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Carbonic anhydrase inhibitors. Inhibition of the cytosolic and tumor-associated carbonic anhydrase isozymes I, II, and IX with a series of 1,3,4-thiadiazole- and 1,2,4-triazole-thiols

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Abstract—A series of heterocyclic mercaptans incorporating 1,3,4-thiadiazole- and 1,2,4-triazole rings have been prepared and assayed for the inhibition of three physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isozymes, the cytosolic human isozymes I and II, and the transmembrane, tumor-associated hCA IX. Against hCA I the investigated thiols showed inhibition constants in the range of 97 nM to 548 μM, against hCA II in the range of 7.9–618 μM, and against hCA IX in the range of 9.3–772 μM. Thiadiazoles were generally more active than triazoles against all investigated isozymes. Generally, the best inhibitors were the simple derivative 5-amino-1,3,4-thiadiazole-2-thiol and its N-acetylated derivative, which were anyhow at least two orders of magnitude less effective inhibitors when compared to the corresponding sulfonamides, acetazolamide, and its deacetylated derivative. An exception was constituted by 5-(2-pyridylcarboxamido)-1,3,4-thiadiazole-2-thiol, which is the first hCA I-selective inhibitor ever reported, possessing an inhibition constant of 97 nM against isozyme I, and being a 105 times less effective hCA II inhibitor, and 3154 times less effective hCA IX inhibitor. Thus, the thiol moiety may lead to effective CA inhibitors targeting isozyme I, whereas it is a less effective zinc-binding function for the design of CA II and CA IX inhibitors over the sulfonamide group.

1. Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1)[1–4] constitute interesting targets for the design of pharmacological agents useful in the treatment or prevention of a variety of disorders such as among others, glaucoma, acid-base disequilibria, epilepsy, and other neuromuscular diseases, altitude sickness, edema, and obesity.[5,6] A quite new and unexpected application of the CA inhibitors (CAIs) regard their potential use in the management (imaging and treatment) of hypoxic tumors,[7–14] since at least two CA isozymes of the 15 presently known in humans,[1–5] that is, CA IX and XII, are predominantly found in tumor cells and lack (or are present in very limited amount) in normal tissues.[6,15–18] The involvement of these enzymes, which catalyze the simplest physiological reaction, CO_2 hydration to bicarbonate and a proton,[1–4] in many physiological/pathological processes as well as the fact that generally different isozymes of the 15 mentioned above are involved in particular such processes, allows for the development of diverse medicinal chemistry applications of their inhibitors.[1,2] Thus, as mentioned above, CA IX and XII are the targets for the development of novel antitumor therapies,[5,7–10] CA II and XII for the development of antiglaucoma drugs,[19–22] CA Va and CA Vb for the design of new antiobesity agents,[6,23,24] CA VII for the development of anticonvulsant/antiepileptic drugs,[25] whereas non-vertebrate CAs such as, for example, the α-CA present in *Plasmodium falciparum* may lead to novel types of antimalaria drugs,[26] to cite only the most important isozymes investigated ultimately for drug design purposes.

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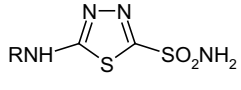
Most of the potent CAIs investigated up to now belong to the aromatic/heterocyclic sulfonamide or sulfamate classes,[1–5,27,28] although compounds incorporating other zinc-binding groups have also been investigated.[1–5,29,30] Indeed, such derivatives directly bind by means of the deprotonated sulfonamide/sulfamate moiety to the catalytically critical Zn(II) ion of the enzyme active site, also participating in a multitude of polar and hydrophobic interactions with amino acid residues of the cavity.[28,31–36] Typically, clinically used sulfonamide/sulfamate CAIs show potencies in the low nanomolar range against the physiologically relevant isozymes, such as CA I, II, V, and IX among others.[1–8]

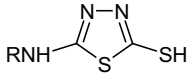
Some years ago we have also investigated the interaction of a small series of heterocyclic mercaptans with isozymes CA I, II, and IV,[37] showing that the SH moiety may act as a zinc-binding function in the design of CAIs, although the potency of these derivatives was lower than that of the corresponding sulfonamides or sulfenamides reported in the same work.[37] Here we extend those investigations, reporting the first inhibition study of the tumor-associated isozyme CA IX with a series of heterocyclic mercaptans incorporating 1,3,4-thiadiazole and 1,2,4-triazole moieties. Besides the interest in investigating novel zinc-binding functions in the design of CAIs, thiols may be important for their interaction with tumors from at least two other points of view: it is well documented that oxidative stress is a feature of many tumors expressing CA IX,[38] probably due to an imbalance between reduced and oxidized thiols such as glutathione, cysteine, etc., present in these tissues.[39,40] On the other hand, the activity of an enzyme critical for the proper folding of proteins, that is, protein disulfide isomerase (PDI), is highly influenced by the presence of this type of compounds,[39,40] and many biomedical applications are presently envisaged for thiols that may enhance the catalytic activity of PDI.[41] Thus, thiols as those described here may interact with such multiple biological targets, which makes them attractive candidates for in vivo studies in order to design novel approaches for the management of tumors.

2. Chemistry

Acetazolamide, 2-acetamido-1,3,4-thiadiazole-5-sulfonamide **1a**, is the sulfonamide CAI *par excellence*, showing low nanomolar activity against most of the investigated isozymes.[1–9] Its deacetylated derivative, 2-amino-1,3,4-thiadiazole-5-sulfonamide **1b**, also acts as a CAI, although with diminished potency when compared to acetazolamide.[1–10] However, its derivatization by means of acyl-, alkyl/arylsulfonyl-, ureido-, thioureido-, or sulfenamido moieties among others greatly enhances the CA inhibitory power of such derivatives.[1–10,27] Considering **1a** and **1b** as lead molecules, we decided to investigate the corresponding thiols **2** and **3**, as well as some derivatives of **2**, for their interaction with various CA isozymes.



1a: R = Ac
1b: R = H



2: R = H
3: R = Ac

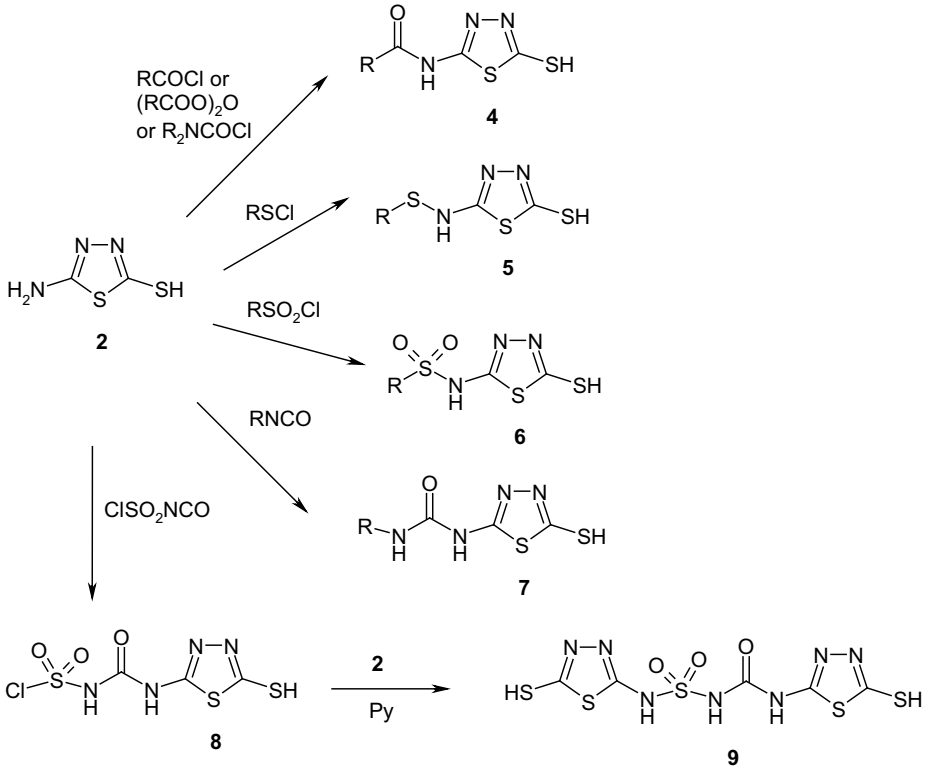
Derivatization of **2** has been performed by acylation with acyl chlorides, anhydrides, or carbamoyl chlorides, leading to compounds of type **4**.[9,27] Alternatively, reaction of **2** with sulfenyl- or sulfonyl chlorides leads to sulfenamides **5**[42,43] or sulfonamides **6**,[9,27] whereas transformation into ureas **7** has been achieved by means of the reaction with aryl isocyanates.[44] Treatment of **2** with chlorosulfonyl isocyanate[45] afforded the intermediate **8** (which was not isolated), which reacted with another equivalent of **2** leading to the bis-thiol **9** (thus, the reactions of **2** and **1b** with chlorosulfonyl isocyanate are quite different, **2** leading to the bis-thiol mentioned above, whereas **1b** leading to an annulated bicyclic derivative, investigated earlier by Katritzky's group).[45] The nature of the moiety R in derivatives **4**–**7** was chosen as varied as possible in order to detect compounds with the best affinity for the various CA isozymes against which they were assayed as inhibitors. It should be noted that the NH_2 moiety of **2** is much more reactive when compared to the SH one,[9,27,42–45] and in all these reactions only the N-substituted derivatives have been obtained (Scheme 1).

In order to obtain heterocyclic thiols possessing a different ring system except the 1,3,4-thiadiazole one, a library of mercapto-triazoles has been prepared by cyclization of the substituted thiosemicarbazides **10** (prepared as reported earlier)[46] in the presence of a strong base (NaOH). The desired sodium salts of the 3-mercapto-1,2,4-triazoles **11**–**19**[46] have thus been obtained, being then converted to the free mercaptans by treatment with hydrochloric acid as shown in Scheme 2. Among the obtained mercapto-triazoles, compounds **14**–**16** were reported earlier,[47] whereas the other derivatives are new.[48]

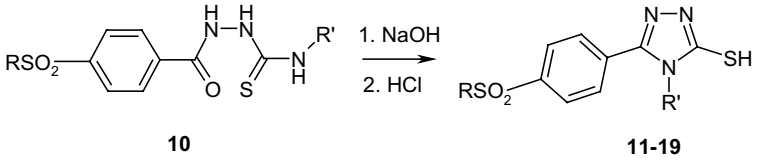
3. Carbonic anhydrase inhibition

Thiols **2**–**19** were investigated for the inhibition of three physiologically relevant CA isozymes, the cytosolic, ubiquitous hCA I and II, as well as the tumor-associated, transmembrane isozyme hCA IX. The lead sulfonamides **1a** and **1b** were also assayed in the same conditions, in order to afford data of some standard inhibitors (Table 1). As far as we know, this is the first report regarding the inhibition of hCA IX with mercaptans.

The following should be noted regarding data presented in Table 1: (i) thiols **2**–**19**, similarly to sulfonamides **1a**,**b** inhibit the slow cytosolic isozyme hCA I, showing inhibition constants in the range of 97 nM to 548 μM. The most potent hCA I inhibitor was unexpectedly the 2-pyridylcarboxamido derivative of 5-amino-1,3,4-thiadiazole-2-thiol **4b**, with a K_I of 97 nM, followed by acetazolamide **1a**, with a K_I of 250 nM. This is a quite



Scheme 1.



Scheme 2.

unexpected result, being the first documented case of a thiol with such a high affinity for a CA isozyme.[1] It is difficult to explain these results at this moment, without an X-ray crystal structure for the adduct of **4b** with hCA I but work is in progress in our laboratory to obtain such data. Good hCA I inhibitory properties were also shown by the simple thiols **2** and **3** as well as the derivatized 5-amino-1,3,4-thiadiazole-2-thiols **4c**,**d**, **5**, **6b**, **7**, and **9**, as well as the mercapto-triazoles **16** and **19**, which showed K_I values in the range of 2.6–9.0 μM, of the same order of magnitude as deacetylated acetazolamide **1b** (K_I of 8.6 μM). Thus, acetylation of **2** or its transformation to the bis-thiol **9** are beneficial for enhancing the hCA I inhibitory properties of these derivatives, whereas introduction of other moieties (such as 3-carboxy-pyridin-2-yl, *N*,*N*-diphenylcarbamoyl, 4-nitrobenzenesulfenyl-, dansyl, or 3,4-dichlorophenylureido) led to compounds with activity quite similar to that of the parent thiol **2**. It should be noted that the large difference of activity between the structurally related derivatives **4b** and **4c** as the introduction of a carboxy moiety in **4b** leads to a 85.5 times diminution of the hCA I inhibitory activity of **4c** when compared to **4b**. Other moieties, such as pentafluorophenylcarboxy- or 2-nitrobenzenesulfonyl-, lead to compounds (**4a** and **6a**) with greatly diminished hCA I inhibitory properties, with K_I-s in the range of 303–308 μM. Weak activity was also shown by most of the mercaptotriazoles **11**–**19** (except the two compounds mentioned above), which presented K_I-s in the range of 273–508 μM. It is at this point difficult to explain the relatively good hCA I inhibitory properties of **16** and **19**, which presents a rather similar substitution pattern as the less active derivatives (**11**–**15**, **17**, and **18**) in the library of prepared compounds; (ii) the rapid cytosolic isozyme hCA II was inhibited by thiols **2**–**19** with inhibition constants in the range of 7.9–618 μM, at least two order of magnitude higher than those of the sulfonamides **1a** and **1b**, which showed K_I-s in the range of 12–60 nM (Table 1). Clearly, the first conclusion is that the thiol moiety is less appropriate for the design of strong hCA II inhibitors, when compared to the sulfonamide one, since all thiols investigated here were moderate or weak inhibitors of this isozyme (comparing the pairs of derivatives **1a**–**3** and **1b**–**2**, the thiols are 153–733 times less effective hCA II inhibitors when compared to the corresponding sulfonamides). Thus,

Table 1. Inhibition data for derivatives **1**–**19** investigated in the present paper and standard sulfonamide CAIs (acetazolamide **1a** and its deacetylated derivative **1b**), against isozymes hCA I, II, and IX[49]

Compound	R	R′	K_I[a] (μM)		
			hCA I[c]	hCA II[c]	hCA IX[d]
1a[b]	—	—	0.250	0.012	0.025
1b[b]	—	—	8.6	0.060	0.041
2	—	—	7.1	9.2	9.3
3	—	—	4.5	8.8	9.5
4a	C_6F_5	—	308	129	312
4b	2-Pyridyl	—	0.097	10.2	306
4c	3-HOOC-pyridin-2-yl	—	8.3	9.1	242
4d	Ph_2N–	—	7.0	9.3	271
5	4-O_2N–C_6H_4	—	7.3	8.3	256
6a	2-O_2N–C_6H_4	—	303	230	315
6b	Dansyl	—	7.6	7.9	296
7	3,4-$Cl_2C_6H_3$	—	8.6	9.1	271
9	—	—	4.0	8.2	322
11	Ph	*n*-Pr	290	276	119
12	4-Cl–C_6H_4	*n*-Pr	548	618	9.8
13	4-Br–C_6H_4	*n*-Pr	508	382	772
14	Ph	*n*-Bu	415	604	310
15	4-Cl–C_6H_4	*n*-Bu	273	227	96.8
16	4-Br–C_6H_4	*n*-Bu	2.6	9.8	11.5
17	4-Br–C_6H_4	4-Me–C_6H_4	249	251	60.4
18	4-Br–C_6H_4	3-Me–C_6H_4	254	301	320
19	4-Br–C_6H_4	4-MeO–C_6H_4	9.0	9.9	192

[a] Errors in the range of 5–10% of the reported value (from three different assays).
[b] From Ref. 12.
[c] Human cloned isozyme, by the CO_2 hydration method.
[d] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.

all thiadiazoles except **4a** and **6a** and two triazoles (**16** and **19**) showed moderate hCA II inhibitory activity, possessing inhibition constants in the range of 7.9–10.2 μM. The other investigated thiols were very weak inhibitors of this isozyme (K_I-s in the range of 129–618 μM). SAR is quite obvious for the 1,3,4-thiadiazoles investigated here, with most substitution patterns at the amino moiety of **2** leading to compounds with comparable activity to that of the parent, underivatized thiol. But it is quite difficult to understand the highly diminished hCA II inhibitory activity of the pentafluorophenyl-carboxamido (**4a**) and 2-nitrophenylsufonamido (**6a**) derivatives of **2**, when compared to the other investigated compounds. For the triazole series just the opposite is true, with only two active derivatives (**16** and **19**), which in fact possess a very similar substitution pattern to those of the less active compounds **11**–**15**, **17**, and **18** (it should be mentioned that these were also the compounds with good hCA I inhibitory activity); (iii) against the tumor-associated isozyme hCA IX, thiols **2**–**19** again showed modest inhibitory activity, with inhibition constants in the range of 9.3–772 M, being at least two order of magnitude less efficient inhibitors when compared to sulfonamides **1a** and **1b** (which possess inhibition constants in the range of 25–41 nM) (Table 1). The best thiol inhibitors were compounds **2**, **3**, **12**, and **16**, which showed K_I-s in the range of 9.3–11.5 μM. All derivatizations of **2** (except acetylation) led to a large diminution of the hCA IX inhibitory properties (compounds **4**–**9** showed inhibition constants in the range of 242–322 μM), whereas the triazoles (except the two derivatives mentioned above, **12** and **16**) were generally even less effective hCA IX inhibitors (K_I-s in the range of 60.4–772 μM).

One of the most interesting results obtained here is the observation that **4b** is a hCA I selective inhibitor. Thus, this compound shows an inhibition constant of 97 nM against hCA I, being a 105 times less effective hCA II inhibitor, and a 3154 times less effective hCA IX inhibitor. Most of the sulfonamide/sulfamate CAIs investigated up to now show a much higher affinity for the rapid (hCA II and hCA IX) isozymes when compared to the slow isozyme hCA I.[1–10] Thus, this first CA I-selective inhibitor may lead to a better understanding of the physiological functions of this isozyme, which is poorly understood presently.[1]

4. Conclusions

A series of heterocyclic mercaptans incorporating 1,3,4-thiadiazole- and 1,2,4-triazole rings has been prepared and assayed for the inhibition of three physiologically relevant CA isozymes, the cytosolic hCA I and II, and the transmembrane, tumor-associated hCA IX. Against hCA I the investigated thiols showed inhibition constants in the range of 97 nM to 548 μM, against hCA II in the range of 7.9–618 μM, and against hCA IX in the range of 9.3–772 μM. Thiadiazoles were generally more active than triazoles against all investigated isozymes. The best inhibitors were the simple derivatives 5-amino-1,3,4-thiadiazole-2-thiol and its N-acetylated derivative, which were anyhow at least two orders of

magnitude less effective when compared to the corresponding sulfonamides, acetazolamide, and its deacetylated derivative. An exception was constituted by 5-(2-pyridylcarboxamido)-1,3,4-thiadiazole-2-thiol, which is the first hCA I-selective inhibitor ever reported, possessing an inhibition constant of 97 nM against isozyme I, being also a 105 times less effective hCA II inhibitor, and 3154 times less effective hCA IX inhibitor. Thus, heterocyclic thiols may lead to effective inhibitors targeting isozyme I, whereas the SH moiety is a less effective zinc-binding function for the design of CA II and CA IX inhibitors over the sulfonamide group.

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48. *4-Substituted-5-mercapto-3-(arylsulfonyl)phenyl-1,2,4-triazoles* ***11–19***: A solution of thiosemicarbazide **10**[46] (1 mmol) in 8 mL 8% NaOH solution was refluxed for 3 h and then filtered. After cooling the filtrate was neutralized with diluted aqueous HCl. The obtained white precipitate was filtered and recrystallized from $CHCl_3$/petroleum ether (1:2). *3-(Phenylsulfonyl)phenyl-4-n-propyl-5-mercapto-1,2,4-triazole (**11**)*: Mp 223 °C; 73% yield. Found: C, 56.59; H, 4.95; S, 17.53; N, 11.47. Anal. Calcd for $C_{17}H_{17}N_3O_2S_2$: C, 56.82; H, 4.73; S, 17.82; N, 11.69. UV spectrum (methanol; λ_{max} nm; ε_{max}): 202 (27,000); 254 (24,000); 320 (5500); IR spectrum (KBr; cm^{-1}): 3439s; 3102m; 2965m; 2934m; 2883w; 1548m; 1449m; 1472s; 1419m; 1320vs; 1256m; 1158vs; ^1H NMR, δ ppm (*J*, Hz): 0.69 t (7.3); 1.51 sx (7.3); 3.98 t (7.3); 7.63, t (8.0); 7.73, tt (8.0; 1.6); 7.92, d (8.4); 8.01, dd (8.0; 1.6); 8.11, d (8.4).
49. hCA I and hCA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog's group.[50] Cell growth conditions were those described in Ref. 50 and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[51] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 mM^{-1} cm^{-1} for CA I and 54 mM^{-1} cm^{-1} for CA II, respectively, based on M_r = 28.85 kDa for CA I, and 29.3 kDa for CA II, respectively. A variant of the previously published[52,53] CA IX purification protocol has been used for obtaining high amounts of hCA IX needed in these experiments. The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[54]) was amplified by using PCR and specific primers for the glutathione S-transferase (GST)-Gene Fusion Vector pGEX-3X. The obtained fusion construct was inserted in the pGEX-3X vector and then expressed in *E. coli* BL21 Codon Plus bacterial strain (from Stratagene). The bacterial cells were sonicated, then suspended in the lysis buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8, 150 mM NaCl, and 0.2% Triton X-100). After incubation with lysozime (approx. 0.01 g/L) the protease inhibitors Complete™ were added to a final concentration of 0.2 mM. The obtained supernatant was then applied to a prepacked Glutathione Sepharose 4B column, extensively washed with buffer and the fusion (GST-CA IX) protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin. The advantage of this method over the previous one,[53,54] is that CA IX is not precipitated in inclusion bodies from which it has to be isolated by denaturing–renaturing in the presence of high concentrations of urea, when the yields in active protein were rather low, and the procedure much longer. The obtained CA IX was further purified by sulfonamide affinity chromatography,[15] the amount of enzyme being determined by spectrophometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[55] The specific activity of the obtained enzyme was the same as the one previously reported,[16,17] but the yields in active protein were 5–6 times higher per liter of culture medium.
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55. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561–2573, An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for measuring the initial velocities for the CO_2 hydration reaction catalyzed by different CA isozymes, by following the change in absorbance of a pH indicator. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. The kinetic constants k_{cat} and k_{cat}/K_m were obtained by non-linear least-squares methods using PRISM 3. Stock solutions of inhibitors were prepared at a concentration of 1–3 mM (in DMSO–water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above. K_I-s of the inhibitors were determined by using Lineweaver–Burk plots, as reported earlier,[12,16,52] and represent the mean from at least three different determinations.