conformation characterized by the rotated plane of benzoisoselenazolone

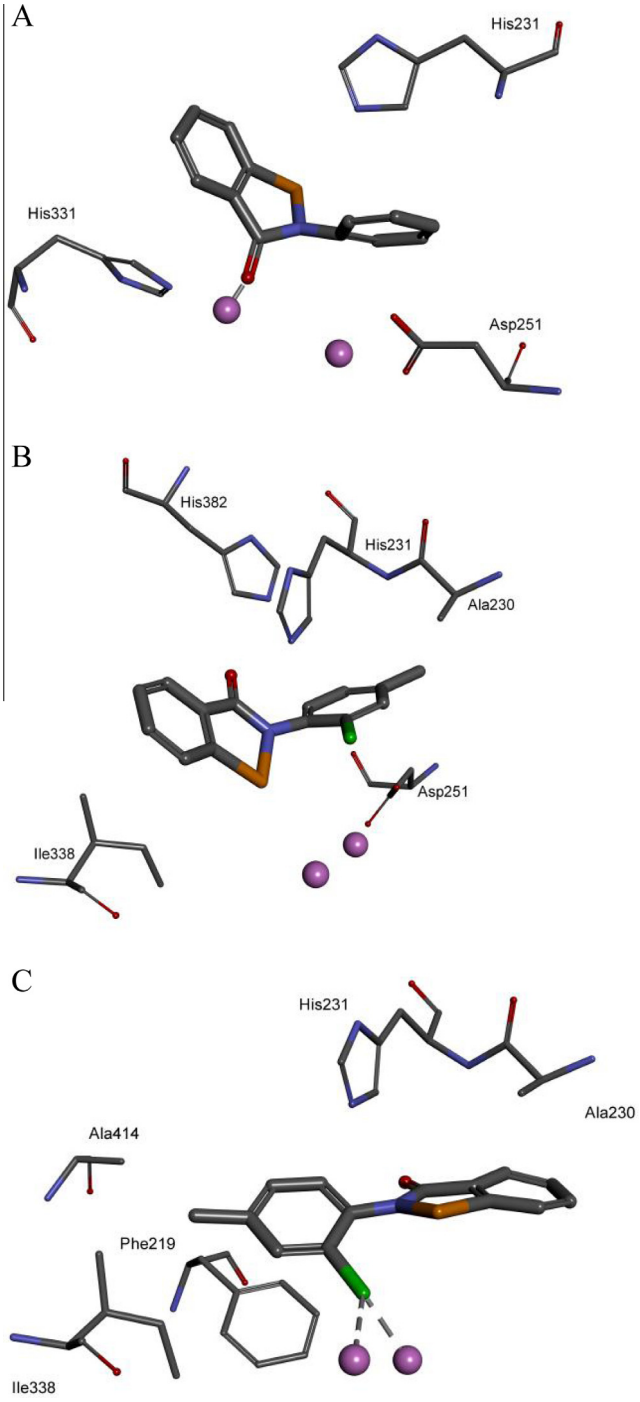


Figure 1. The privilege conformation of ebselen (A) and alternative conformations in the model of the complex of ebselen derivative **1l** (B and C) with human MetAP2[19] (PDB ID code 5D6E). Metal–oxygen interactions are marked in gray.

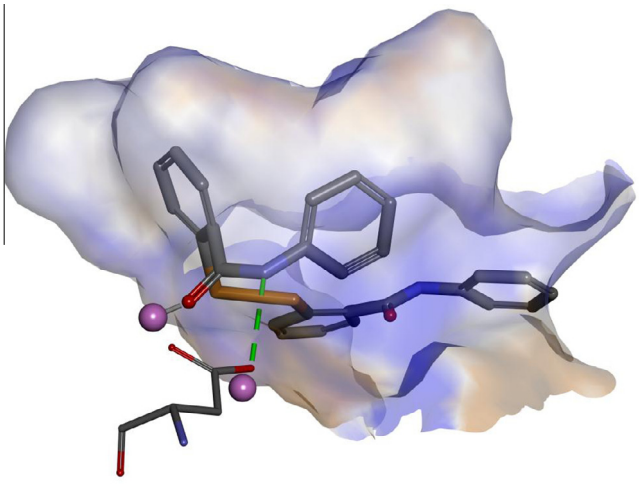


Figure 2. A model of the complex of compound **2a** with human MetAP2[19] (PDB ID code 5D6E). The hydrophobic active site surface is marked in blue (hydrophilic in brown), metal–oxygen interactions are marked in gray, intermolecular hydrogen bond ligand–NH···Asp262 carboxylate is marked in green.

ring was identified as the second, almost equally favorable option (Fig. 1B). In this case the selenium side of the bicyclic system is pointed toward cobalt ions. However, the minimal energy was calculated for a reversed arrangement, with the selenazolone fragment buried deeper in the binding pocket (Fig. 1C). This conformation does not demand any fold of the structure, the rings are only slightly twisted to each other. The key hydrophobic contacts with His231 and His331 are well conserved in both the rotated and inversed version. An increased lipophilic surface (2-chloride and 4-methyl groups) of the inhibitor gives rise to further stacking involving mainly Ile338, and additionally with Phe219 for the most privileged conformation depicted in Figure 1C. Importantly, in the last-mentioned arrangement, chloride coordinates with the cobalt ions (2.26 Å and 3.01 Å). These favorable structural features seems to be responsible for an improved activity of **1l** compared with ebselen (IC_{50} = 0.121 vs 2.43 μM).

The opening of the selenazole ring at the selenium-nitrogen bond and dimerization increased the inhibitory potency of ebselen by one order of magnitude (IC_{50} = 0.23 μM for diselenide **2a**). In the arrangement predicted by molecular modeling, the typical coordination between the cobalt ions and the oxygen of the carbonyl group are well reproduced (compare Fig. 1A). The distances are shorter than for **1a**, 1.14 Å and 3.01 Å, respectively. Moreover, a hydrogen bond was identified between the nitrogen atom of the ligand amide group and the carboxyl of Asp262 (2.10 Å). Aromatic rings interact tightly with the surrounding residues (Ala141, Phe219, His231, Ala230, Asp251, Leu328, Ile338 and Tyr444) employing the whole range of π–π, π–alkyl and π–anion contacts (outlined in Fig. 2 as a fit to the hydrophobic surface, see also Graphical Abstracts). For diselenides even the simplest analogue **2a** fits extremely tightly to the MetAP2 binding site, thus, any further extension can alter the complementarity. Indeed, only **2c** and **2d**, containing an oxygen atom in the *ortho* position, slightly improved the potency. The improvement was correlated with hydrogen bonding formation, e.g. with Asn329 (2.72 Å) for **2c**, however, to achieve this contact the diselenide backbone is forced to adopt a sterically demanded arrangement.

The inhibition of human methionine aminopeptidase 2 has been identified as a crucial way to inhibit angiogenesis during the growth and metastasis of solid tumors.[9,15] Here, we reported a well-known antioxidant drug ebselen[21] and its derivatives/analogues as inhibitors of MetAP2 with IC_{50} values in the low micromolar and submicromolar range. The structure optimization of the lead compound resulted in an improvement of the inhibitory potency from 2.43 μM to 0.121 μM. Increasing the size of the *N*-phenyl fragment of ebselen by disubstitution with a 2-chloro and a 4-methyl group was so beneficial. Extension of *N*-phenyl group to *N*-benzyl also resulted in a very potent inhibitor with an inhibition constant of 0.32 μM. Among the group of diselenides, the acyclic forms of the benzisoselenazolones, the opened form of ebselen was the most active: the IC_{50} value decreased by one order of magnitude compared to the lead, reaching 0.23 μM. Our discovery may result in a widening range of applications of the antioxidant drug ebselen as an antiangiogenic agent. Ebselen has the following two undoubted advantages: (1) it has passed clinical trials, and there are no barriers to using it in humans; (2) it is selective toward MetAP2 versus other neutral aminopeptidases such as APNs and LAP. It must be remembered that in the human organism, benzisoselenazolones, glutathione peroxidase mimetics, are transformed to the corresponding diselenides[31,32] which, as we showed here, can also bind to MetAP2 even with higher affinity. Thus, ebselen can be considered as the drug and a prodrug at the same time. The identified active organoselenium compounds may be useful tools in basic studies on the function of MetAP2 and profitable in the development of new generations of therapeutic agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.09.050.

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