Endothelin-Converting Enzyme-1 Inhibition and Growth of Human Glioblastoma Cells

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Endothelin-1 (ET-1) is mitogenic and/or antiapoptotic in human cancers, and antagonists to ET-1 receptors are under evaluation for cancer treatment. Inhibition of ET-1 activation by the endothelin-converting enzymes 1_{a-d} (ECE-1_{a-d}; EC 3.4.24.71) represents another approach to block the ET-1 effect in cancer. To evaluate this potential, we synthesized and characterized a series of low nanomolar nonpeptidic thiol-containing ECE-1 inhibitors, and evaluated their effect, as well as the effect of inhibitors for the related metalloproteases neprilysin (NEP; EC 3.4.24.11) and angiotensin-converting enzyme (ACE; EC 3.4.15.1), on human glioblastoma cell growth. Only ECE-1 inhibitors inhibited DNA synthesis by human glioblastoma cells. Exogenous addition of ET-1 or bigET-1 to glioblastoma cells did not counterbalance the growth inhibition elicited by ECE-1 inhibitors, suggesting that ECE-1 inhibitors block the proliferation of human glioblastoma cells most likely via a mechanism not involving extracellular production of ET-1. This class of molecules may thus represent novel therapeutic agents for the potential treatment of human cancer.

Introduction

Endothelin-1 (ET-1) is a 21-amino acid peptide enzymatically activated from its inactive precursor, bigET-1, by endothelin-converting enzyme-1 (ECE-1; EC 3.4.24.71) to yield the active peptide.[1] At least four ECE-1 isoforms, ECE-1_{a-d}, have been characterized and are localized in different subcellular compartments.[2] ET-1 acts on two distinct high-affinity receptor subtypes, ET_A and ET_B, which belong to the family of seven transmembrane G-protein-coupled receptors (GPCR). In addition to its potent vasoconstrictor activity, ET-1 is an autocrine/paracrine comitogen in many cell types, including human brain[3] and cancers.[4,5] We have previously demonstrated the expression of the complete endothelin system, at the mRNA and protein levels, in human glioblastoma and human colon carcinoma.[6−8] Our results in both cancers suggested a potential function for ET-1 as a survival factor for human tumor cells, and blockade of ET-1 receptors by a dual ETA/ETB receptor antagonist either induced apoptosis or sensitized cells to induced apoptosis.

Another approach to achieve the blockade of ET-1 effect in cancer might be to control its activation by inhibiting ECE-1. Neither ECE-1 inhibition in human cancer cells nor the evaluation of the effect of this inhibition on cell function has hitherto been described. To achieve ECE-1 inhibition, we prepared and characterized a series of very potent nonpeptidic ECE-1 inhibitors whose effects were evaluated on the proliferation of human glioblastoma cells in culture.

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Results

Synthesis of ECE-1 Inhibitors. Synthesis of Carbamate and Pyrimidine Pyrrolidine Ethers (Scheme 1). The synthesis started with the readily available chiral *N*-BOC-trans-4-hydroxy-L-proline methyl ester **1**. Introduction of the essential thiol group in position 4 was achieved using an efficient two-step double-inversion procedure. First, chloride **2** was synthesized with carbon tetrachloride and triphenylphosphine in dichloromethane followed by reaction of potassium triphenylmethylthiolate in DMF to give the trityl-protected **3**.[9] Reduction with lithium aluminum hydride gave alcohol **4**, which was deprotonated with sodium hydride and benzylated with the fluoro-substituted benzyl bromide to give ether **5**. BOC deprotection gave the secondary amine **6**, which was a key building block to evaluate the scope and limitations of the R^1 residues. Reaction with trichloromethyl chloroformate and quinoline as a base gave an intermediate carbamoyl chloride, which was reacted with the sodium salt of methyl salicylate to give the carbamate **7**. Trityl deprotection with triethylsilane in trifluoroacetic acid gave the free thiol **8**. 2,3-Dihydro-benzo[1,4]dioxin-5-yl-substituted derivative **10** was synthesized in a similar way. Reaction of intermediate **6** with 1,4-benzodioxan-5-yl chloroformate[10] and pyridine followed by trityl deprotection in trifluoroacetic acid and triethylsilane gave thiol **10**. Pyrimidine-substituted derivative **11** was synthesized from amine **6** in 2-chloro-5-*n*-propylpyrimidine and *N*,*N*-diisopropylethylamine with a catalytic amount of copper(I) iodide. In this case, attempts to deprotect the thiol using the same reaction conditions as described before (triethylsilane in trifluoroacetic acid) resulted in the reduction of the pyrimidine. Therefore, the reaction was performed using milder conditions with triisopropylsi-

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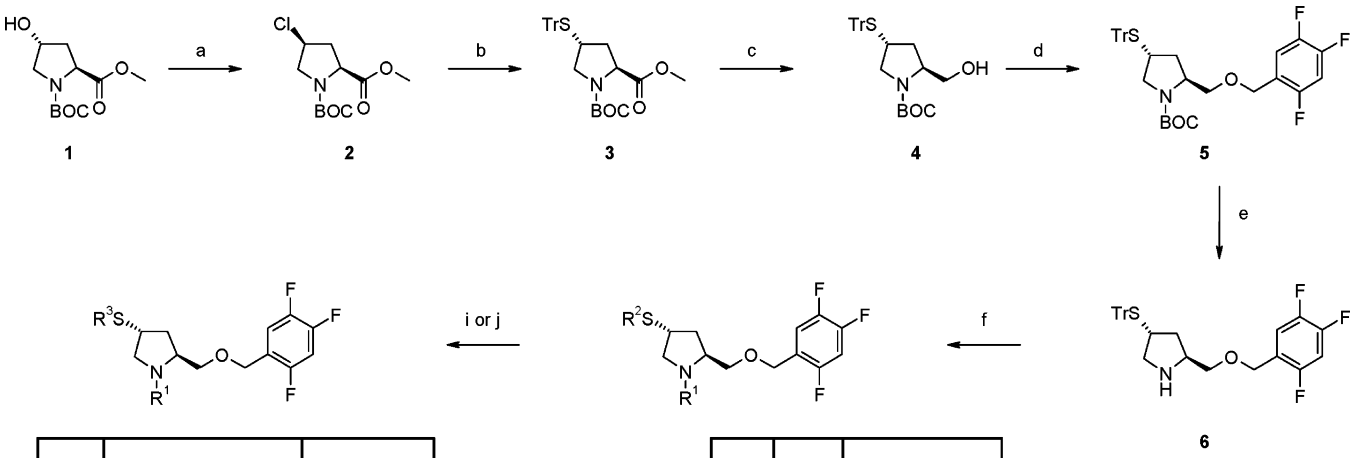
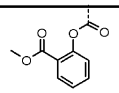
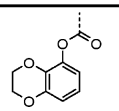
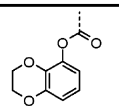
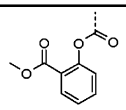
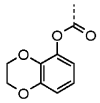
Scheme 1. Synthesis of Pyrrolidine Ethers ECE Inhibitors[a]



	R1	R3
13		Acetyl
14		Acetyl
15		Benzoyl

	R2	R1
7	Tr	
8	H	
9	Tr	
10	H	
11	Tr	
12	H	

[a] Reagents and conditions: (a) PPh_3/CCl_4, CH_2Cl_2, 3 °C to rt; (b) Ph_3CSH/KO*t*-Bu in DMF, 0 °C to rt; (c) $LiAlH_4$, THF, −20 °C; (d) NaH, 2,4,5-trifluorobenzyl bromide, DMF, 0 °C to rt; (e) TFA, CH_2Cl_2, −20 °C to rt; (f) for **7**, (1) trichloromethyl chloroformate/quinoline, CH_2Cl_2, 0 °C, (2) methyl salicylate/NaH/NaI, THF, 0 °C to reflux; for **9**, 1,4-benzodioxan-5-yl chloroformate/pyridine, THF, 0 °C; for **11**, 2-chloro-5-*n*-propylpyrimidine/EtN(*i*-Pr)/CuI, 80°C; (g) TFA/Et_3SiH, 0 °C to rt; (h) TFA/*i*-Pr_3SiH CH_2Cl_2, 0 °C to rt; (i) for **13** and **14**: acetyl chloride, pyridine, 0 °C to rt; (j) for **15**: benzoyl chloride, pyridine, 0 °C to rt.

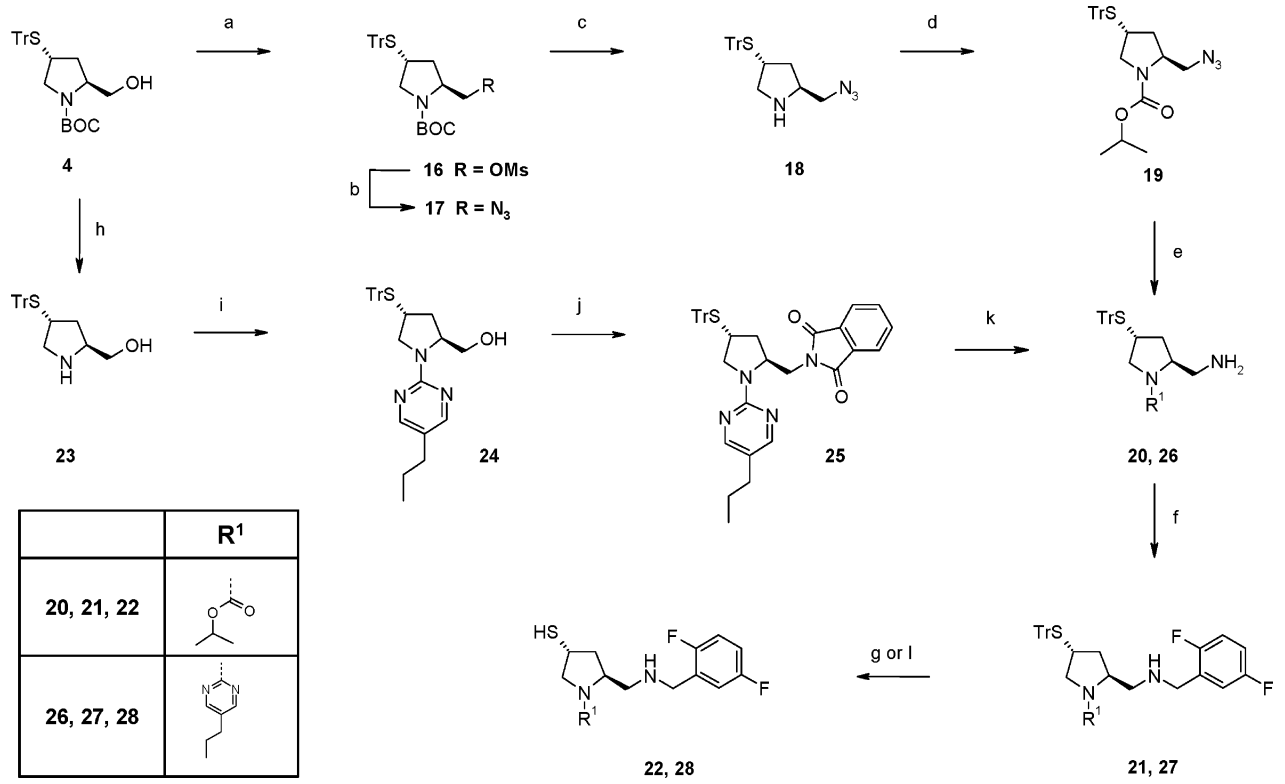
lane and trifluoroacetic acid in dichloromethane to give the thiol **12**. Acetyl prodrug **13** was synthesized from compound **8** with acetyl chloride in pyridine. The same procedure was applied to thiol derivative **10** to give the acetyl prodrug **14**. In a similar manner the benzoyl prodrug **15** was prepared from **10** with benzoyl chloride.

Synthesis of Carbamate and Pyrimidine Pyrrolidine Amines (Scheme 2). The synthesis of the pyrrolidine amine derivatives **22** and **28** started from the common intermediate **4**. For the introduction of the amine moiety a two-step procedure was used. Mesylation of the alcohol **4** with methanesulfonyl chloride in the presence of DMAP [4-(dimethylamino)pyridine] yielded the mesylate **16**, which was converted to the azide **17** by treatment with sodium azide. BOC deprotection and subsequent conversion of the amine **18** by reaction with isopropyl chloroformate in the presence of *N*,*N*-diisopropylethylamine and DMAP gave compound **19**. Staudinger reduction of the azide residue of **19** gave the amine derivative **20**, which was subjected to a reductive alkylation with 2,5-difluorobenzaldehyde ($ZnCl_2$, $NaBH_3CN$) to yield **21**. Trityl deprotection with trifluoroacetic acid in the presence of triethylsilane as scavenger[11] provided **22**.

An alternative synthetic route was used for the preparation of **28**. Cleavage of the BOC protecting group was achieved by treatment of **4** with trifluoroacetic acid. The resultant amine **23** was treated with 2-chloro-5-*n*-propylpyrimidine in *N*,*N*-diisopropylethylamine in the presence of CuI to yield **24**. Mitsunobu reaction with phthalimide as a nucleophile[12,13] provided **25** which was converted to the amino derivative **26** by hydrazinolysis of the phthalimide protecting group. Reductive alkylation of **26** with 2,5-difluorobenzaldehyde ($ZnCl_2$, $NaBH_3$-CN), analogously to compound **20**, yielded **27**. Trityl deprotection in the presence of triisopropylsilane as scavenger gave pyrrolidine amine **28**.

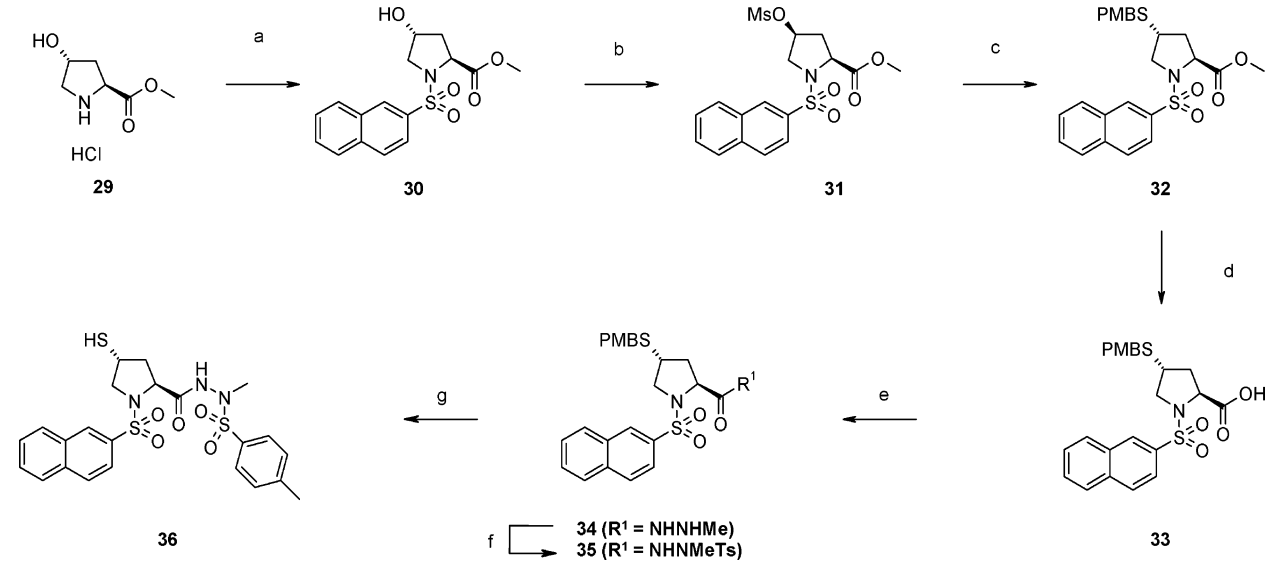
Synthesis of a Proline Hydrazide (Scheme 3). The starting point for the synthesis of the proline hydrazide **36** was the commercially available L-4-hydroxyproline methyl ester·hydrochloride **29**, which was first treated with hexamethyldisilazane at reflux. Then the in situ protected derivative was reacted with 2-naphthylsulfonyl chloride in the presence of diisopropylethylamine to give compound **30**. Introduction of the mercapto group in the 4-position under net retention of the configuration was accomplished by a two-step procedure. Sulfonation using Mitsonubu conditions with methanesulfonic acid, triphenylphosphine, triethylamine, and diethylazodicarboxylate yielded **31** with inversion of the configuration.[14] The second inversion of the configuration was achieved by treatment of the mesylate **31** with the potassium salt of 4-methoxybenzylmercaptane, previously prepared from potassium *tert*-butylate and 4-methoxybenzylmercaptane. Saponification of the methyl ester moiety of **32** using lithium

Scheme 2. Synthesis of Pyrrolidine Amines ECE Inhibitors[a]



[a] Reagents and conditions: (a) $MeSO_2Cl$, pyridine, DMAP, CH_2Cl_2, 0 °C to rt; (b) NaN_3, DMF, 80 °C; (c) (1) TFA, CH_2Cl_2, 0 °C to rt; (2) aq $NaHCO_3$, EtOAc; (d) isopropyl chloroformate, $EtN(i\text{-}Pr)_2$, DMAP, CH_2Cl_2, 0 °C to rt; (e) Ph_3P, THF, rt; (f) 2,5-difluorobenzaldehyde, $ZnCl_2$, $NaBH_3CN$, MeOH, rt; (g) Et_3SiH, TFA/CH_3CN, 50 °C; (h) (1) TFA, CH_2Cl_2, 0 °C to rt; (2) aq $NaHCO_3$, EtOAc; (i) 2-chloro-5-*n*-propylpyrimidine, CuI, $EtN(i\text{-}Pr)_2$, 80 °C; (j) phthalimide, Ph_3P, DEAD, THF, 0 °C to rt; (k) $NH_2NH_2{\cdot}H_2O$, EtOH, Δ; (l) $i\text{-}Pr_3SiH$, TFA/CH_2Cl_2, 0 °C.

Scheme 3. Synthesis of Proline Hydrazide ECE Inhibitors[a]

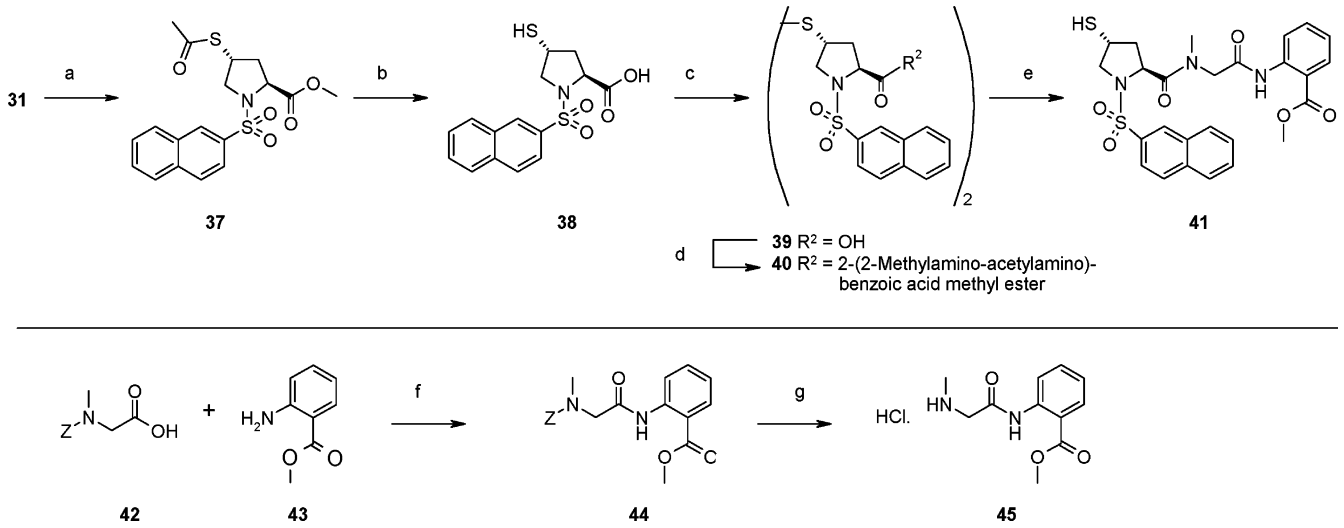


[a] Reagents and conditions: (a) (1) hexamethyldisilazane, reflux; (2) 2-naphthylsulfonyl chloride, $EtN(i\text{-}Pr_2)$, THF, rt; (b) $MeSO_3H$, NEt_3, Ph_3P, DEAD, toluene/THF, 80 °C; (c) KO*t*-Bu, 4-MeOPhCH_2SH, DMF, 100 °C; (d) (1) LiOH, THF, rt; (2) 1 M $KHSO_4$, EtOAc; (e) N-hydroxy-2-pyridone, DCC, NH_2NHMe, NEM, CH_2Cl_2, rt; (f) toluene-4-sulfonyl chloride, $EtN(i\text{-}Pr)_2$, DMAP, CH_2Cl_2, rt; (g) Et_3SiH, TFA, 80 °C.

hydroxide followed by workup under acidic conditions gave the desired acid **33**. Carboxylic acid **33** was coupled to methylhydrazine by treatment with *N*-hydroxy-2-pyridone, DCC, and *N*-ethylmorpholine.[15,16] Tosylation of **34** using toluene-4-sulfonyl chloride and diisopropylethylamine in the presence of DMAP provided **35**. Deprotection was accomplished by treatment with trifluoroacetic acid in the presence of triethylsilane as scavenger[11] to give the pyrrolidine hydrazide **36**.

Synthesis of a Proline Amide (Scheme 4). Treatment of the mesylate **31** with potassium thioacetate gave **37** with inversion of the configuration at the 4-position.[17] Saponification of the methyl ester moiety of **37** and concomitant hydrolysis of the S-acetate yielded

Scheme 4. Synthesis of Proline Amide ECE Inhibitors[a]



[a] Reagents and conditions: (a) CH_3COSK, DMF, 100 °C; (b) (1) LiOH, THF, rt; (2) 1 M $KHSO_4$, EtOAc; (c) (1) I_2, EtN(*i*-Pr)$_2$, CH_2Cl_2, room temp; (2) 1 M $KHSO_4$, EtOAc; (d) **45**, NMM, TPTU, CH_2Cl_2, rt; (e) (1) MeOH, 2 M K_2CO_3, 1,4-dithiothreitol, rt; (2) 1 M $KHSO_4$, EtOAc; (f) NMM, TPTU, CH_2Cl_2, room temp; (g) (1) H_2, Pd/C, MeOH; (2) HCl.

38. The disulfide derivative of compound **38** was obtained by treatment with iodide and diisopropylethylamine. The dicarboxylic acid **39** and the amine **45** were converted to the diamide **40** using the coupling agent TPTU [*O*-(2-oxo-1(2*H*)pyridyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate][18] in the presence of *N*-methylmorpholine. Compound **45** was prepared by a three step procedure involving the coupling of **42** with **43** in the presence of TPTU and *N*-methylmorpholine followed by cleavage of the Z protecting group by hydrogenation (Pd/C, MeOH) and treatment of the amine with hydrochloric acid. Reductive cleavage of the disulfide bond was accomplished by treatment of **40** with 1,4-dithiothreitol under alkaline conditions (2 M K_2CO_3) and acidic work up (1 M $KHSO_4$, ethyl acetate), yielding **41**.

Biological Assays. The endothelin-converting enzyme-1 (ECE-1; EC 3.4.24.71) screening assay was first validated by determining the inhibitory constants of phosphoramidon (IC_{50} 0.8 ± 0.2 μM) and CGS 314447 (IC_{50} 20 ± 4 nM)[19] for bigET-1 hydrolysis. The IC_{50} values for soluble ECE-1 found for these two inhibitors did not significantly differ from those described in the literature. Under more physiological conditions in the MDCK-ECE-1_c-transfected cell-based assay, an IC_{50} of 4 μM was found for phosphoramidon. Thiorphan, a selective NEP inhibitor, was not tested, as no neprilysin (NEP; EC 3.4.24.11) activity was present in MDCK-ECE-1_c-transfected cells. The ECE-1-inhibitors from four different classes, displaying low nanomolar activities against ECE-1 in binding assay and in the MDCK-ECE-1_c-cell based assay (listed in Table 1, ECE RIA, ECE cell, respectively), were selected for further evaluation of their effect in human glioblastoma cells.

The evaluation of these ECE-1 inhibitors on [^{3}H]thymidine (^{3}HT) incorporation, to quantify DNA synthesis in the human LN18 and LNZ308 glioblastoma cells, demonstrated the efficacy of these molecules (Figure 1); however, compounds **14**, **28**, and **41** displayed a plateau. An evaluation of the relationship between structure and efficacy demonstrated that both the acetyl thiol-protected prodrug **13** and the unprotected **8** derivatives were equally potent, suggesting that the thiol was deprotected and that hydrolysis of the prodrug was not a limiting factor for the inhibition of DNA synthesis by this class of compounds. This information was further supported by comparison of the derivatives **14** (acetyl), **15** (benzoyl), and **10** (thiol, not shown). However, in LN18 cells, but not in LNZ308 cells, **15** was less efficient than **14**, suggesting that the cellular enzyme(s) required for hydrolysis of the prodrug of the thiol may be differently expressed or selective in cells of a similar phenotype. The replacement of the ether- or amine-based spacer with an amide or hydrazide spacer was not detrimental for inhibition of DNA synthesis, since **36** and **41** gave comparable inhibition. However, these two inhibitors were the weakest inhibitors of this series of thiol-based ECE-1 inhibitors. The comparison between the pyrimidine ether **12** and pyrimidine amine **28** showed that neither the ether nor the amine significantly changed the potency of these compounds. However, **28** was more efficient in LN18 cells than in LNZ308 cells. The most potent inhibitor, the acetyl prodrug **14**, and the corresponding free thiol **10** were selected for further investigations.

Table 1. Biological Characteristics of the ECE-1 Inhibitors

	IC_{50} (μM)[a]				DNA synthesis inhibition IC_{50} (μM)	
compd	ECE RIA	ECE cell	ACE	NEP	LN18	LNZ308
8	0.0012	0.0670	13.4	>100	19	40
10	0.0017	0.1370	12.2	>100	28	28
12	0.0012	0.0198	15.7	>100	17	33
13	0.0011	0.0215	>100	>100	15	22
14	0.0069	0.0765	>100	>100	14	16
15	0.0497	0.4805	>100	>100	18	20
22	0.0160	1.0900	>100	>100	27	50
28	0.0104	0.0729	31.5	>100	19	40
36	0.0613	2.5300	14.6	39.2	25	24
41	0.0808	10.890	36.6	81.9	12	16

[a] The inhibition of ECE-1 activity (ECE RIA) was determined by measuring the ET-1 released from bigET-1 by membrane-solubilized ECE-1 or by ECE-1-transfected MDCK cells (ECE cell). The inhibition of ACE and NEP activity was determined with soluble enzymes. The inhibition of DNA synthesis (DNA synthesis inhibition) in human glioblastoma cells was determined by quantifying [^{3}H]thymidine incorporation.

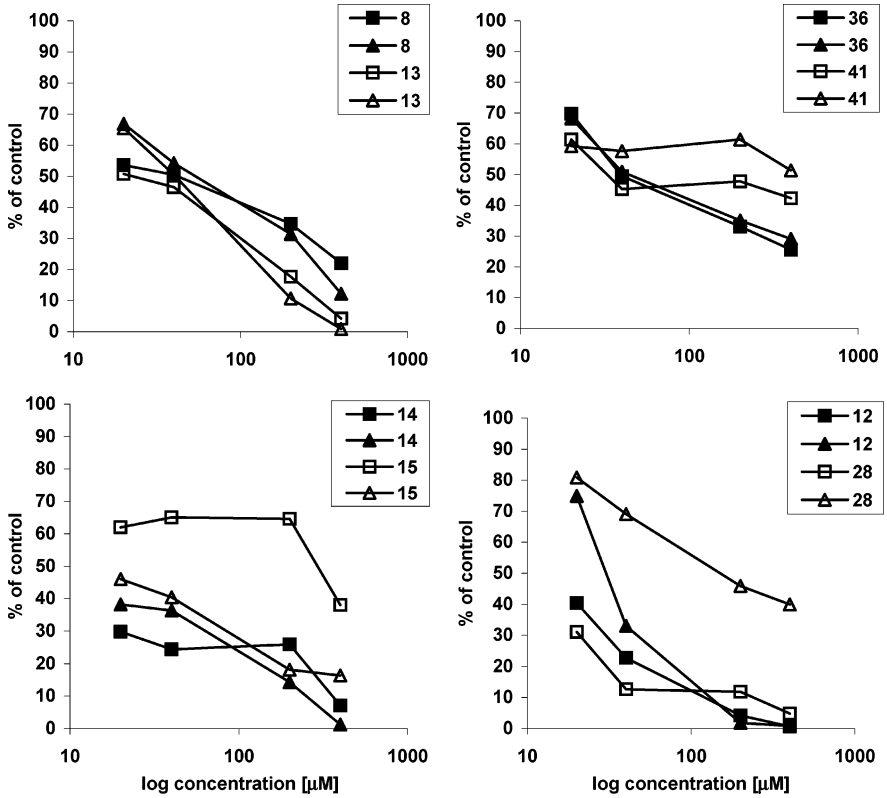


Figure 1. Dose−response effect on DNA synthesis of long-term (18 h) exposure of human glioblastoma cells to ECE-1-inhibitors. LN18 or LNZ308 cells were grown to confluence in culture medium containing FCS and then exposed for 18 h to the ECE-1 inhibitors at increasing concentration (0−400 μM) in complete fresh medium. [^{3}H]Thymidine (^{3}HT) incorporation to quantify DNA synthesis was performed during the last 2 h of incubation: ■, □, LN18; ▲, △, LNZ308 cells.

We first determined whether these inhibitors were fast-acting or needed a long period of cell exposure to be active. DNA synthesis inhibition by **14** at 100 and 200 μM was already maximal after 2 h and did not further increase for up to 7 h of cell exposure, in the LN18, LN229, or LNZ308 cells (Figure 2). A comparison of the dose−response curves of DNA synthesis inhibition measured after 2 h (Figure 3) or 18 h (Figure 1) showed that the curves were similar. These results demonstrate the rapid inhibitory effect of compounds **10** and **14** on DNA synthesis in human glioblastoma cells.

To exclude a possible nonselective effect of these inhibitors on two ECE-1-related enzymes, NEP and ACE, we also evaluated the effect of inhibitors of these enzymes on glioblastoma cells. The NEP inhibitors phosphoramidon (an inhibitor with low nanomolar activity on NEP and low micromolar activity on ECE-1) or D,L-thiorphan (a thiol-based inhibitor of this enzyme) did not modify DNA synthesis in human LN18, LN229, and LNZ308 glioblastoma cells (Figure 4), even at high inhibitor concentrations. A similar result was obtained using the ACE inhibitors captopril (a thiol-based inhibitor of this enzyme) or lisinopril in human LN18 and LNZ308 glioblastoma cells, measuring either DNA synthesis (^{3}HT incorporation) or the number of metabolically active cells (3,4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium/MTT reduction) (Figure 5).

We then evaluated if the blockade of extracellular production of ET-1 was involved in the inhibition of DNA synthesis by ECE-1 inhibitors in human glioblastoma cells. First we determined the ETA and/or ETB receptor-mediated ET-1 binding capacity of human glioblastoma cells, by measuring the displacement of radioactive ET-1 ([3-^{125}I-Tyr]-endothelin-1) by exogenous ET-1 added to cells, in the presence of an ETA-selective (BQ123) or ETB-selective (BQ788) receptor antagonist (Figure 6). The results showed that only LN229 cells (Figure 6), but not LN18 and LNZ308 cells (results not shown), expressed measurable ET-1 binding mediated exclusively by the ETB receptor. This result suggested that ET-1 production and ET-1 binding to its membrane receptor were not involved in the inhibition of DNA synthesis by ECE-1 inhibitors in human glioblastoma cells. This finding was further confirmed by exogenous ET-1 or bigET-1 (results not shown) added at 1, 10, or 100 nM to LN18, LN229, and LNZ308 cells together with the ECE-1 inhibitor **14** at 50, 100, or 300 μM for 18 h (Figure 7). Experiments were repeated with a shorter exposure (2 h) of cells to compound **14**, in the presence of ET-1 and thiorphan to inhibit ET-1 degradation by NEP (Figure 8), demonstrating that inhibition of DNA synthesis was comparable under all experimental conditions. Thus the presence of exogenous ET-1 did not counterbalance the inhibitory effect of ECE-1 inhibitors on cell growth, independent of the expression level of functional cell membrane ET-1 receptor. Moreover, extracellular ET-1 at high concentration rather increased glioblastoma cell death after 18 h exposure, but not after 2 h (results not shown).

Finally, the expression of ECE-1 and bigET-1/ET-1 was determined using immunohistochemistry in a human glioblastoma surgical sample and immunocytochemistry in human glioblastoma LN18 and LNZ308 cells in culture. The expression of ECE-1 (Figure 9) was robust in most cells of surgical specimens of human glioblastoma or human glioblastoma cells in culture,

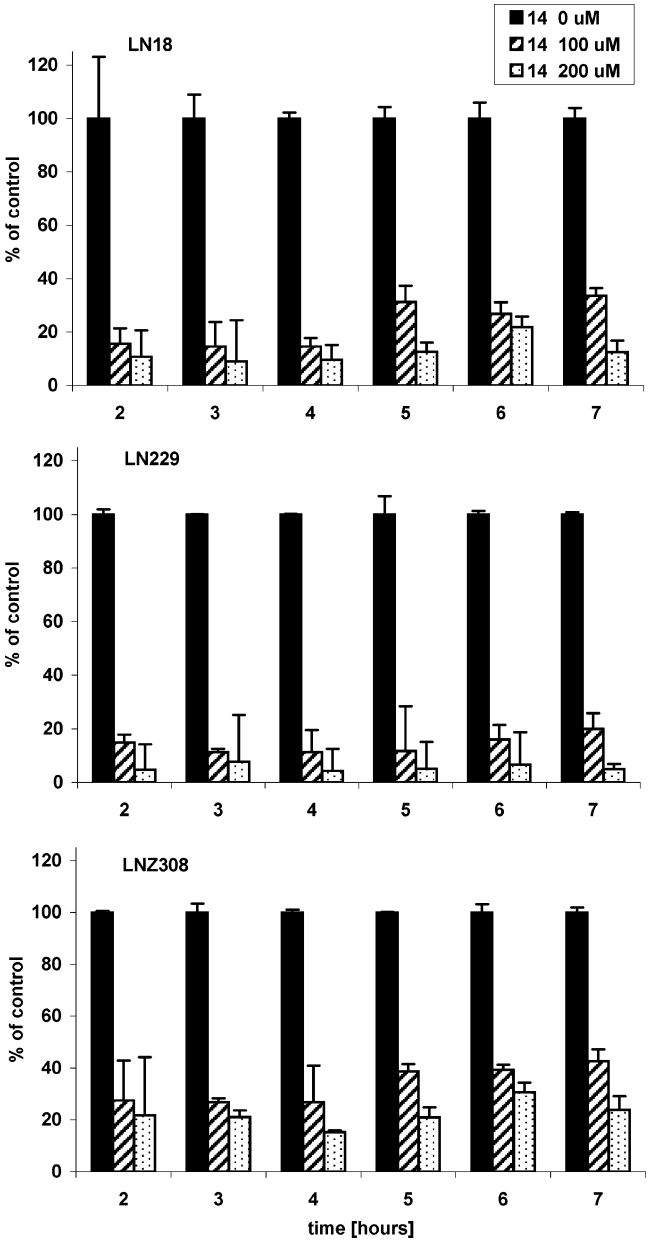


Figure 2. Short-term (2−7 h) effect on DNA synthesis of exposure of human glioblastoma cells to ECE-1 inhibitors. LN18, LN229, or LNZ308 cells were grown to confluence in culture medium containing FCS and then exposed for 2−7 h to the ECE-1 inhibitor **14** at 0 μM (black bars), 100 μM (stripped bars) or 200 μM (dotted bars) in complete fresh medium. [^{3}H]Thymidine (^{3}HT) incorporation to quantify DNA synthesis was performed during the last 2 h of incubation. Means ± SD were calculated.

whereas ET-1 expression was weak or absent in most cells, for all samples.

Discussion

ECE-1 (EC 3.4.24.71) belongs to a family of several related metalloproteases, of which neprilysin (NEP, EC 3.4.24.11) and angiotensin-converting enzyme (ACE, EC 3.4.15.1) are the best studied. Inhibitors have been developed against these two proteases and have reached clinical use. Of these related metalloproteases, NEP displays the closest sequence and structural similarities to ECE-1. On the basis of structural, site-directed mutagenesis and substrate characteristics of NEP[20,21]

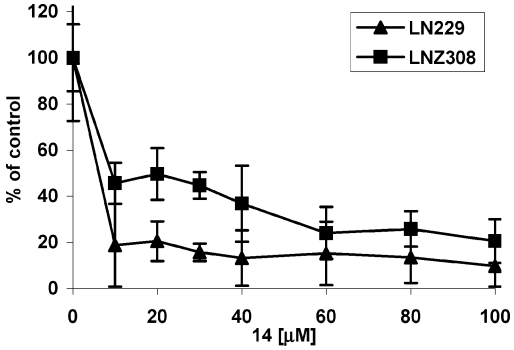


Figure 3. Dose−response effect on DNA synthesis of short term (2 h) exposure of human glioblastoma cells to ECE-1 inhibitors. LN229 or LNZ308 cells were grown to confluence in culture medium containing FCS and then exposed for 2 h to the ECE inhibitor **14** at increasing concentration (0−100 μM) in complete fresh medium. [^{3}H]Thymidine (^{3}HT) was added together with the inhibitors, and radioactivity incorporation was measured to quantify DNA synthesis. Means ± SD were calculated.

and on the structure of the NEP inhibitors phosphoramidon (a phosphono derivative) or the thiol derivative thiorphan, several groups have synthesized nonpeptidyl phosphono-based or thiol-based ECE-1 inhibitors.[19,22−31] Our lead structure for nonpeptidic ECE inhibitors contains the rigid [2*S*,4*R*]-4-mercaptoproline core. The mercapto moiety forms a very strong interaction with the catalytic zinc of the ECE-1 and the two additional exit vectors of the proline scaffold are amenable to rapid variation. During the process of lead identification we have shown that the respective activities of the isomers were [2*S*,4*R*] > [2*S*,4*S*] > [2*R*,4*R*] for the 4-mercaptoproline core (results not shown). We have not further evaluated isomers of the less active molecules at this stage of lead identification. Therefore, we have built on the most active [2*S*,4*R*]-isomer. Modification around this core led to the identification of low nanomolar ECE-1 inhibitors, which can be subdivided into four classes: the pyrrolidine ethers **8**, **10**, **12** (Scheme 1); the pyrrolidine amines **22**, **28** (Scheme 2); the proline hydrazide **36** (Scheme 3); and the proline amide **41** (Scheme 4). We tested representatives of each class or their prodrugs in cellular assays.

ECE-1 inhibitors have been evaluated only in the context of cardiovascular or pulmonary disorders and only for a limited number of these derivatives.[19,23,24,29,32] For one of the ECE-1 inhibitors of the present series (compound **13**), a diminution in Ang II-dependent cardiac damage and fibrosis was observed[32] in an experimental model of rats expressing human renin and angiotensinogen. To our knowledge, no study has evaluated the effects of ECE-1 inhibitors in cancer models, although the endothelin system is widely distributed in human tissues, including the brain,[3] and is expressed in many human cancers.[5] We[6−8] and others[4,5] have shown that blocking ET-1 binding to its receptors may result in growth inhibition and apoptosis induction in cancer cells. Depending on the particular ECE-1 isoform expressed or on external factors,[2] ECE-1 may be secreted or expressed at the cell surface (ECE-1_a and ECE-1_c) and thus be directly accessible to inhibitors. Alternatively, ECE-1 may be intracellular (ECE-1_b and ECE-1_d), thus necessitating that inhibitors traverse the cell membrane. To identify the desired properties of an

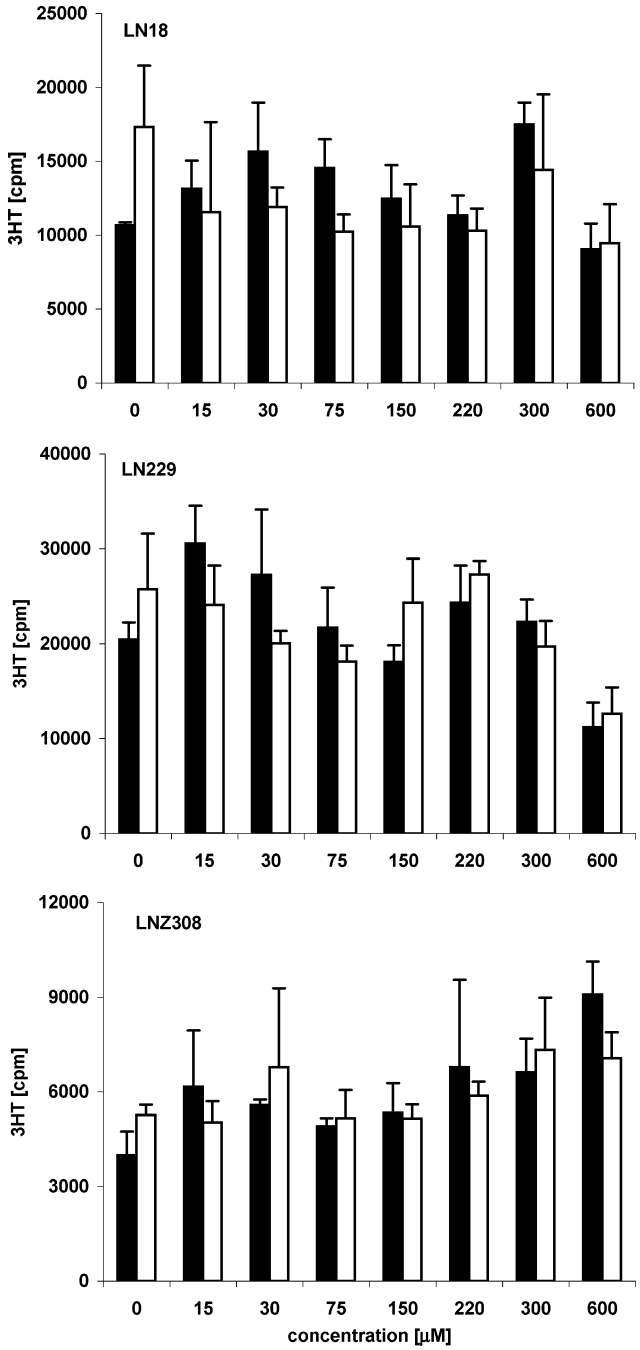


Figure 4. Effect of the NEP inhibitors phosphoramidon and D,L-thiorphan on human glioblastoma cell growth. LN18, LN229, or LNZ308 cells were grown to confluence in culture medium containing FCS and then exposed for 24 h to phosphoramidon or D,L-thiorphan at increasing concentration (0−600 μM), and [^{3}H]thymidine (^{3}HT) incorporation was performed during the last 2 h of incubation to quantify DNA synthesis. Means + SD were calculated. Black bars, phosphoramidon; white bars, D,L-thiorphan.

ECE-1 inhibitor able to control the proliferation of human cancer cells, we prepared a series of ECE-1 inhibitors and their prodrugs and evaluated their activities in human glioblastoma cells expressing ECE-1 isoforms.[7]

Our results demonstrate that potent thiol-based ECE-1 inhibitors have the potential to inhibit the proliferation of human glioblastoma cells, whereas NEP or ACE inhibitors, either the thiol derivatives thiorphan or captopril, as well as the nonthiol phosphoramidon (an inhibitor with low nanomolar activity on NEP and low micromolar activity on ECE-1) or lisinopril (an ACE inhibitor), do not show this antiproliferative activity. Phosphoramidon, but not thiorphan, in addition to inhibiting NEP, is also an inhibitor of ECE-1.[22,24,28] While glioblastoma cells may express NEP,[33] they do not express ACE,[34,35] and in an experimental model of glioblastoma, ACE inhibition did not modulate tumor growth.[34] Therefore, growth inhibition by ECE-1 inhibitors is not simply related to a nonspecific effect of protease inhibitors on cells.

The growth-inhibitory effect of ECE-1-inhibitors in human glioblastoma cells was rapid and already maximal after 2-h exposure of cells to these compounds. No obvious correlation was observed between the growth-inhibitory effect of this series of ECE-1 inhibitors and their potency to inhibit soluble ECE-1 or the ET-1 production by ECE-1$_c$-transfected MDCK cells when ECE-1 was expressed at the cell membrane and freely accessible to inhibitors or the prodrugs of these molecules. This suggests that inhibition of bigET-1 activation by cell-surface ECE-1 may not be the main pathway targeted by ECE-1 inhibitors in the context of cell growth. Our previous experiments, using either human glioblastoma or colon carcinoma surgical specimens,[6−8] suggested that while ECE-1 expression was significantly elevated in tumors, compared to the nontumoral associated tissue, no significant increase in ET-1 expression was found. We had previously shown[6] that exogenous addition of ET-1 or bigET-1 (unpublished results) to human glioblastoma cells did not induce cell proliferation, while antagonists to receptors, but only at high concentration, induced apoptosis. Here we show that the addition of exogenous ET-1 or bigET-1 (results not shown) together with the ECE-1 inhibitors did not reverse the growth inhibitory effect of these inhibitors in human glioblastoma cells. Therefore, our results suggest that the growth-regulatory role of ET-1 is intracellular and that only cell-permeable inhibitors can achieve this role. Alternatively, other enzymes or other peptides[36−38] may be involved, suggesting that ECE-1 inhibitors may act by mechanisms different from bigET-1 activation.

The treatment options of human glioblastoma are very limited, due to the high potential for local invasion of the brain and numerous mechanisms of resistance to chemotherapeutic drugs of this cancer. Therefore, several chemotherapeutic agents are under clinical reevaluation[39,40] and new potential therapeutic agents are actively investigated,[41,42] to which ECE-1 inhibitors may belong.

In conclusion, we demonstrated that a series of thiol ECE-1 inhibitors displayed growth inhibitory properties for human glioblastoma cells, involving a rapid mechanism. This effect was not linked to a deficit in extracellular production of ET-1 and a decrease of ET-1 binding to its membrane receptor(s) but may depend on either alternate substrates for ECE-1 or an intracellular role of ECE-1 and/or ET-1 in the control of cell growth.

Experimental Section

Synthesis. General Synthetic Methods. Reactions were carried out under an atmosphere of argon. Unless otherwise noted, NH_4Cl, $NaHCO_3$, and Na_2CO_3 solutions were saturated aqueous solutions. All compounds were characterized by 250

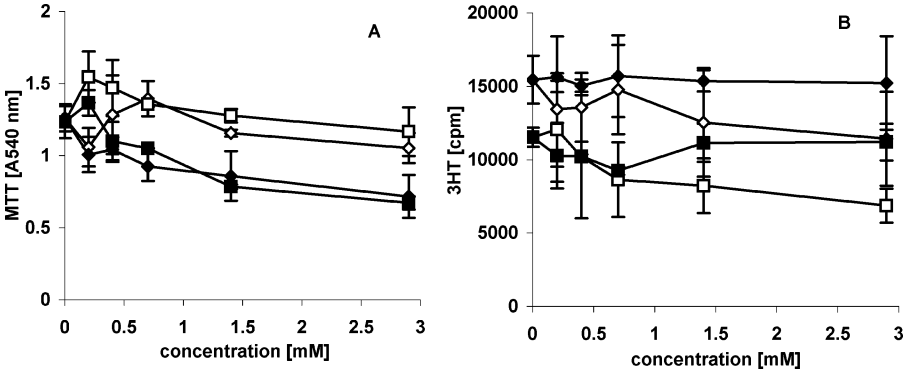


Figure 5. Effect of the ACE inhibitors captopril and lisinopril on human glioblastoma cell growth. LN18 or LNZ308 cells were grown to confluence in culture medium containing FCS, deprived of FCS for 24 h, and exposed for another 24 h to the ACE inhibitors captopril or lisinopril at increasing concentration (0−3 mM) in complete fresh medium. MTT reduction to determine viable cell number and [^{3}H]thymidine (^{3}HT) incorporation to quantify DNA synthesis were performed during the last 2 h of incubation. (A) MTT assay; (B) thymidine incorporation. Means ± SD were calculated. LN 18: ◆, lisinopril; ◇, captopril. LNZ308: ■, lisinopril; □, captoril.

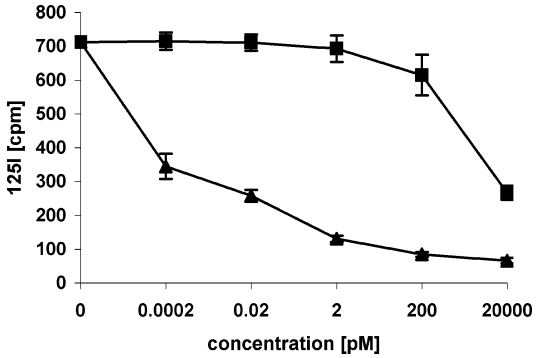


Figure 6. ET-1 binding is mediated by ETB-receptor in LN229 cells. LN229 cells were grown to confluence in culture medium containing FCS and incubated for 15 min at 37 °C with medium containing BQ123 or BQ788. Then 50 pM ^{125}I-ET-1 per well was added. After 1 h incubation at 37 °C, cell-bound ^{125}I-ET-1 was quantified. Means ± SD were calculated. ■, BQ123; ▲, BQ788.

MHz ^{1}H NMR, IR, and MS and the final compounds also by microanalyses. Melting points (uncorrected) were determined using a Büchi 510 apparatus and are uncorrected. Unless otherwise indicated, proton NMR spectra were recorded on a Bruker AC250 spectrometer, and δ values are given in ppm relative to tetramethylsilane. When indicated, proton NMR spectra were recorded on a Bruker AVANCE300 or Bruker AVANCE400 spectrometer. IR spectra of KBr pellets or film on NaCl plates were recorded using a Nicolet 7199-FT IR spectrometer. ATR (attenuated total reflection) spectra were recorded with an FT-IR spectrometer equipped with an IR microscope and a ZnSe ATR objective. The spectrometer was a Nicolet Magna 550/860 or equivalent (resolution 2 cm^{-1}, 200 or 500 coadded scans, MCT detector). Mass spectra (MS) were obtained using the pneumatically assisted electrospray technique (Perkin-Elmer Sciex, type API-III). Results of elemental analyses were within 0.4% of theoretical values. Unless otherwise indicated, commercially available chemicals and solvents were used without further purification. Silica gel 60 (0.04−0.063 mm; Merck) was used for flash chromatography.

(2*S*,4*S*)-4-Chloropyrrolidine-1,2-dicarboxylic Acid 1-*tert*-Butyl 2-Methyl Diester (2). A solution of **1** (374 g, 1.48 mol) in dichloromethane (1.6 L) was treated with triphenylphosphine (680 g, 2.6 mol), cooled to 3−5 °C, and treated in 10 min with CCl_4 (1.24 L, 12.8 mol) and maintained for 2 h at this temperature. Then temperature was raised to 35 °C for 2 h and cooled to 20 °C, and the mixture was stirred for a further 45 min. After addition of *n*-heptane (4 L), the reaction was evaporated to 2.9 L, cooled to 0 °C, and filtered; the residue was treated twice in the same way, and the residue was dissolved in dichloromethane (2 L). The solvents were evaporated, and the compound was filtered through silica gel with *n*-hexane/*tert*-butyl-methyl ether 9:1 as eluent. Evaporation of the solvents gave **2** (347 g, 89%) as a colorless oil: IR (ATR microscope) 1752, 1696 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz, two rotamers) δ 1.35 and 1.41 (s, 9H), 2.10−2.20 (m, 1H), 2.75−2.88 (m, 1H), 3.43 (dd, J_1 = 11.6 Hz, J_2 = 3.6 Hz, 1H), 3.64 and 3.67 (s, 3H), 3.81−3.91 (m, 1H), 4.30−4.39 (m, 1H), 4.57−4.72 (m, 1H); MS *m/z* 246 (M + H$^+$, 1 Cl).

(2*S*,4*R*)-4-Tritylsulfanylpyrrolidine-1,2-dicarboxylic Acid 1-*tert*-Butyl 2-Methyl Diester (3). A solution of potassium-*tert*-butylate (52.8 g, 200 mmol) in DMF (800 mL) was cooled (−3 °C) and slowly treated (1.5 h) with triphenylmethanethiol (66.4 g, 240 mmol) at a maximum of 1 °C. After 20 min at room temperature, the reaction was cooled (0 °C) and a solution of **2** (52.8 g, 200 mmol) in DMF (800 mL) was added. The reaction was stirred for 15 min at 0 °C and 3 h at room temperature. The solvent was evaporated, dissolved in ethyl acetate (1 L), poured into water-saturated NH_4Cl solution (1 L), and extracted with ethyl acetate (2×). The organic phase was washed with aqueous saturated $NaHCO_3$ and aqueous 10% NaCl, dried (Na_2SO_4), and evaporated. Flash column chromatography on silica gel with *n*-hexane/ethyl acetate (9:1 to 1:1) gave **3** (83.5 g, 83%) as a colorless oil: IR (film) 1749, 1704 cm^{-1}; ^{1}H NMR (DMSO-d_6, two rotamers) δ 1.26 and 1.34 (s, 9H), 1.76−1.92 (m, 1H), 2.68−3.19 (m, 2H), 3.40 (dd, J_1 = 9 Hz, J_2 = 6 Hz, 1H), 3.51 and 3.53 (s, 3H), 3.61−3.72 (m, 1H), 4.00−4.14 (m, 1H), 7.22−7.45 (m, 15H); MS *m/z* 504 (M + H$^+$, 1 Br).

(2*S*,4*R*)-2-Hydroxymethyl-4-tritylsulfanylpyrrolidine-1-carboxylic Acid *tert*-Butyl Ester (4). A solution of **3** (30.22 g, 60 mmol) in THF (600 mL) was slowly treated (20 min) at −20 °C with lithium aluminum hydride (1 M in THF, 72 mL, 72 mmol). After 20 min at −20 °C, the solution was cooled (−78 °C) and hydrolyzed with a suspension of 20 g of $MgSO_4 \cdot 7H_2O$ and 20 g of silica gel in aqueous 10% $KHSO_4$ (70 mL). The cooling bath was removed, THF was added, and the mixture was filtered. The residue was dissolved in dichloromethane, dried (Na_2SO_4), and evaporated to give **4** (30.4 g, quantitative) as a colorless oil: IR (film) 3431, 1694, 1672 cm^{-1}; ^{1}H NMR (DMSO-d_6, partially in the coalescence point) δ 1.32 and 1.39 (s, 9H), 1.65−1.95 (m, 2H), 2.45−3.15 (m, 3H), 3.18−3.45 (m, 2H), 3.52−3.65 (m, 1H), 3.59 and 3.66 (t, J_1 = 5.7 Hz, 1H), 7.12−7.45 (m, 15H); MS *m/z* 476 (M + H$^+$).

(2*S*,4*R*)-2-(2,4,5-Trifluorobenzyloxymethyl)-4-tritylsulfanylpyrrolidine-1-carboxylic Acid *tert*-Butyl Ester (5). A solution of **4** (20.2 g, 42.47 mmol) and 2,4,5-trifluorobenzyl bromide (32.42 g, 144.4 mmol) in DMF (900 mL) at 0 °C was treated with NaH (55% in oil, 2.96 g, 67.95 mmol) in four portions (20 min) and warmed to room temperature overnight. The reaction was cooled to 5 °C and poured on aqueous saturated NH_4Cl solution (600 mL)/ice (100 g). The water phase was extracted with ethyl acetate (3×) and the organic

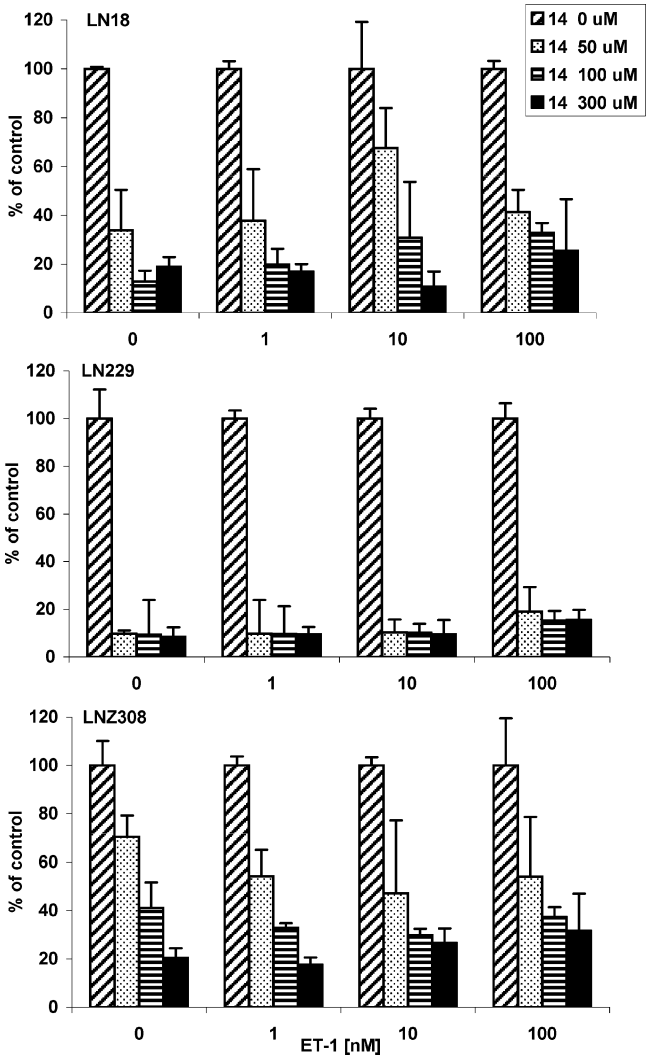


Figure 7. Extracellular ET-1 does not counterbalance cell growth inhibition mediated by ECE-1 inhibition in human glioblastoma cells after long-term (18 h) exposure. LN18, LN229, or LNZ308 cells were grown to confluence in culture medium containing FCS and then exposed for 18 h to the ECE-1 inhibitor **14** at increasing concentration (0−300 μM) in the presence of ET-1 (0−100 nM) in complete fresh medium. [^{3}H]thymidine (^{3}HT) incorporation to quantify DNA synthesis was performed during the last 2 h of incubation. Means + SD were calculated. Diagonal stripped bars, no **14**; dotted bars, 50 μM **14**; horizontal stripped bars, 100 μM **14**; black bars, 300 μM **14**.

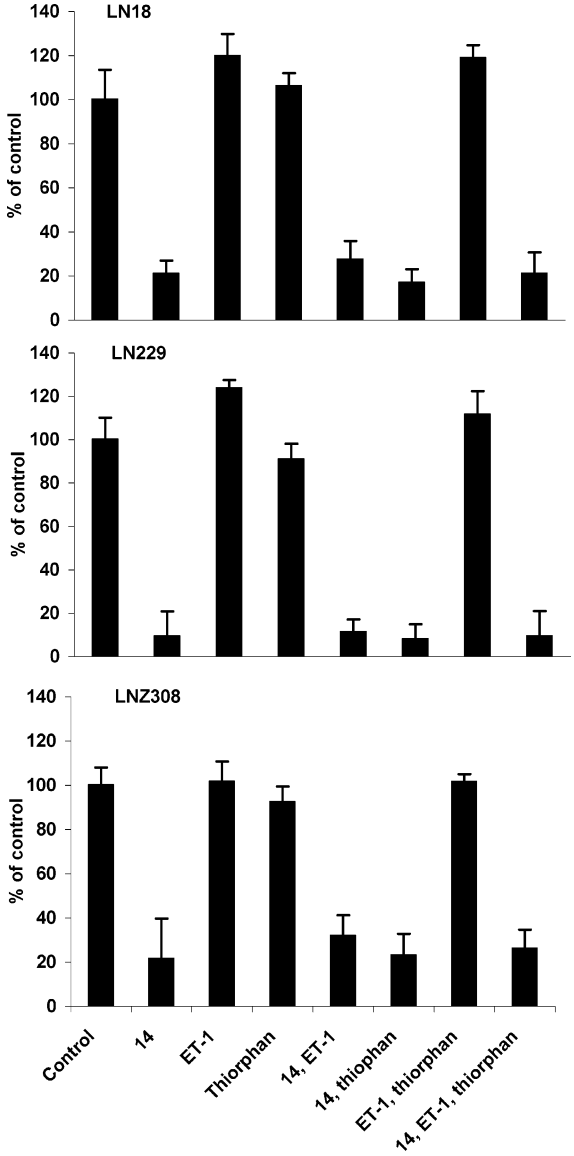


Figure 8. Extracellular ET-1 and thiorphan do not counterbalance cell growth inhibition mediated by ECE-1 inhibition in human glioblastoma cells after short-term (2 h) exposure. LN18, LN229, or LNZ308 cells were grown to confluence in culture medium containing FCS and then exposed for 2 h to 50 μM of the ECE-1 inhibitors **14** in the presence of 10 nM ET-1 and/or 150 μM thiorphan in complete fresh medium. [^{3}H]Thymidine (^{3}HT) was added together with the effectors, and radioactivity incorporation was measured to quantify DNA synthesis. Means + SD were calculated.

phase was washed with 10% NaCl, dried (Na_2SO_4), and evaporated. Flash column chromatography on silica gel with dichloromethane gave **5** (18.5 g, 70%): IR (ATR microscope) 1690 cm^{-1}; ^{1}H NMR (DMSO-d_6, partially in the coalescence point) δ 1.30 and 1.32 (s, 9H), 1.60−1.85 (m, 2H), 2.55−3.08 (m, 3H), 3.14−3.42 (m, 2H), 3.65−3.78 (m, 1H), 4.25−4.40 (m, 2H), 7.15−7.40 (m, 16H), 7.52 (td, J_1 = 9.6 Hz, J_2 = 6.6 Hz, 1H); MS *m/z* 620 (M + H$^+$).

(2*S*,4*R*)-2-(2,4,5-Trifluorobenzyloxymethyl)-4-tritylsulfanylpyrrolidine (6). A solution of **5** (9.37 g, 15.11 mmol) in dichloromethane (30 mL) was treated at −20 °C with trifluoroacetic acid (34 mL) and the temperature was raised to room temperature during 5.5 h. The solvent was evaporated and treated with aqueous saturated $NaHCO_3$ solution/ethyl acetate (3×) to give **6** (7.77 g, quantitative) as a light brown oil: IR (film) 3270, 1633, 1122 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.270−1.51 (m, 2H), 2.29 (dd, J_1 = 10.0 Hz, J_2 = 7.9 Hz, 1H), 2.46−2.68 (m, 2H), 3.05−3.45 (m, 4H), 4.37 (s, 2H), 7.15−7.40 (m, 16H), 7.51 (td, J_1 = 10.8 Hz, J_2 = 6.7 Hz, 1H); MS *m/z* 520 (M + H$^+$).

(2*S*,4*R*)-2-(2,4,5-Trifluorobenzyloxymethyl)-4-tritylsulfanylpyrrolidine-1-carboxylic Acid 2-Methoxycarbonylphenyl Ester (7). A solution of trichloromethyl chloroformate (3.46 mL, 28.72 mmol) in dichloromethane (200 mL) was treated at 0 °C with quinoline (6.79 mL, 57.4 mmol) and after 15 min with **6** (27.13 g, 52.21 mmol) in dichloromethane (160 mL). After 2 h at 0 °C the dichloromethane was evaporated. The residue was dissolved in THF (365 mL) and treated at 0 °C with methyl salicylate (14.09 mL, 109.64 mmol) and NaH (55%, 5.47 g, 125.30 mmol) in 10 portions and after 10 min with potassium iodide (8.67 g, 52.21 mmol). The reaction was stirred at room temperature overnight and cooled (0 °C), and after the addition of 1 equiv of NaH (55%, 2.28 g, 52.2 mmol) the mixture was refluxed for 3 h. The reaction was partitioned between aqueous 10% $KHSO_4$/ethyl acetate (3 × 300 mL). The organic phases were washed with aqueous 10% NaCl, dried (Na_2SO_4), and evaporated. Flash chromatography on silica gel (*n*-hexane/ethyl acetate 9:1 to 2:1) gave **7** (34.43 g, 95%) as a

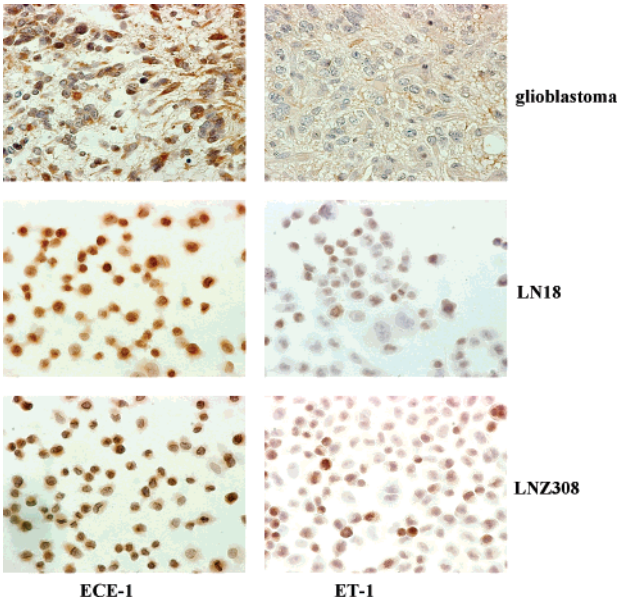


Figure 9. Immunohistochemistry and immunocytochemistry for ECE-1 and ET-1 in human glioblastoma and glioblastoma cells. ECE-1 and ET-1 expression by immunohistochemistry or immunocytochemistry was performed using specific antibodies and histological slides of surgical specimen of human glioblastoma or human glioblastoma cells in culture. Magnification 100×.

viscous colorless oil: IR (ATR microscope) 1720 cm^{-1}; ^1H NMR (DMSO-d_6, two rotamers) δ 1.60−1.95 (m, 2H), 2.75−2.92 (m, 1H), 2.99−3.15 (m, 2H), 3.28−3.42 (m, 1H), 3.45−3.52 (m, 1H), 3.69 (s, 3H), 3.80−3.92 and 4.01−4.12 (m, 1H), 4.35 and 4.38 (s, 2H), 7.10−7.39 (m, 18H), 7.40−7.55 (m, 1H), 7.56−7.68 (m, 1H), 7.80−7.90 (m, 1H); MS *m/z* 698 (M + H^+).

(2*S*,4*R*)-4-Mercapto-2-(2,4,5-trifluorobenzyloxymethyl)-pyrrolidine-1-carboxylic Acid 2-Methoxycarbonylphenyl Ester (8). A solution of **7** (31.34 g, 44.91 mmol) in trifluoroacetic acid (300 mL) was treated at 0 °C with triethylsilane (71.3 mL, 449.10 mmol) for 15 min at room temperature, evaporated, and purified by flash chromatography on silica gel (*n*-hexane/ethyl acetate 4:1) to give **8** (17.48 g, 85%) as a colorless, viscous oil: IR (ATR microscope) 2480, 1716 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz, two rotamers) δ 1.84 and 1.87 (d, J = 7.5 Hz, 1H), 1.98−2.27 (m, 1H), 2.30−2.62 (m, 1H), 3.40−3.91 and 3.91−4.14 and 4.17−4.33 and 4.33−4.46 (m, 6H), 3.84 (s, 3H), 4.54 and 4.56 (s, 2H), 6.80−6.98 (m, 1H), 7.09−7.36 (m, 3H), 7.55 (td, J_t = 7.7 Hz, J_d = 1.7 Hz, 1H), 7.99 (dd, J_1 = 7.8 Hz, J_2 = 1.6 Hz, 1H); MS *m/z* 454 (M − H^-). Anal. ($C_{21}H_{20}F_3NO_5S$) C, H, F, N, S.

(2*S*,4*R*)-2-(2,4,5-Trifluorobenzyloxymethyl)-4-tritylsulfanylpyrrolidine-1-carboxylic Acid 2,3-Dihydrobenzo-[1,4]dioxin-5-yl Ester (9). A solution of **6** (6.91 g, 13.29 mmol) in THF (40 mL) at 0 °C was treated with 1,4-benzodioxan-5-yl chloroformate [18] (2.99 g, 13.96 mmol) and pyridine (1.34 mL, 16.62 mmol). After 1 h at 0 °C, the solvent was evaporated and the compound was partioned between aqueous 10% $KHSO_4$/ether (3×). The organic phases were washed with H_2O and aqueous saturated $NaHCO_3$, dried (Na_2SO_4), and evaporated. Purification by flash chromatography on silica gel (toluene/ethyl acetate 2.5:97.5) gave **9** (5.94 g, 64%) as a white foam: IR (ATR microscope) 1719 cm^{-1}; ^1H NMR (DMSO-d_6, two rotamers) δ 1.40−1.95 (m, 2H), 2.85−2.94 (m, 1H), 2.96−3.14 (m, 2H), 3.25−3.35 (m, 1H), 3.42−3.52 (m, 1H), 3.79−3.90 and 3.94−4.05 (m, 1H), 4.10 and 4.25 (m, 4H), 4.28 and 4.38 (m, 2H), 6.52−7.62 (m, 1H), 6.65−6.78 (m, 1H), 7.08−7.38 (m, 16H), 7.42−7.57 (m, 1H); MS *m/z* 698 (M + H^+).

(2*S*,4*R*)-4-Mercapto-2-(2,4,5-trifluorobenzyloxymethyl)-pyrrolidine-1-carboxylic Acid 2,3-Dihydrobenzo[1,4]-dioxin-5-yl Ester (10). As described for **8**, treatment of compound **9** with **9** in trifluoroacetic acid and triethylsilane gave **10** as a colorless viscous oil in 91% yield: IR (ATR microscope) 2555, 1716 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.73 (d, J = 6.9 Hz, 1H), 1.97−2.21 (m, 1H), 2.39−2.55 (m, 1H), 3.40−3.92 (m, 4H), 3.99 (q, J = 3.6 Hz, 1H), 4.15−4.40 (m, 5H), 4.48−4.60 (m, 2H), 6.64−6.84 (m, 3H), 6.91 (td, J_1 = 9.7 Hz, J_2 = 6.5 Hz, 1H), 7.21 (ddd, J_1 = 6.7 Hz, J_2 = 8.9 Hz, J_3 = 10.2 Hz, 1H); MS *m/z* 456 (M + H^+). Anal. ($C_{21}H_{20}F_3NO_5S$) C, H, F, N, S.

(2*S*,4*R*)-5-Propyl-2-[2-(2,4,5-trifluorobenzyloxymethyl)-4-tritylsulfanylpyrrolidin-1-yl]pyrimidine (11). A mixture of **6** (2 g, 3.85 mmol), 2-chloro-5-*n*-propylpyrimidine (1.2 g, 7.7 mmol), *N*-ethyldiisopropylamine (1.98 mL, 11.55 mmol), and a catalytic amount of copper(I) iodide was heated for 10 h at 80 °C. The reaction was cooled and partitioned between H_2O (100 mL) and ether (3 × 300 mL). The organic phases were washed with aqueous saturated $NaHCO_3$ and aqueous 10% NaCl, dried (Na_2SO_4), and evaporated. Flash chromatography on silica gel (toluene/ether 99:1) gave **11** (2 g, 81%) as a light brown viscous oil: IR (ATR microscope) 1632, 1602, 1540, 1518, 1493 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.92 (t, J = 7.3 Hz, 3H), 1.59 (m, 2H), 1.75 (m, 1H), 2.32−2.46 (m, 3H), 3.09 (m, 1H), 3.21−3.40 (m, 2H), 3.50 (dd, J_1 = 2.4 Hz, J_2 = 8.4 Hz, 1H), 3.68 (dd, J_1 = 6.6 Hz, J_2 = 9.6 Hz, 1H), 4.16 (m, 1H), 4.34 (q, J = 8.4 Hz, 2H), 6.84 (td, J_1 = 9.7 Hz, J_2 = 6.5 Hz, 1H), 7.05 (ddd, J_1 = 6.7 Hz, J_2 = 8.9 Hz, J_3 = 10.2 Hz, 1H), 7.14−7.35 (m, 10H), 7.41−7.54 (m, 5H), 8.11 (s, 2H); MS *m/z* 640 (M + H^+).

(3*R*,5*S*)-1-(5-Propylpyrimidin-2-yl)-5-(2,4,5-trifluorobenzyloxymethyl)pyrrolidine-3-thiol Trifluoroacetate (1:0.17) (12). A solution of **11** (1.95 g, 3.05 mmol) in dichloromethane (30 mL) was treated at 0 °C with trifluoroacetic acid (8 mL) and triisopropylsilane (6.26 mL, 30.5 mmol). After 20 min at room temperature the solution was evaporated. Flash chromatography on silica gel (toluene/ethyl acetate 95:5 to 9:1) gave **12**·trifluoroacetate (1:0.17) (1.27 g, 97%) as a light yellow viscous oil: IR (ATR microscope) 1632, 1601 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.94 (t, J = 7.2 Hz, 3H), 1.59 (sextet, J = 7.2 Hz, 2H), 1.71 (d, J = 7.2 Hz, 1H), 2.07 (ddd, J_1 = 8.6 Hz, J_2 = 9.4 Hz, J_3 = 12.9 Hz, 1H), 2.43 (t, J = 7.2 Hz, 2H), 2.52 (ddd, J_1 = 2.4 Hz, J_2 = 6.5 Hz, J_3 = 12.9 Hz, 1H), 3.48 (dd, J_1 = 8.1 Hz, J_2 = 11.2 Hz, 1H), 3.65−3.75 (m, 3H), 4.04 (dd, J_1 = 7.0 Hz, J_2 = 11.2 Hz, 1H), 4.40−4.47 (m, 1H), 4.49 (q, J = 12.9 Hz, 2H), 6.88 (td, J_1 = 9.7 Hz, J_2 = 6.5 Hz, 1H), 7.16 (ddd, J_1 = 6.7 Hz, J_2 = 8.9 Hz, J_3 = 10.2 Hz, 1H), 8.22 (s, 2H); MS *m/ z* 398 (M + H^+). Anal. ($C_{19}H_{22}F_3N_3OS$·0.64H_2O·0.17TFA) C, H, F, N, S.

(2*S*,4*R*)-4-Acetylsulfanyl-2-(2,4,5-trifluorobenzyloxymethyl)pyrrolidine-1-carboxylic Acid 2-Methoxycarbonylphenyl Ester (13). A solution of **8** (3.3 g, 7.25 mmol) in pyridine (30 mL) was treated at 0 °C with acetyl chloride (1.14 mL, 14.49 mmol) and stirred for 6 h at room temperature. The reaction was evaporated and poured onto aqueous 1 N HCl/ether (3×). The organic phases were washed with 10% NaCl, dried (Na_2SO_4), and evaporated. Flash chromatography on silica gel (*n*-hexane/ethyl acetate 4:1) gave **13** (3.09 g, 98%) as colorless oil: IR (ATR microscope) 1720, 1691, 1632, 1607, 1516 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz, two rotamers) δ 2.08−2.23 (m, 1H), 2.36 (s, 3H), 2.42−2.53 (m, 1H), 3.48−3.98, 4.08−4.28 and 4.32−4.45 (m, 6H), 3.85 (s, 3H), 4.56 and 4.58 (s, 2H), 6.85−6.92 (m, 1H), 7.10−7.17 (m, 1H), 7.26−7.35 (m, 1H), 7.53 (td, J_1 = 8.5 Hz, J_2 = 1.5 Hz, 1H), 7.53 (dd, J_1 = 8.7 Hz, J_2 = 1.5 Hz, 1H); MS *m/z* 498 (M + H^+). Anal. ($C_{23}H_{22}F_3N_3O_6S$·0.25EtOAc) C, H, F, N, S.

(2*S*,4*R*)-4-Acetylsulfanyl-2-(2,4,5-trifluorobenzyloxymethyl)pyrrolidine-1-carboxylic Acid 2,3-Dihydrobenzo-[1,4]dioxin-5-yl Ester (14). Compound **14** was synthesized as described for **13**. Compound **10** gave **14** as a colorless viscous oil in 91% yield: IR (ATR microscope) 1720, 1690 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz, two rotamers) δ 2.05−2.21 (m, 1H), 2.35 (s, 3H), 2.39−2.62 (m, 1H), 3.45−3.82, (m, 3H), 3.90 and 4.06 (dd, J_1 = 7.6 Hz, J_2 = 11.2 Hz, 1H), 4.15−4.35 (m, 5H), 4.48−4.60 (m, 2H), 4.55 and 4.57 (s, 2H), 6.65−6.71 (m, 1H), 6.72−6.85 (m, 2H), 6.90 (td, J_1 = 9.7 Hz, J_2 = 6.5 Hz, 1H),

7.28 (ddd, $J_1 = 6.7$ Hz, $J_2 = 8.9$ Hz, $J_3 = 10.2$ Hz, 1H); MS *m/z* 497 (M^+). Anal. ($C_{23}H_{22}F_3NO_6S \cdot 0.09H_2O \cdot 0.15CH_2Cl_2$) C, H, F, N, S.

Benzoylsulfanyl-2-(2,4,5-trifluorobenzyloxymethyl)-pyrrolidine-1-carboxylic Acid 2,3-Dihydrobenzo[1,4]-dioxin-5-yl Ester (15). Compound **10** and benzoyl chloride gave **15** as colorless oil in 94% yield: IR (ATR microscope) 1719, 1661 cm^{-1}; ^1H NMR ($CDCl_3$, two rotamers) δ 2.15−2.35 (m, 1H), 2.50−2.72 (m, 1H), 3.58−3.92, (m, 3H), 3.96−4.45 (m, 7H), 4.58 and 4.60 (s, 2H), 6.64−6.82 (m, 3H), 6.92 (td, $J_1 = 9.7$ Hz, $J_2 = 6.5$ Hz, 1H), 7.32 (ddd, $J_1 = 6.7$ Hz, $J_2 = 8.9$ Hz, $J_3 = 10.2$ Hz, 1H), 7.41−7.50 (m, 2H), 7.54−7.82 (m, 1H), 7.94 (d, $J = 7.2$ Hz, 2H); MS *m/z* 560 ($M + H^+$). Anal. ($C_{28}H_{24}F_3NO_6S$) C, H, F, N, S.

(2*S*,4*R*)-2-Methanesulfonyloxymethyl-4-tritylsulfanylpyrrolidine-1-carboxylic Acid *tert*-Butyl Ester (16). A solution of **4** (20 g, 42 mmol) in dichloromethane (500 mL) was treated at 0 °C with methanesulfonyl chloride (3.6 mL, 46 mmol), pyridine (4.9 mL, 63 mmol), and DMAP (5.1 g, 42 mmol), and the mixture was stirred at room temperature for 4 h. The reaction mixture was poured into ethyl acetate/H_2O and adjusted to pH 3 with 1 M HCl. The organic phase was washed with 10% aqueous NaCl, dried (Na_2SO_4), and evaporated to give **16** (21 g, 90%) as a white foam: IR (ATR microscope) 1693, 1593, 1488, 1176, 744, 702 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.48 (m, 1H), 1.53−1.72 (m, 2H), 1.8 (m, 1H), 2.84 (s, 3H), 2.94 (m, 1H), 3.13 (m, 1H), 3.91 (m, 1H), 4.05 (m, 1H), 7.28 (m, 9H), 7.46 (m, 6H); MS *m/z* 554 ($M + H^+$).

(2*S*,4*R*)-2-Azidomethyl-4-tritylsulfanylpyrrolidine-1-carboxylic Acid *tert*-Butyl Ester (17). To a solution of **16** (21 g, 38 mmol) in DMF (130 mL) was added sodium azide (3.5 g, 53.2 mmol). The solution was stirred at 80 °C overnight and cooled, and water was added. The phases were separated, the aqueous phase was extracted with ether, and the combined organic layers were washed with water and brine, dried (Na_2SO_4), and evaporated to yield **17** as yellow oil: IR (ATR microscope) 2102, 1695, 1488, 1391, 767, 702 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.42 (s, 9H), 1.46−1.62 (m, 1H), 1.77 (m, 1H), 2.79−3.05 (m, 3H), 3.05−3.2 (m, 1H), 3.2−3.42 (m, 1H), 3.70−3.9 (m, 1H), 7.2−7.35 (m, 9H), 7.46 (m, 6H); MS *m/z* 501 ($M + H^+$).

(2*S*,4*R*)-2-Azidomethyl-4-tritylsulfanylpyrrolidine (18). Trifluoroacetic acid (17 mL) was added to **17** (8.85 g, 17.68 mmol) in dichloromethane (35 mL) at 0 °C. The mixture was stirred at room temperature until no starting material could be detected by TLC. The solution was concentrated and the residue was poured into a saturated aqueous solution of $NaHCO_3$. The inorganic phase was extracted with ethyl acetate and the combined organic phases were washed with a saturated aqueous solution of $NaHCO_3$ and brine, dried (Na_2SO_4), filtered, and evaporated. Purification by column chromatography with ethyl acetate yielded **18** (5.77 g, 81%) as a light yellow oil: IR (ATR microscope) 2094, 1600, 1488, 1444, 743, 700 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.42 (m, 1H), 1.65 (m, 1H), 1.71 (m, 1H), 2.5 (dd, 1H, $J_1 = 6$ Hz, $J_2 = 12$ Hz), 2.73 (dd, 1H, $J_1 = 6$ Hz, $J_2 = 12$ Hz), 2.81 (m, 1H), 3.05 (dd, 1H, $J_1 = 6.6$ Hz, $J_2 = 12.6$ Hz), 3.13 (dd, 1H, $J_1 = 5.4$ Hz, $J_2 = 12$ Hz), 3.30 (m, 1H), 7.16−7.33 (m, 9H), 7.46 (m, 6H); MS *m/z* 400 (M^+).

(2*S*,4*R*)-2-Azidomethyl-4-tritylsulfanylpyrrolidine-1-carboxylic Acid Isopropyl Ester (19). *N*,*N*-Diisopropylethylamine (0.16 mL, 0.96 mmol) was added at 0 °C to a solution of **18** (320.4 mg, 0.8 mmol) in dichloromethane (5 mL), followed by isopropyl chloroformate (0.96 mL, 0.96 mmol). The solution was stirred at room temperature for 2 days, then 1 M $KHSO_4$ (2 mL) was added, and the inorganic layer was extracted with dichloromethane. The combined organic layers were washed with water and brine, dried (Na_2SO_4), and evaporated to yield **19** (390 mg, quantitative) as a colorless oil: ^1H NMR ($CDCl_3$) δ 1.22 (d, 6H), 1.50 (m, 1H), 1.75 (m, 1H), 2.78−3.2 (m, 4H), 3.2−3.46 (m, 1H), 3.74−3.94 (m, 1H), 4.74−4.97 (m, 1H), 7.14−7.36 (m, 9H), 7.47 (m, 6H); MS *m/z* 487 ($M + H^+$).

(2*S*,4*R*)-2-Aminomethyl-4-tritylsulfanylpyrrolidine-1-carboxylic Acid Isopropyl Ester (20). 19 (389.3 mg, 0.8 mmol) was treated with triphenylphosphine (630 mg, 2.4 mmol) in THF (4 mL) and H_2O (0.12 mL) at room temperature overnight. The solution was diluted with ethyl acetate, dried (Na_2SO_4), and evaporated. The residue was purified by ion exchange chromatography (Varian, Mega Bond Elut, SCX) to give **20** (238 mg, 65%) as white foam: ^1H NMR ($CDCl_3$) δ 1.26 (d, 6H), 1.28 (m, 1H), 1.65 (m, 1H), 2.31 (m, 1H), 2.54 (m, 1H), 2.82 (m, 1H), 2.96−3.43 (m, 2H), 3.64 (m, 1H), 4.84 (m, 1H), 7.19−7.36 (m, 9H), 7.46 (m, 6H); MS *m/z* 461 ($M + H^+$).

(2*S*,4*R*)-2-[(2,5-Difluorobenzylamino)methyl]-4-tritylsulfanylpyrrolidine-1-carboxylic Acid Isopropyl Ester (21). A solution of zinc chloride (36 mg, 0.26 mmol) and $NaBH_3CN$ (33 mg, 0.05 mmol) in methanol (1 mL) was added to **20** (232 mg, 0.5 mmol) and 2,5-difluorobenzaldehyde (48 μL, 0.44 mmol) in methanol (1 mL). The solution was stirred for 2 days, concentrated, and resuspended in dichloromethane and a saturated solution of $NaHCO_3$. The inorganic layer was extracted with dichloromethane. The combined organic layers were washed with water and brine, dried (Na_2SO_4), and evaporated. Purification with column chromatography yielded **21** (210 mg, 81%) as a light yellow foam: IR (ATR microscope) 3336, 1697, 1182, 743, 701 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.04−1.32 (m, 6H), 1.43 (m, 1H), 1.56 (m, 1H), 1.71 (m, 1H), 2.30 (m, 1H), 2.53 (m, 1H), 2.82−3.31 (m, 3H), 3.68 (s, 2H), 3.72−3.91 (m, 1H), 4.83 (m, 1H), 6.83−7.04 (m, 3H), 7.17−7.35 (m, 9H), 7.45 (m, 6H); MS *m/z* 587 ($M + H^+$).

(2*S*,4*R*)-2-[(2,5-Difluorobenzylamino)methyl]-4-mercaptopyrrolidine-1-carboxylic Acid Isopropyl Ester (22). At 0 °C, trifluoroacetic acid (3 mL) was added to a solution of **21** (208 mg, 0.35 mmol) in acetonitrile (5 mL), followed by triethylsilane (0.6 mL). The mixture was heated to 50 °C for 4 h, concentrated, and added to a saturated solution of $NaHCO_3$. The inorganic phase was extracted with dichloromethane, and the combined organic phases were washed with water and brine, dried (Na_2SO_4), and concentrated. Flash chromatography on silica gel (ethyl acetate/hexane 1:1) gave **22** (90 mg, 74%) as a colorless oil: IR (ATR microscope) 3340, 2545, 1681, 1179, 812, 771 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.24 (d, 6H, $J = 6$ Hz), 1.46 (m, 1H), 1.65 (d, 1H, $J = 7.2$ Hz), 1.96 (dt, 1H, $J_1 = 8.4$ Hz, $J_2 = 12$ Hz), 2.28 (m, 1H), 2.63−2.86 (m, 2H), 3.22−3.39 (m, 1H), 3.49 (m, 1H), 3.71 (dd, 1H, $J_1 = 6$ Hz, $J_2 = 12$ Hz), 3.82 (s, 2H), 3.94−4.16 (m, 1H), 4.89 (m, 1H), 6.84−7.02 (m, 2H), 7.07 (m, 1H); MS *m/z* 345 ($M + H^+$). Anal. ($C_{16}H_{22}F_2N_2O_2S$) C, H, N, S, F.

(2*S*,4*R*)-(4-Tritylsulfanylpyrrolidin-2-yl)methanol (23). At 0 °C, trifluoroacetic acid (40 mL) was added to a solution of **4** (25.0 g, 52.6 mmol) in dichloromethane (80 mL) and the mixture was stirred at room temperature overnight. The solution was concentrated under reduced pressure, and the residue was redissolved in ethyl acetate, washed with a saturated solution of $NaHCO_3$ and brine, and dried (Na_2SO_4) to give **23** (21.64 g, quantitative) as a light brown foam: ^1H NMR ($CDCl_3$) δ 1.68 (m, 1H), 1.71 (m, 1H), 2.46 (dd, 1H, $J_1 = 6$ Hz, $J_2 = 12$ Hz), 2.61 (dd, 1H, $J_1 = 6$ Hz, $J_2 = 12$ Hz), 2.87 (m, 1H), 2.95 (m, br, 2H), 3.17 (dd, 1H, $J_1 = 6$ Hz, $J_2 = 12$ Hz), 3.37 (m, 1H), 3.45 (dd, 1H), 7.16−7.33 (m, 9H), 7.44 (m, 6H); MS *m/z* 376 ($M + H^+$).

(2*S*,4*R*)-[1-(5-Propylpyrimidin-2-yl)-4-tritylsulfanylpyrrolidin-2-yl]methanol (24). Crude **23** was suspended in 2-chloro-5-*n*-propylpyrimidine (16.46 g, 105.1 mmol) and *N*-ethyldiisopropylamine (30 mL, 175 mmol), and the mixture was heated to 80 °C. When dissolution was achieved, copper iodide (350 mg, 1.84 mmol) was added, and the reaction mixture was stirred at 80 °C overnight. After cooling to room temperature, the mixture was diluted with ethyl acetate/H_2O, and the aqueous solution was extracted with ethyl acetate. The combined organic layers were washed with 1 M $KHSO_4$, 1 M HCl, and brine and dried (Na_2SO_4). Purification by flash chromatography with ethyl acetate/*n*-hexane (1:4 to 1:1) yielded **24** (19.21 g, 74%, 2 steps) as a light yellow foam: IR (ATR microscope) 3290, 1602, 1032, 740, 696 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.92 (t, 3H, $J = 7.2$ Hz), 1.1 (m, 1H), 1.58 (m, 2H),

1.73 (dt, 1H, J_1 = 9 Hz, J_2 = 12 Hz), 2.39 (t, 2H, J = 7.2 Hz), 2.9 (m, 1H), 3.32 (m, 1H), 3.4 (dd, 1H), 3.52 (dd, 1H, J_1 = 7.2 Hz, J_2 = 13.2 Hz), 3.69 (dd, 1H, J_1 = 7.2 Hz, J_2 = 13.2 Hz), 4.15 (m, 1H), 5.74 (s, 1H), 7.17−7.34 (m, 9H), 7.47 (m, 6H), 8.1 (s, 2H); MS *m/z* 496 ($M + H^+$).

(2*S*,4*R*)-2-[1-(5-Propylpyrimidin-2-yl)-4-tritylsulfanylpyrrolidin-2-ylmethyl]isoindole-1,3-dione (25). Triphenyl phosphine (764 mg, 2.82 mmol) and phthalimide (420 mg, 2.82 mmol) were added to a solution of **24** (1.0 g, 2.0 mmol) in THF (15 mL) at room temperature. The reaction mixture was cooled to 0 °C and diethylazo dicarboxylate (615 μL, 3.83 mmol) in THF (3 mL) was added. The solution was stirred at room temperature overnight, H_2O was added, and the inorganic layer was extracted with ethyl acetate. The combined layers were washed with 1 M NaOH, a saturated solution of $NaHCO_3$, and brine and dried (Na_2SO_4). Column chromatography with ethyl acetate/*n*-hexane 1:2 as eluent yielded **25** (1.20 g, 95%) as a white solid: IR (ATR microscope) 1711, 1144, 742, 697 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.78 (t, 3H), 1.37 (m, 2H), 1.54 (dd, 1H, J_1 = 7.2 Hz, J_2 = 12 Hz), 1.8 (m, 1H), 2.19 (t, 2H), 3.06 (m, 1H), 3.27 (t, 1H), 3.45 (dd, 1H, J_1 = 9.2 Hz, J_2 = 10 Hz), 3.61 (m, 2H), 4.58 (m, 1H), 7.16−7.33 (m, 9H), 7.40 (dd, 6H), 7.58−7.90 (m, 6H); MS *m/z* 625 ($M + H^+$).

(2*S*,4*R*)-[[1-(5-Propylpyrimidin-2-yl)-4-tritylsulfanylpyrrolidin-2-yl]methyl]amine (26). 25 (960 mg, 1.54 mmol) in ethanol (95 mL) was treated with hydrazine hydrate (2.4 mL, 49.4 mmol) under reflux. After cooling to room temperature, the solution was filtered and concentrated, and the crude product was purified by flash chromatography with dichloromethane/methanol/NH_4OH 90:10:0.25 to yield **26** (659 mg, 88%) as a white foam: IR (ATR microscope) 1600, 1486, 1443, 740, 696 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.92 (t, 3H, J = 7.2 Hz), 1.17 (dd, 1H, J_1 = 6 Hz, J_2 = 13.2 Hz), 1.47−1.76 (m, 3H), 1.55 (m, 1H), 2.33 (m, 1H), 2.37 (m, 2H), 2.70 (dd, 1H, J_1 = 4.8 Hz, J_2 = 13.2 Hz), 2.95 (m, 1H), 3.39 (t, 1H, J = 12 Hz), 3.77 (dd, 1H, J_1 = 7.2 Hz, J_2 = 12 Hz), 3.95 (m, 2H), 7.19−7.38 (m, 9H), 7.49 (m, 6H), 8.11 (s, 2H); MS *m/z* 495 ($M + H^+$).

(2*S*,4*R*)-(2,5-Difluorobenzyl)-[1-(5-propylpyrimidin-2-yl)-4-tritylsulfanylpyrrolidin-2-ylmethyl]amine (27). 2,5-Difluorobenzaldehyde (158 μL, 1.43 mmol) and methanol (5 mL) were added to **26** (643 mg, 1.3 mmol) in methanol (3 mL) to partially dissolve the compound, followed by a solution of zinc chloride (108 mg, 0.78 mmol) and $NaBH_3CN$ (109 mg, 1.56 mmol) in methanol (3 mL). The solution was stirred overnight and concentrated, and the residue was dissolved in ethyl acetate/saturated $NaHCO_3$. The inorganic layer was extracted with ethyl acetate, and the combined organic layers were washed with a solution of $NaHCO_3$ and brine, dried (Na_2SO_4), and evaporated. Purification with column chromatography yielded **27** (750 mg, 93%) as a light yellow gum: IR (ATR microscope) 3330, 1600, 1443, 840, 740, 696 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.91 (t, 3H),1.33 (m, 2H), 1.55 (m, 2H), 1.73 (m, 1H), 2.30 (dd, 1H), 2.36 (t, 2H), 2.64 (dd, 1H), 3.0 (m, 1H), 3.36 (t, 1H), 3.64 (s, 2H), 3.68 (m, 1H), 4.11 (m, 1H), 6.79−7.02 (m, 3H), 7.15−7.33 (m, 9H), 7.48 (m, 6H), 8.1 (s, 2H); MS *m/z* 621 ($M + H^+$).

(2*S*,4*R*)-5-[(2,5-Difluorobenzylamino)methyl]-1-(5-propylpyrimidin-2-yl)pyrrolidine-3-thiol (28). As described for **12**, compound **28** was prepared from **27**, as a colorless gum: IR (ATR microscope) 3305, 2470, 1603, 1541, 1494, 874 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.93 (t, 3H, J = 7.4 Hz), 1.57 (m, 2H), 1.64 (m, 1H), 1.69 (d, 1H, J = 7.2 Hz), 2.05 (dt, 1H, J_1 = 8.4 Hz, J_2 = 12 Hz), 2.39 (t, 2H, J = 7.8 Hz), 2.45 (m, 1H), 2.77 (dd, 1H, J_1 = 6 Hz, J_2 = 12 Hz), 2.9 (dd, 1H, J_1 = 4.8 Hz, J_2 = 13.2 Hz), 3.49 (dd, 1H, J_1 = 6 Hz, J_2 = 12 Hz), 3.64 (m, 1H), 3.83 (s, 2H), 3.97 (dd, 1H, J_1 = 6 Hz, J_2 = 12 Hz), 4.36 (m, 1H), 6.82−7.02 (m, 2H), 7.15 (m, 1H), 8.14 (s, 2H); MS *m/z* 379 ($M + H^+$). Anal. ($C_{19}H_{24}F_2N_4S$) C, H, N, S, F.

(2*S*,4*R*)-4-Hydroxy-1-(naphthalene-2-sulfonyl)pyrrolidine-2-carboxylic Acid Methyl Ester (30). 29 (40 g, 220 mmol, twice suspended in toluene and evaporated under reduced pressure to remove water) was suspended in hexamethyldisilazane (600 mL) and refluxed for 2 h. The solution was evaporated under reduced pressure and dissolved in THF (100 mL). 2-Naphthalenesulfonyl chloride (49.9 g, 220 mmol) in THF (200 mL) was added slowly and the mixture was stirred at room temperature for 16 h. Water (150 mL) was added and after 1 h the solvents were evaporated. The residue was partitioned between water/ethyl acetate (3×), the organic phases were washed with 10% NaCl and dried (Na_2SO_4) to give **30** (60.4 g, 82%): IR (ATR microscope) 3509, 1729, 1337, 1238, 1197, 1026 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.48 (d, 1H, J = 6 Hz), 2.12 (m, 1H), 2.22 (m, 1H), 3.47 (dt, 1H, J = 12 Hz), 3.66 (dd, 1H, J_1 = 4.8 Hz, J_2 = 12 Hz), 3.74 (s, 3H), 4.46 (m, 1H), 4.53 (t, 1H, J = 9 Hz), 7.64 (m, 2H), 7.9 (m, 2H), 7.99 (m, 2H), 8.47 (s, 1H); MS *m/z* 335 (M^+).

(2*S*,4*S*)-4-Methanesulfonyloxy-1-(naphthalene-2-sulfonyl)pyrrolidine-2-carboxylic Acid Methyl Ester (31). A solution of **30** (34.5 g, 120.8 mmol) in toluene (250 mL) and THF (150 mL) was added to a suspension of methanesulfonic acid (8 mL, 123.4 mmol), triethylamine (17.2 mL, 123.4 mmol), and triphenylphosphine (33.7 g, 128.5 mmol) in toluene (150 mL). After the addition of diethyl azodicaboxylate (20.8 mL, 133.6 mmol), the solution was heated to 80 °C for 3 h and stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate (350 mL), and water (250 mL) was added. The phases were separated, and the inorganic phase was extracted with ethyl acetate (3 × 300 mL). The organic phase was washed with aqueous 1 M $KHSO_4$ and brine, dried (Na_2SO_4), and evaporated. Column chromatography gave **31** (37.5 g, 88%) as a colorless solid: IR (ATR microscope) 1747, 1600, 1333, 1184, 821, 751 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.35 (m, 1H), 2.56 (dd, 1H, J = 13.2 Hz), 2.96 (s, 3H), 3.71 (s, 3H), 3.74 (m, 2H), 4.67 (dd, 1H, J_1 = 3.6 Hz, J_2 = 9 Hz), 5.17 (m, 1H), 7.66 (m, 2H), 7.91 (dt, 2H), 8.0 (d, 2H), 8.47 (s, 1H); MS *m/z* 414 ($M + H^+$).

(2*S*,4*R*)-4-(4-Methoxybenzylsulfanyl)-1-(naphthalene-2-sulfonyl)pyrrolidine-2-carboxylic Acid Methyl Ester (32). Potassium *tert*-butylate (157 mg, 1.4 mmol) in DMF (5 mL) was treated with 4-methoxybenzylmercaptane (0.2 mL, 1.4 mmol) at 0°C. The solution was stirred at room temperature for 20 min before **31** (532 mg, 1.3 mmol) in DMF (5 mL) was added. The reaction was stirred at 100 °C for 1 h and cooled to room temperature, and a saturated solution of NH_4Cl was added. The layers were separated, and the inorganic phase was extracted with ethyl acetate, washed with a solution of $NaHCO_3$ and brine, dried (Na_2SO_4), and evaporated. The crude oil was purified by flash chromatography on silica gel with hexane/ethyl acetate (2:1) as eluent to yield **32** (295 mg, 48%) as a colorless oil: ^1H NMR ($CDCl_3$) δ 1.93 (dt, 1H, J_1 = 8.4 Hz, J_2 = 12 Hz), 2.19 (m, 1H), 3.18 (dd, 1H, J_1 = 6.6 Hz, J_2 = 11.4 Hz), 3.29 (m, 1H), 3.55 (s, 2H), 3.70 (s, 3H), 3.74 (m, 1H), 3.77 (s, 3H), 4.41 (dd, 1H, J_1 = 4.8 Hz, J_2 = 7.2 Hz), 6.78 (d, 2H), 7.07 (d, 2H), 7.65 (m, 2H), 7.84 (dd, 1H), 7.94 (m, 1H), 7.99 (m, 2H), 8.41 (s, 1H); MS *m/z* 472 ($M + H^+$).

(2*S*,4*R*)-4-(4-Methoxybenzylsulfanyl)-1-(naphthalene-2-sulfonyl)pyrrolidine-2-carboxylic Acid (33). At 0 °C, 0.1 M LiOH (950 mL, 95 mmol) was added to a solution of **32** (14.8 g, 31.6 mmol) in THF (950 mL). The solution was stirred at room temperature for 2 h and diluted with ice-cold water. Then 1 M $KHSO_4$ was added (pH 2), and the inorganic phase was extracted with ethyl acetate. The combined organic phases were washed with brine, dried (Na_2SO_4), and evaporated. The product was crystallized from ethyl acetate/*n*-hexane to yield **33** (13.15 g, 90%) as a colorless solid: ^1H NMR ($CDCl_3$) δ 1.89 (dt, 1H, J_1 = 9 Hz, J_2 = 12 Hz), 2.31 (m, 1H), 3.13 (dd, 1H, J_1 = 6 Hz, J_2 = 9 Hz), 3.29 (m, 1H), 3.55 (s, 3H), 3.76 (m, 1H), 3.77 (s, 3H), 4.4 (dd, 1H, J_1 = 4.8 Hz, J_2 = 7.2 Hz), 6.79 (d, 2H, J = 7.2 Hz), 7.13 (d, 2H, J = 7.2 Hz), 7.67 (m, 2H), 7.84 (dd, 1H), 7.94 (dd, 1H), 8.01 (dd, 2H), 8.44 (s, 1H); MS *m/z* 456 ($M-H^-$).

(2*S*,4*R*)-4-(4-Methoxybenzylsulfanyl)-1-(naphthalene-2-sulfonyl)pyrrolidine-2-carboxylic Acid *N*′-Methylhydrazide (34). At 0 °C, *N*-hydroxy-2-pyridone (0.26 g, 2.7 mmol) was added to a solution of **33** (1 g, 2.1 mmol) in dichloromethane (10 mL), followed by *N*,*N*-dicyclohexylcarbodiimide (0.48 mg, 2.3 mmol) in dichloromethane (10 mL) over a period

of 30 min. The suspension was stirred for an additional 4 h at that temperature before *N*-ethylmorpholine (1 mL, 7.8 mmol) and methylhydrazine (0.2 mL, 3.7 mmol) were added. The reaction mixture was stirred at room temperature overnight. The suspension was treated with glacial acetic acid (0.1 mL) in water (25 mL), stirred for 2 h, diluted with a solution of $NaHCO_3$ (5%), and extracted with dichloromethane. The combined organic phases were washed with 1 M $KHSO_4$, water, and brine; dried (Na_2SO_4); and evaporated. Column chromatography yielded **34** (930 mg, 90%) as a white crystalline material: ^{1}H NMR ($CDCl_3$) δ 1.49 (m, 1H), 2.4 (m, 1H), 2.63 (s, 3H), 3.05 (m, 2H), 3.55 (s, 2H), 3.74 (m, 1H), 3.77 (s, 3H), 4.24 (dd, 1H), 6.79 (d, 2H, J = 7.2 Hz), 7.07 (d, 2H, J = 7.2 Hz), 7.68 (m, 2H), 7.8 (dd, 1H), 8.0 (m, 3H), 8.4 (s, 1H); MS *m/z* 486 (M + H$^+$).

(2*S*,4*R*)-4-(4-Methoxybenzylsulfanyl)-1-(naphthalene-2-sulfonyl)pyrrolidine-2-carboxylic Acid *N*′-Methyl-*N*′-(4-methylphenylsulfonyl)hydrazide (35). 34 (200 mg, 0.41 mmol) in dichloromethane (15 mL) was treated with *N*,*N*-diisopropylethylamine (0.28 mL, 1.68 mmol), *p*-toluene sulfonyl chloride (157 mg, 0.82 mmol), and DMAP (5 mg, 0.04 mmol) at 0 °C. The mixture was stirred at room temperature overnight. Additional *p*-toluenesulfonyl chloride (157 mg, 0.82 mmol) and DMAP (5 mg, 0.04 mmol) were added, and stirring was continued for 15 h. Potassium sarcosinate (180 mg, 1.44 mmol) was added, the solution was stirred at room temperature for 1 h, 1 M $KHSO_4$ was added, and the phases were separated. The organic layer was extracted with a saturated solution of $NaHCO_3$, and the combined inorganic layers were washed with dichloromethane and brine, dried (Na_2SO_4), and evaporated. Purification of the crude residue by flash chromatography with *n*-hexane/ethyl acetate 1:1 as eluent yielded **35** (190 mg, 89%), which was directly subjected to the next step.

(2*S*,4*R*)-4-Mercapto-1-(naphthalene-2-sulfonyl)pyrrolidine-2-carboxylic Acid *N*′-Methyl-*N*′-(4-methylphenylsulfonyl)hydrazide (36). Triethylsilane (0.06 mL, 0.38 mmol) was added to **35** (85 mg, 0.13 mmol) in trifluoroacetic acid (10 mL). The mixture was heated to 80 °C for 1.5 h and cooled to room temperature, and the solution was concentrated under reduced pressure. The residue was purified by flash chromatography with *n*-hexane/ethyl acetate 1:1 to yield **36** (36.1 mg, 53%) as a white crystalline: IR (ATR microscope) 3274, 1706, 1596, 1348, 1164, 817, 750 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.45 (d, 1H, J = 8 Hz), 1.59 (m, 1H), 2.37 (m, 1H), 2.45 (s, 3H), 2.99 (t, 1H, J = 8 Hz), 3.16 (s, 3H), 3.24 (m, 1H), 3.85 (dd, 1H), 4.21 (dd, 1H), 7.37 (d, 2H), 7.69 (m, 2H), 7.81 (m, 3H), 7.95 (d, 1H), 8.05 (m, 2H), 8.29 (s, 1H), 8.43 (s, 1H); MS *m/z* (M + H$^+$). Anal. ($C_{23}H_{25}N_3O_5S_3$·0.13H_2O) C, H, N, S, F, H_2O.

(2*S*,4*R*)-4-Acetylsulfanyl-1-(naphthalene-2-sulfonyl)-pyrrolidine-2-carboxylic Acid Methyl Ester (37). 30 (36.5 g, 90.8 mmol) in DMF (620 mL) was treated with potassium thioacetate (14.5 g, 127 mmol) at 100 °C for 2.5 h. The solution was concentrated under reduced pressure and the residue was dissolved in ethyl acetate and a saturated aqueous solution of $NaHCO_3$. The phases were separated, and the inorganic layer was extracted with ethyl acetate. The combined organic phases were washed with water and brine and dried (Na_2SO_4). Column chromatography gave **37** (25.1 g, 72%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 2.08 (s, 3H), 2.12 (m, 1H), 2.4 (m, 1H), 3.39 (dd, 1H, J_1 = 5.4 Hz, J_2 = 11.4 Hz), 3.71 (s, 3H), 3.93 (m, 1H), 3.97 (m, 1H), 4.47 (dd, 1H, J_1 = 6 Hz, J_2 = 7.2 Hz), 7.65 (m, 2H), 7.86 (dd, 1 H, J_1 = 1.2 Hz, J_2 = 8.4 Hz), 7.94 (dd, 1H, J_1 = 1.2 Hz, J_2 = 9 Hz), 8.0 (d, 2H, J = 8.4 Hz), 8.43 (s, 1H); MS *m/z* (M + H$^+$).

(2*S*,4*R*)-4-Mercapto-1-(naphthalene-2-sulfonyl)pyrrolidine-2-carboxylic Acid (38). At 0°C, 0.33 M LiOH (1000 mL) was added to **37** (24.84 g, 63.13 mmol) in THF (1780 mL). The solution was stirred at room temperature for 1.5 h, $KHSO_4$ solution was added, and the inorganic phase was extracted with ethyl acetate. The combined organic phases were washed with brine, dried (Na_2SO_4), and evaporated to give **38** (22.2 g, quantitative): ^{1}H NMR ($CDCl_3$) δ 1.55 (s, 1H, J = 6 Hz), 1.97 (m, 1H), 2.53 (m, 1H), 3.17 (dd, 1H, J_1 = 6 Hz, J_2 = 12 Hz), 3.53 (m, 1H), 3.94 (dd, 1H), 4.49 (dd, 1H), 7.67 (m, 2H), 7.84−8.07 (m, 4H), 8.48 (s, 1H); MS *m/z* 336 (M − H$^-$).

(2*S*,4*R*)-4-[(3R,5S)-5-Carboxy-1-(naphthalene-2-sulfonyl)-pyrrolidin-3-yldisulfanyl]-1-(naphthalene-2-sulfonyl)-pyrrolidine-2-carboxylic Acid (39). *N*,*N*-Diisopropylethylamine (22.12 mL, 8.8 mmol) was added to crude **38** in dichloromethane (350 mL), followed by a solution of iodide (8.2 g, 32.4 mmol) in dichloromethane (300 mL) until no more iodide was consumed. The excess of iodide was degraded by the addition of an aqueous solution of $NaHSO_3$. The phases were separated, and the organic phase was extracted with a solution of $NaHCO_3$ (3×). The combined inorganic phases were adjusted to pH 3 with $KHSO_4$ and were extracted with ethyl acetate, washed with brine, dried (Na_2SO_4), and evaporated. The crude product was triturated in *n*-hexane to yield **39** (16.9 g, 79%, 2 steps) as a white solid: ^{1}H NMR (DMSO-d_6) δ 1.84 (m, 2H), 2.04 (m, 2H), 3.04 (dd, 2H, J_1 = 5 Hz, J_2 = 10.9 Hz), 3.36 (m, 2H), 3.63 (dd, 2H, J_1 = 6 Hz, J_2 = 10.9 Hz), 4.2 (dd, 2H, J_1 = 6 Hz, J_2 = 8 Hz), 7.71 (m, 6H), 8.06 (t, 4H, J = 8.8 Hz), 8.16 (d, 2H, J = 7.2 Hz), 8.44 (s, 2H), 12.97 (s, 2H); MS *m/z* 673 (M + H$^+$).

2-[2-[[(2*S*,4*R*)-4-[(3*R*,5*S*)-5-[[(2-Methoxycarbonylphenylcarbamoyl)methyl]methylcarbamoyl]-1-(naphthalene-2-sulfonyl)pyrrolidin-3-yldisulfanyl]-1-(naphthalene-2-sulfonyl)pyrrolidine-2-carbonyl]methylamino]-acetylamino]benzoic Acid Methyl Ester (40). 4-Methylmorpholine (5.8 mL, 52.8 mmol) was added to **39** (8.05 g, 11.95 mmol) in dichloromethane (450 mL), followed by TPTU (8.9 g, 29.9 mmol) and 2-(2-methylaminoacetylamino)benzoic acid methyl ester·hydrochloride **(45)** (6.19 g, 23.9 mmol). The solution was stirred overnight at room temperature; washed with 1 M $KHSO_4$, 5% aqueous $NaHCO_3$, and brine; dried (Na_2SO_4); filtered; and evaporated. The crude product was purified by flash chromatography to yield **40** (8.7 g, 67%) as a white amorphous solid: ^{1}H NMR (DMSO-d_6, 150°C) δ 2.09 (m, 2H), 2.24 (m, 2H), 2.64 (s, 2H), 3.1 (s, 6H), 3.36 (dd, 2H), 3.63 (m, 2H), 3.73 (m, 2H), 3.85 (s, 6H), 4.06 (d, 2H), 4.22 (d, 2H), 4.95 (m, 2H), 7.15 (t, 2H), 7.50−7.68 (m, 6H), 7.76 (d, 2H), 7.89−8.03 (m, 8H), 8.29 (d, 2H), 8.37 (s, 2H); MS *m/z* 1098 (M + NH_4^+). Anal. ($C_{52}H_{52}N_6O_{12}S_4$.0.65EtOAc) C, H, N, S.

(2*S*,4*R*)-2-[2-[[4-Mercapto-1-(naphthalene-2-sulfonyl)-pyrrolidine-2-carbonyl]methylamino]acetylamino]-benzoic Acid Methyl Ester (41). K_2CO_3 (2 M, 1 mL) and DTT (2.72 g, 17.6 mmol) were added to **40** (7.6 g, 7.05 mmol) in methanol (200 mL). The solution was stirred at room temperature for 2 h, the solution was adjusted to pH 2 with 1 M aqueous $KHSO_4$, and the inorganic phase was extracted with ethyl acetate. The combined organic phases were washed with brine, dried (Na_2SO_4), filtered, and evaporated. The crude product was triturated in diethyl ether to give **41** (6.95 g, 91%) as a white solid: IR (ATR microscope) 3261, 1720, 1687, 1605, 1377, 1266, 1154, 756 cm^{-1}; ^{1}H NMR ($CDCl_3$, rotamers) δ 1.56 and 1.66 (d, 1H), 1.91 and 2.16 (dt, 1H), 2.50 and 2.70 (m, 1H), 3.08 and 3.30 (s, 3H), 3.37 (m, 1H), 3.64 (d, 1H, J = 16 Hz), 3.69−3.86 (m, 2H), 3.90 (s, 3H), 4.74 and 4.78 (d, 1H, J = 16 Hz), 4.85 and 5.18 (dd, 1H, J1 = 3.2 Hz, J2 = 8.8 Hz), 7.09 and 7.15 (t, 1H), 7.50−7.68 (m, 3H), 7.79−8.09 (m, 5H), 8.42 and 8.46 (s, 1H), 8.70 and 8.73 (d, 1H), 11.28 and 11.56 (s, 1H); MS *m/z* 542 (M + H$^+$). Anal. ($C_{26}H_{27}N_3O_6S_2$) C, H, N.

2-[2-(Benzyloxycarbonylmethylamino)acetylamino]-benzoic Acid Methyl Ester (44). At 0°C *N*-methylmorpholine (23.9 mL, 212 mmol) was added to Z-Sar-OH **(42)** (43.5 g, 193 mmol) and TPTU (73.85 g, 244 mmol) in dichloromethane (720 mL), followed by methyl anthranilate **(43)** (28.05 mL, 212 mmol) in dichloromethane (80 mL). The solution was stirred for 2 h at 0 °C and warmed to room temperature overnight. Additional methyl anthranilate **(43)** (5 mL, 38.7 mmol) was added and stirring was continued for 4 h. The solution was poured on ice water, the layers were separated, and the aqueous layer was extracted with dichloromethane. The combined organic layers were washed with water, dried ($MgSO_4$), filtered, and evaporated. Flash chromatography on silica gel with a gradient hexane/ethyl acetate (4:1 to 1:1 to

ethyl acetate) yielded **44** (64.8 g, 94%) as a light yellow oil: IR (ATR microscope) 3267, 1698, 1528, 1268, 758, 700 cm^{-1}; ^1H NMR ($CDCl_3$, rotamers) δ 3.13 (s, 3H), 3.86 (s, 3H), 4.10 and 4.18 (s, 2H), 5.17 and 5.25 (s, 2H), 7.12 (t, 1H), 7.17−7.50 (m, 5H), 7.6 (dt, 1H), 8.05 (dd, 1H), 8.74 (m, 1H), 11.48 and 11.55 (s, 1H); MS *m/z* 356 (M^+).

2-(2-Methylaminoacetylamino)benzoic Acid Methyl Ester Hydrochloride (1:1) (45). 44 (64.8 g, 182 mmol) dissolved in methanol (1 L) was hydrogenated in the presence of 10% Pd/C (9.8 g, 9.2 mmol) for 7 h. The catalyst was removed by filtration and the pH of the solution was adjusted to pH 1−2 by the addition of HCl. Evaporation of the solvent and crystallization from methanol/diethyl ether yielded **45** (39.9 g, 83%) as white crystals: IR (ATR microscope) 3408, 2775, 1683, 1544, 1267, 765 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.60 (s, 3H), 3.8 (s, 3H), 4.03 (s, 2H), 7.29 (dt, 1H, J = 6.6 Hz), 7.65 (dt, 1H, J = 8.4 Hz), 7.88 (dd, 1H, J = 6.4), 7.94 (dd, 1H, J = 8.1 Hz), 9.2 (s, 2H), 10.85 (s, 1H); MS *m/z* 222 (M^+).

Soluble ECE Activity Determination. Cell pellets of confluent cultures of the ECV304 human umbilical vein endothelial cell line (ATCC, Manassas, VA) were prepared as previously described[43] and stored at −80 °C until used. All procedures were performed at 0−4°C if not stated otherwise. The cell pellet (1×10^9 cells) was suspended in 50 mL of buffer A [20 mM Tris/HCl, pH 7.5, containing 5 mM $MgCl_2$, 100 μM PMSF (Sigma P7626), 20 μM E64 (Sigma E3132), 20 μM leupeptin (Sigma)] and sonicated. The resulting cell homogenate was centrifuged at 100 000*g* for 60 min. The supernatant was discarded and the resulting membrane pellet was homogenized in 50 mL of buffer A, centrifuged, and washed twice in buffer A. The final membrane preparation was homogenized in 50 mL of buffer B [buffer A + 0.5% Tween 20 (Bio-Rad) (v/v), 0.5% CHAPS (Serva) (w/v), 0.5% digitonin (Serva) (w/v)], stirred at 4 °C for 2 h, and centrifuged, and the supernatant containing the solubilized ECE was stored at −120 °C until use.

The assay measured the production of ET-1 from human bigET-1 (Novabiochem). Fluoronunc Maxisorp White 96-well plates (code 437796) were irradiated with 1 J for 30 min in a UV Stratalinker 2400 (Stratagene). A 300 μL portion of protein A solution (2 μg/mL in 0.1 M Na_2CO_3 pH 9.5) per well was added, and the plates were incubated for 48 h at 4 °C. Before use the plates were blocked for 2 h at 4 °C with 0.5% BSA in 0.1 M Na_2CO_3, pH 9.5, and were washed with water. Synthetic ECE-1 inhibitors were dissolved and diluted in DMSO, and 10 μL of this solution, 125 μL of assay buffer [50 mM Tris/HCl, pH 7.0, 1 μM Thiorphan (Sigma T6031), 0.1% NaN_3, 0.1% bovine serum albumin (BSA, Sigma A7888)] containing 200 ng of bigET-1, and 50 μL of solubilized ECE (prepared in house) [diluted in assay buffer 1:30- to 1:60-fold (v/v)] were incubated for 30 min at 37 °C. The enzyme reaction was stopped by addition of 10 μL of a solution of 150 mM ETDA, pH 7.0, and ET-1 generated was quantified by radioimmunoassay (RIA) (cf. below).

Cell-Based ECE Activity Determination. MDCK cells stably transfected with human ECE-1_c[43] were grown to confluence in 24-well plates in DMEM supplemented with 10% FCS, 0.8 mg/mL Geneticin (G418), 100 IU/mL penicillin, and 100 μg/mL streptomycin, and then the medium was replaced by 0.5 mL of DMEM−HBSS 1:1, 10 mM HEPES (all from Gibco), pH 7.0, supplemented with 0.1% (w/v) BSA (Sigma, A7888). The ECE-1 inhibitors (either the synthetic ECE-1 inhibitors under evaluation, phosphoramidon, or GCS 314447)[19] dissolved in DMSO (final concentration 1%) were added to cells. The enzymatic reaction was initiated by the addition of 0.42 μM human bigET-1, and the cells were incubated for 1.5 h at 37 °C. The amount of ET-1 generated was quantified in the supernatants by RIA as described below.

ET-1 Radioimmunoassay (RIA). The RIA for ET-1 was performed in triplicate wells essentially as previously described.[44] Briefly, 25 μL of assay buffer containing 20 000 cpm (3-[^{125}I]Tyr)-endothelin-1 (Anawa) and 25 μL of the polyclonal anti-ET-1 antiserum AS-3 (BRL-Füllinsdorf) (dilution in assay buffer 1:1000) were added to each well, and the plates were incubated overnight at 4 °C under mixing, and then 200 μL per well of scintillation cocktail (Microscint 40 LSC−Cocktail, Packard) was added, and the plates were counted for 2 min per well in a Topcount (Packard). The amount of ET-1 produced was calculated from a standard curve prepared with synthetic ET-1. Non-ECE-1-dependent ET-1 generation was determined in the presence of 10 mM EDTA or 100 μM phosphoramidon.

NEP and ACE Activity Determination. The NEP assay was based on a method described by Carvahlo et al.[45] with minor differences in the substrate. The internally quenched fluorogenic substrate (Abz-GG$_D$FLRRVQEDDnp, synthesized at Hoffmann La Roche, Basel, Switzerland, containing an additional glutamate between the valine and the fluorescent group), at concentrations ranging from 100 μM to 1 nM, NEP solution (rat crude kidney membranes prepared as in ref 45), and the inhibitors under evaluation at concentrations ranging from 100 μM to 10 nM were incubated for 1 h at 37 °C in triplicate wells. The reaction was stopped with 0.1 M EDTA, and the plates were read at λ_{ex} 320 nm/λ_{em} 420 nm in a Perkin-Elmer luminescence spectrometer (LS50B). IC_{50} was calculated after logit/log transformation of the percent inhibition data with a best fit regression model. Thiorphan as reference inhibitor gave an IC_{50} of 0.007 ± 0.0002 μM (n = 6) in this assay.

The ACE assay was based on the method described by Carmel et al.,[46] which quantifies the fluorescence generated by ACE-dependent hydrolysis of *o*-aminobenzoylglycyl-*p*-nitro-L-phenylalanyl-L-proline (Bachem). ACE (Sigma) and the inhibitors under evaluation at concentrations ranging from 100 μM to 10 nM were incubated for 1 h at 40 °C in triplicate wells. The reaction was stopped by EDTA, and the plates were read at λ_{ex} 330 nm/λ_{em} 425 nm in a Perkin-Elmer luminescence spectrometer (LS50B). IC_{50} were calculated after logit/log transformation of the percent inhibition data with a best fit regression model. Captopril as reference compound gave an IC_{50} of 0.010 ± 0.002 μM in this assay. All synthetic inhibitors were checked for intrinsic fluorescence or quenching properties and the results were corrected if needed.

Treatment of Human Glioblastoma Cells by Synthetic ECE-1 Inhibitors. The human glioblastoma cell lines LN18, LN229, and LNZ308 were a kind gift of AC Diserens, Neurosurgery Division, CHUV, Lausanne.[33] The cells were grown in DMEM containing 4.5 g/L glucose supplemented with 10% FCS and antibiotics. The cells were grown to confluence in complete medium and then incubated at 37 °C in complete fresh medium containing either the ECE-1, NEP, or ACE inhibitors and/or ET-1 or bigET-1 (both from Bachem, Bubendorf, Switzerland) at the concentration indicated. DNA synthesis was determined by measuring the incorporation of thymidine and cell viability by the MTT test (cf. below). The NEP inhibitor dl-thiorphan was purchased from Fluka, and the dual ECE-1/NEP inhibitor phosphoramidon and the ACE inhibitor captopril were from Sigma. Lisinopril was a gift from Merck, Sharp, and Dohme.[34]

Evaluation of Cell Growth. Thymidine incorporation was used to assess DNA synthesis. Following cell treatment with the effectors, cell were exposed to 1 μCi/mL [^{3}H]thymidine (Amersham Pharmacia) for the last 2 h and washed, and high molecular weight molecules were precipitated with 10% trichloroacetic acid and then solubilized in 0.1 N NaOH. Incorporated radioactivity was quantified in a β-counter (Rackbeta, LKB), as previously described.[7,8]

Alternatively, MTT reduction was used to quantify metabolically active cells. Briefly, following treatment, cells were exposed to 0.25 mg/mL MTT [3,4,5-dimethylthiazol-yl)-2,5-diphenyl tetrazolium (Sigma)] for 2 h. The cells were examined under an inverted microscope to ascertain the density of violet spots corresponding to active mitochondria in order to exclude a potential mitochondrial toxicity of the compounds. The supernatant was then removed and the precipitated formazan was dissolved in 0.1 N HCl in 2-propanol and quantified at 540 nm in a multiwell plate reader (iEMS, Labsystems).

Evaluation of ET-1 Binding to Cells. Cells were grown to confluence in the presence of fetal calf serum (FCS) and washed with phosphate-buffered saline (PBS), and medium was changed to fresh medium containing 10% FCS and 50 pM [^{125}I]ET-1 (Anawa Trading) and either BQ123 or BQ788 [2×10^{-7} to 20×10^{-3} M] (Bachem). After 1 h at room temperature, cells were washed with PBS and extracted in 1% SDS in 0.1 N NaOH, and supernatants and cell extracts were counted in a γ-counter (Cobra Packard).

Immunohistochemistry and Immunocytochemistry. Immunohistochemistry for ET-1 and ECE-1 of human paraffin-embedded surgical specimen of glioblastoma was performed essentially as previously described.[7] Human glioblastoma cells were grown on glass slides for 2−3 days, washed in PBS, fixed for 15 min in 4% buffered paraformaldehyde, washed, and air-dried. Immunocytochemical detection of ET-1 and ECE-1 was performed as for human glioblastoma.

Calculations of Results. Each experiment was repeated at least three times unless otherwise stated. Means and standard deviation were calculated. Statistical significance was assessed using an unpaired two-tailed Student's *t*-test. IC_{50} values for growth inhibition were calculated using the experimental dose−response curves at the inhibitor concentration inhibiting by 50% the incorporation of thymidine for each inhibitor and cell line, respectively.

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Supporting Information Available: Elemental analysis of ECE-1 inhibitors. This material is available free of charge via the Internet at http://pubs.acs.org.

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