Cloning, Expression, Post-Translational Modifications and Inhibition Studies on the Latest Mammalian Carbonic Anhydrase Isoform, CA XV

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We have cloned and purified to homogeneity the latest member of the mammalian α-carbonic anhydrase (CA, EC 4.2.1.1) family, the mouse CA XV (mCA XV) protein. An investigation on the post-translational modifications of the enzyme has also been performed. The enzyme shows a moderate catalytic activity for the physiologic reaction, similarly to the physiologically relevant isoforms CA I, IV, VI, XII, and XIV, and it is susceptible to inhibition by sulfonamides and sulfamates. Best mCA XV inhibitors were celecoxib, sulfanilyl-sulfonamides, methazolamide, ethoxzolamide, dorzolamide, brinzolamide, and sulthiame, with K_Is in the range of 45−65 nM. Due to the presence of this enzyme in rather high amounts in the rodent kidneys, the contribution of this isoform to the overall drug response should be taken into account when animal models are used to investigate CA inhibitors.

Introduction

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide and water to bicarbonate and a proton ($CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$).[1] The CA proteins can be found in organisms all over the phylogenetic tree, and the mammalian enzymes belong to the so-called α-CA family, as it was the first one to be described.[2] In mammals, the members of the α-CA family participate in several physiological processes, such as pH regulation, CO_2 and HCO_3^- transport, production of biological fluids, bone resorption, metabolic processes, as well as cell adhesion and cell proliferation.[3,4] The mammalian CA family consists of thirteen active members that differ in their kinetic and inhibitory properties, cell and tissue distribution, and function. The isozymes can be classified according to their subcellular localization: CAs I, II, III, VII, and XIII are cytosolic enzymes,[3,5,6] CAs VA and VB are located in the mitochondria,[7] CA VI is the only secreted isozyme,[8] CAs IX, XII, and XIV are transmembrane proteins,[9−11] and CAs IV and XV are GPI-anchored to the cell membrane (Table 1).[12,13]

The latest member of the mammalian CA enzyme family was characterized in 2005, when the isozyme XV was reported for the first time.[13] CA XV appeared to be an exceptional member of this family because it is expressed in several species in different vertebrates, whereas in the primates, such as humans and chimpanzees, it has become a nonfunctional pseudogene. CA XV is a GPI-anchored enzyme like CA IV, and it is likely that CA IV or some other membrane-bound isozyme has taken the functional role of CA XV in primates. Nevertheless, CA XV has a high relevance for the biomedical research, because it is expressed[13] in the widely used model organisms, such as the rodents (mice and rats). This issue has to be taken into particular account when the results from these organisms are inferred to human physiology. In the first publication, the activity of mouse CA XV was measured for a recombinant protein form produced in the bacterial expression system, and it appeared to be very low.[13] Recently, we have obtained preliminary experimental indications suggesting that mouse CA XV possesses a catalytic activity comparable to those of the physiologically relevant isoforms CA XII and XIV, with k_{cat} of 4.7×10^5 s^{-1} and k_{cat}/K_M of 3.3×10^7 M^{-1} s^{-1} (Table 1).[14] In the present study we describe in detail the cloning and protein production of CA XV in the baculovirus-insect cell expression system and use mass spectrometric analyses to characterize the obtained recombinant product, particularly its post-translational modifications. CA XV is the only isozyme whose inhibition profile has not been published earlier (apart from acetazolamide, which showed K_I of 72 nM),[14] and therefore, we have investigated

Table 1. Kinetic Parameters for CO_2 Hydration Reaction Catalyzed by the 13 Vertebrate Catalytically Active α-CA Isozymes, at 20 °C and pH 7.5, and Their Subcellular Localization

isozyme[a]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \times s^{-1}$)	subcellular localization
hCA I	2.0×10^5	4.0	5.0×10^7	cytosol
hCA II	1.4×10^6	9.3	1.5×10^8	cytosol
hCA III	1.3×10^4	52.0	2.5×10^5	cytosol
hCA IV	1.1×10^6	21.5	5.1×10^7	membrane-bound
hCA VA	2.9×10^5	10.0	2.9×10^7	mitochondria
hCA VB	9.5×10^5	9.7	9.8×10^7	mitochondria
hCA VI	3.4×10^5	6.9	4.9×10^7	secreted into saliva/milk
hCA VII	9.5×10^5	11.4	8.3×10^7	cytosol
hCA IX[b]	1.1×10^6	7.5	1.5×10^8	transmembrane
hCA XII	4.2×10^5	12.0	3.5×10^7	transmembrane
hCA XIII	1.5×10^5	13.8	1.1×10^7	cytosol
hCA XIV	3.1×10^5	7.9	3.9×10^7	transmembrane
mCA XV	4.7×10^5	14.2	3.3×10^7	membrane-bound

[a] h = human; *m* = mouse enzyme. [b] Proteoglycan and CA catalytic domains.[15]

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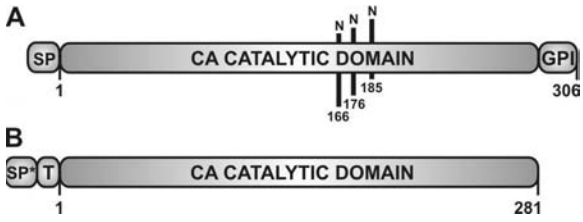


Figure 1. Native mouse and recombinant CA XV sequences are illustrated in panels A and B, respectively. In panel A are shown the putative N-linked glycosylation sites present in the protein sequence; these correspond to residues Asn184, Asn194, and Asn203 of SwissProt entry Q99N23. Abbreviations: SP = signal peptide, SP* = signal peptide optimized for *Spodoptera frugiperda*, *T* = octahistidine tag and a thrombin cleavage site (LVPRGS, the cutting site is underlined).

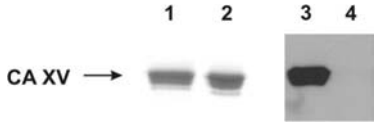


Figure 2. SDS-PAGE shows 2.4 μg of purified protein without the His-tag under reducing (lane 1) and nonreducing conditions (lane 2). Western blot (lanes 3 and 4) was perfomed with His-tag antibody: 900 ng of protein before (lane 3) or after (lane 4) thrombin treatment was applied to the blot and the result confirmed that the removal of the His-tag was successful.

here its inhibition with a panel of sulfonamide and sulfamate derivatives, some of which are clinically used and investigational drugs.

Results

Production of Recombinant Mouse CA XV. The recombinant mouse CA XV (mCA XV) protein was produced in the baculovirus-insect cell expression system. Figure 1A illustrates the domain structure of the native mCA XV, together with its N-glycosylation consensus sequences (Asn_{166}-Phe-Ser, Asn_{176}-Leu-Ser and Asn_{185}-Leu-Thr); Figure 1B shows the nature of the investigated recombinant protein. The latter is missing the C-terminal GPI signal sequence, and therefore, it was possible to isolate the protein directly from the cell culture medium. The protein was successfully purified to homogeneity in one step, as is visible from the SDS-PAGE analysis shown in Figure 2 (lanes 1 and 2). The His-tag was successfully removed from the protein, as confirmed by Western blotting (Figure 2, lanes 3 and 4).[15,16]

Recombinant Mouse CA XV (mCA XV) Contains Three N-Linked Glycosylation Sites. To investigate possible post-translational modifications present in mCA XV, the purified protein was submitted to ESI-IT-MS and MALDI-TOF-MS. Only in the second case was a mass spectrum obtained that, however, showed a nonresolved and broad signal (data not shown), typical of a polypeptide species bearing multiple modifications. Modification type and the assignment of modified residue(s) were elucidated on an mCA XV sample resolved by reducing SDS-PAGE. The protein band was alkylated and digested with trypsin directly into the gel. The peptide digest was extracted and resolved by μLC into different fractions, which were then analyzed by MALDI-TOF-MS. Fractions eluting at 49.8 and 62.0 min showed a similar pattern of multiple signals in the mass spectrum (Figure 3A,B). On the basis of measured masses and known pathways of glycoprotein biosynthesis, these peaks were assigned to peptides (154−175) and (145−175) having a pentasaccharide core N-linked to the putative N-glycosylation site (Asn166), bearing 0−5 hexose units (theor. MH^+ values: *m*/*z* 3284.5, 3608.8, 3770.9, 3933.1, 4095.2 and 4213.5, 4537.8, 4699.9, 4862.1, 5024.2, respectively). The first glycopeptides resulted from the second ones by aspecific hydrolysis at Leu153. Glycopeptides in each fraction collapsed to a unique component after PNGase treatment, which showed a single MH^+ signal *m*/*z* 2392.5 and 3321.9 for peptide (154−175) and (145−175), respectively. These peptide species were submitted for PSD analysis, confirming the identity of the glycopeptides and the expected Asn166 > Asp conversion in the deglycosylated peptide (Figure 3D). Multiple signals associated to glycopeptides were also detected in the mass spectrum of the fraction eluting at 72.3 min (Figure 3C). On the basis of measured mass values, these peaks were tentatively associated to peptides (154−190) and (154−201) having three pentasaccharide cores N-linked to all putative N-glycosylation sites (Asn166, Asn176, and Asn185) present within mCA XV sequence and globally bearing two N-acetylhexosamine and 5−10 hexose units (theor. MH^+ values: *m*/*z* 7775.8, 8100.1, 8262.3, 8424.4, 8586.5 and 8869.2, 9193.5, 9355.6, 9517.7, 9679.9, respectively). The first glycopeptides resulted from the second ones by aspecific hydrolysis at Phe190. This assignment was confirmed by MS analysis of this fraction after PNGase treatment, which showed two components with MH^+ signals at *m*/*z* 3881.5 and 4971.9 for peptides (154−190) and (154−201), respectively. Peptide nature was proved by PSD analysis of the deglycosylated species (data not shown). Also in this case, it was not clear if the complex pattern of peaks measured was generated from real glycopeptides heterogeneity or the well-known partial loss of carbohydrate groups during MALDI-TOF-MS analysis. No signals associated with the nonglycosylated peptides were detected in any LC fractions from mCA XV digest, thus suggesting that this protein was completely modified. This result was in agreement with the heterogeneity pattern observed in SDS-PAGE. In conclusion, our experiments allowed covering 80.1% of the protein sequence and demonstrated that all putative N-glycosylation sites of mCA XV were modified either by high mannose or hybrid-type glycans, also excluding the occurrence of other post-translational modifications.

CA XV Activity and Inhibition Studies. The kinetic parameters for the CO_2 hydration reaction catalyzed by the 13 active mammalian isoforms CA I−XV as well as the subcellular localization of these CAs are shown in Table 1. A series of 38 sulfonamides and sulfamates, some of which are clinically used drugs (**AZA**−**SLT**)[1] have then been tested for their interaction with mCA XV (Chart 1).[17] Inihibition constants of these compounds against mCA XV as well as four other mammalian CA isozymes (the cytosolic hCA I and II, the secreted hCA VI and the transmembrane CA IX) are given in Table 2 for comparison.

Discussion

CA XV Preparation, Post-Translational Modifications, and Activity. All mammalian CA isozymes were cloned and purified at this moment. Genome analysis data for many vertebrate taxa showed CA XV to be the last member of this enzyme family. There is also a high degree of heterogeneity in the subcellular localization of these enzymes, with cytosolic, mitochondrial, membrane-associated, transmembrane, and secreted such proteins being known (Table 1). Thus, considerable problems are encountered when some of them are being cloned and produced in bacterial expression systems (*E. coli*), because formation of inclusion bodies containing unfolded protein usually occurs. Generally it is difficult to obtain catalytically

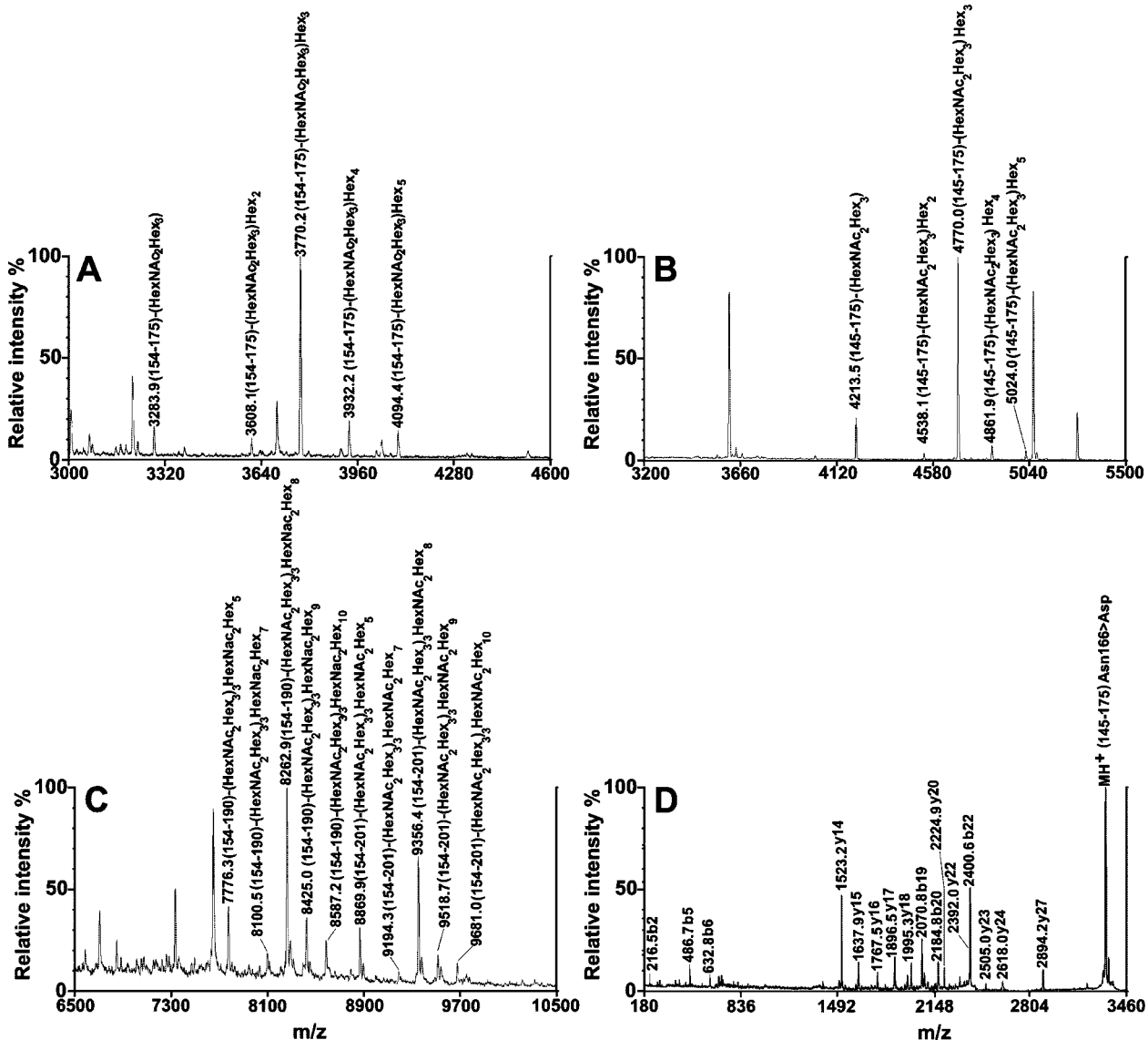


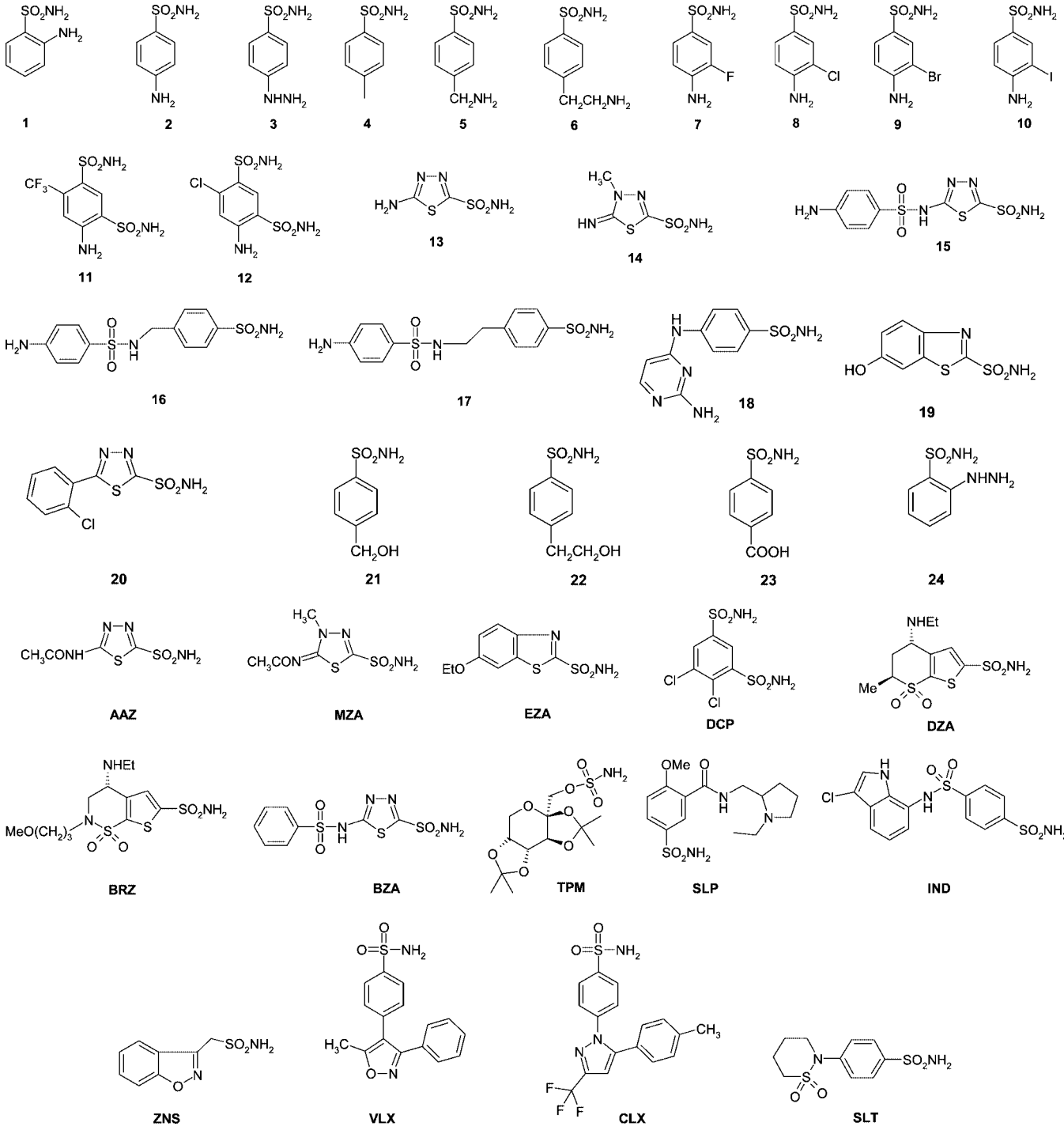
Figure 3. MALDI-TOF-MS analysis of the purified glycopeptides from recombinant mouse CA XV having high mannose and hybrid-type N-linked structures. Mass spectra of glycopeptide (154−175) and (145−175) derivatives eluting in the CA XV tryptic map at 49.8 and 62.0 min are reported in panel A and B, respectively. Mass spectra of glycopeptide (154−190) and (154−201) derivatives eluting at 72.3 min are reported in panel C. Panel D shows the PSD analysis of the deglycosylated peptide (145−175) with MH^+ at *m/z* 3321.9, which was obtained after PNGase treatment of the glycopeptides reported in panel B.

active, properly folded enzymes from such expression systems.[15,18] For this reason, we have developed a procedure for producing membrane-bound CA isozymes using an eukaryotic expression system, the baculovirus-insect cell expression system,[15] and here we have used this method to produce the latest isozyme that was cloned, mCA XV. The yield, the simplicity of the purification procedure, and the purity of the obtained protein when using this system are quite favorable (as compared to the bacterial expression systems)[15,18] to propose it for a wider use (see Figures 1 and 2 and Experimental Section for details).

Detailed MALDI-TOF-MS analysis of the recombinant mCA XV (Figure 3) obtained in this way allowed us to discover three N-linked glycosylation sites present in this protein. Thus, we demonstrated that N-glycosylation of mCA XV occurs at residues Asn166, Asn176, and Asn185, which are modified either by high mannose or hybrid-type glycans. In the case of human CA IX, we showed that the nature of the N-linked glycans present in the protein from baculovirus-insect cell expression system differed slightly with respect to that from mammalian cells.[15] We could also exclude the occurrence of other post-translational modifications in recombinant mCA XV. Information about such modifications, although not directly affecting the enzyme active site (as residues 166, 176, and 185 are outside the active site cavity and exposed on protein surface),[13] may be important when studying CAs both in vivo (e.g., for generation of antibodies and investigation of expression patterns) and in vitro (e.g., for testing enzyme inhibitors/activators, as well as the stability and conformation of the protein). These experiments also showed that the protein used in this work might be very similar (with respect to folding, glycosylation pattern, and enzyme activity) to the mCA XV present in the living organisms.[13]

The enzyme activity of mCA XV was determined for the physiologic reaction, that is, CO_2 hydration to bicarbonate, by means of a stopped-flow technique (Table 1).[17] Data of Table 1 show the k_{cat} and K_M values for all members of the α-CA family, that is, isoforms hCA I−XIV (of human origin) and mCA XV. mCA XV can be classified together with isoforms hCA I, hCA IV, hCA VI, hCA XII, and hCA XIV, among the medium activity CAs, with k_{cat}/K_M values in the range of

Chart 1



(3.3−5.1) × 10^7 M^{-1} s^{-1} (Table 1). Several isozymes, such as hCA II, hCA VB, hCA VII, and hCA IX show higher catalytic activity (the so-called "high speed" CAs) as compared to the medium-activity isoforms mentioned above, with k_{cat}/K_M in the range of 8.3 × 10^7−1.5 × 10^8 M^{-1} s^{-1}. Two isozymes, hCA VA and hCA XIII, are low activity CAs, with k_{cat}/K_M in the range of of (1.1−2.9) × 10^7 M^{-1} s^{-1}, whereas hCA III is a very inefficient catalyst for the hydration of CO_2 to bicarbonate, with a k_{cat}/K_M of only 2.5 × 10^5 M^{-1} s^{-1}.

CA XV Inhibition. CAs are involved in numerous physiological and pathological processes, including respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiological and pathological processes in vertebrates, as well as the growth and virulence of various fungal/bacterial pathogens.[1−3,18,19] In addition to the established role of carbonic anhydrase inhibitors (CAIs) as diuretics and antiglaucoma drugs, it has recently emerged that CAIs could have therapeutic potential as novel antiobesity, anticancer, and anti-infective drugs.[1,4] Many of the CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited or activated to treat a wide range of disorders.[1,4] Two main classes of CAIs are known: the metal-complexing anions and the unsubstituted sulfonamides and their bioisosteres (sulfamates, sulfamides), which bind to the metal ion of the enzyme either by substituting the nonprotein

Table 2. hCA I, II, VI, IX, and mCA XV Inhibition Data with Sulfonamides **1**−**24** and the Clinically used Derivatives **AAZ**−**SLT**, by a Stopped Flow CO_2 Hydrase Assay Method (ref 17)[a]

	K_I[b] (nM)				
inhibitor	hCA I[c]	hCA II[c]	hCA VI[d]	hCA IX[e]	mCA XV[f]
1	45400	295	772	33	8805
2	25000	240	941	238	9610
3	28000	300	1275	294	9540
4	78500	320	1582	460	7990
5	25000	170	4800	103	970
6	21000	160	813	33	783
7	8300	60	96	245	79
8	9800	110	1097	264	62
9	6500	40	4680	269	83
10	6000	70	1024	285	72
11	5800	63	955	24	926
12	8400	75	608	39	757
13	8600	60	798	41	48
14	9300	19	740	30	49
15	6	2	73	38	48
16	164	46	55	31	49
17	185	50	24	24	63
18	109	33	86	16	67
19	95	30	103	14	64
20	690	12	114	30	60
21	55	80	6680	21	4560
22	21000	125	4150	22	4660
23	23000	133	887	26	4650
24	24000	125	1090	176	6895
AAZ	250	12	11	25	72
MZA	50	14	10	27	65
EZA	25	8	43	34	58
DCP	1200	38	79	50	95
DZA	50000	9	10	52	61
BRZ	45000	3	0.9	37	61
BZA	15	9	93	47	70
TPM	250	10	45	58	78
SLP	12000	40	0.8	46	73
IND	31	15	47	24	72
ZNS	56	35	89	5.1	634
VLX	54000	43	572	27	66
CLX	50000	21	94	16	45
SLT	374	9	134	43	65

[a] Data for Isoforms I, II, VI, and IX are from refs 1 and 18. [b] Errors in the range of 5−10% of the shown data from three different assays. [c] Human recombinant isozymes.[1] [d] Full length, human recombinant enzyme.[18d] [e] Catalytic domain of human, recombinant enzyme.[15] [f] Mouse recombinant enzyme.

zinc ligand to generate a tetrahedral adduct or by addition to the metal coordination sphere, generating trigonal-bipyramidal species.[1] However, a critical problem in the design of CAIs is related to the high number of isoforms (16 of which 13 are catalytically active, see Discussion above and Table 1), their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors.[1] Thus, it is critically important to test clinically used compounds as well as simpler sulfonamide building blocks used for designing CAIs against all known mammalian isozymes, as this may have consequences for the side effect profiles of some of these drugs or even for designing novel types of applications for these pharmacological agents.[1] As there are quite scarce literature data regarding the inhibition of CA XV,[14,20] here we present an exhaustive study in which all the clinically used sulfonamides/sulfamates,[1] such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, topiramate **TPM**, sulpiride **SLP**, indisulam **IND**, zonisamide **ZNS**, valdecoxib **VLX**, celecoxib **CLX**, and sulthiame **SLT**, are included, together with simpler aromatic and heterocyclic sulfonamides of types **1**−**24** (generally used in the design of CAIs possessing more complicated scaffolds).[1,21]

The following structure−activity relationship (SAR) can be deduced from data of Table 2, for the 38 compounds **1**−**24** and **AAZ**−**SLT** investigated as mCA XV inhibitors in the present study.

(i) Weak inhibitory properties, with inhibition constants in the range of 4560−9610 nM were observed for derivatives **1**−**4** and **21**−**24**. All these are simple benzenesulfonamides possessing 2- or 4-substituents of the amino-, hydrazino-, methyl-, hydroxyl-methyl/-ethyl, or carboxyl type. Generally, these compounds are also ineffective CA I and CA VI inhibitors, but many times they show much better inhibition of the cytosolic CA II and the transmembrane CA IX isozymes (Table 2).

(ii) A second group of derivatives, including **5**, **6**, **11**, **12**, and **ZNS**, showed more effective mCA XV inhibitory activity, with K_Is in the range of 634−970 nM. These sulfonamides include homosulfanilamide **5** and its longer chain congener **6** (which are much better mCA XV inhibitors as compared to the structurally related, corresponding alcohols **21** and **22** mentioned above), the 1,3-benzenedisulfonamides **11** and **12**, as well as the clinically used antiepileptic zonisamide **ZNS**.[22] As for the compounds mentioned above, these sulfonamides are ineffective inhibitors of hCA I and hCA VI and generally show good to moderate activity as inhibitors of hCA II and IX.

(iii) Most of the sulfonamides/sulfamates investigated here, for example, **7**−**10**, **13**−**20**, **AAZ**, **MZA**, **EZA**, **DCP**, **DZA**, **BRZ**, **BZA**, **TPM**, **SLP**, **IND**, **VLX**, **CLX**, and **SLT**, show effective mCA XV inhibitory activity, with K_Is in the range of 45−95 nM, which probably may elicit physiologic responses in vivo (Table 2). Several interesting SAR was observed by analyzing these data. These compounds are chemically quite heterogeneous, with both aromatic (benzenesulfonamide), heterocyclic, and sugar scaffolds present in their molecules. Especially good inhibition (K_Is > 50 nM) has been observed for **13**−**16** and celecoxib **CLX**. These scaffolds have little in common, as **13** and **14** are the deacetylated precursors of **AAZ** and **MZA** (which are 1.3−1.5 times less inhibitory as compared to **13** and **14**), whereas **15** and **16** are sulfanylated sulfonamides (similarly to **17**, which is a less effective inhibitor). Furthermore, the celecoxib scaffold (this was the best mCA XV inhibitor detected so far) is completely different from all other sulfonamides/sulfamates investigated here. All these data prove that as for other CA isoforms,[15,18,19,23] even small structural variations in the inhibitor molecule lead to important differences of inhibitory power and that probably it should also be possible to point out interactions, which may lead to the design of inhibitors with a certain degree of selectivity for inhibiting mCA XV over other isoforms such as, for example, hCA II. This goal has not been achieved for most isoforms, apart from for hCA IX, for which many highly selective inhibitors have been described.[24] It is also interesting to note that mCA XV has a very different inhibition profile as compared to hCA IV,[24g] the only isozyme that has a GPI-anchoring to the plasma membrane, in addition to CA XV. Thus, except for acetazolamide and ethoxzolamide, which show inhibition constants of 74−93 nM against hCA IV,[24g] all other sulfonamides/sulfamates of types **1**−**24** as well as clinically used drugs employed in the present study, generally show a quite weak inhibitory activity against hCA IV, with inhibition constants in the range of 910 nM−15.33 μM.[24g] Thus, there is a net difference between these two extracellular CAs in their susceptibility to be inhibited by sulfonamides/sulfamates, with mCA XV showing a better affinity for these inhibitors as compared to hCA IV. An explanation of this phenomenon is not available at this moment

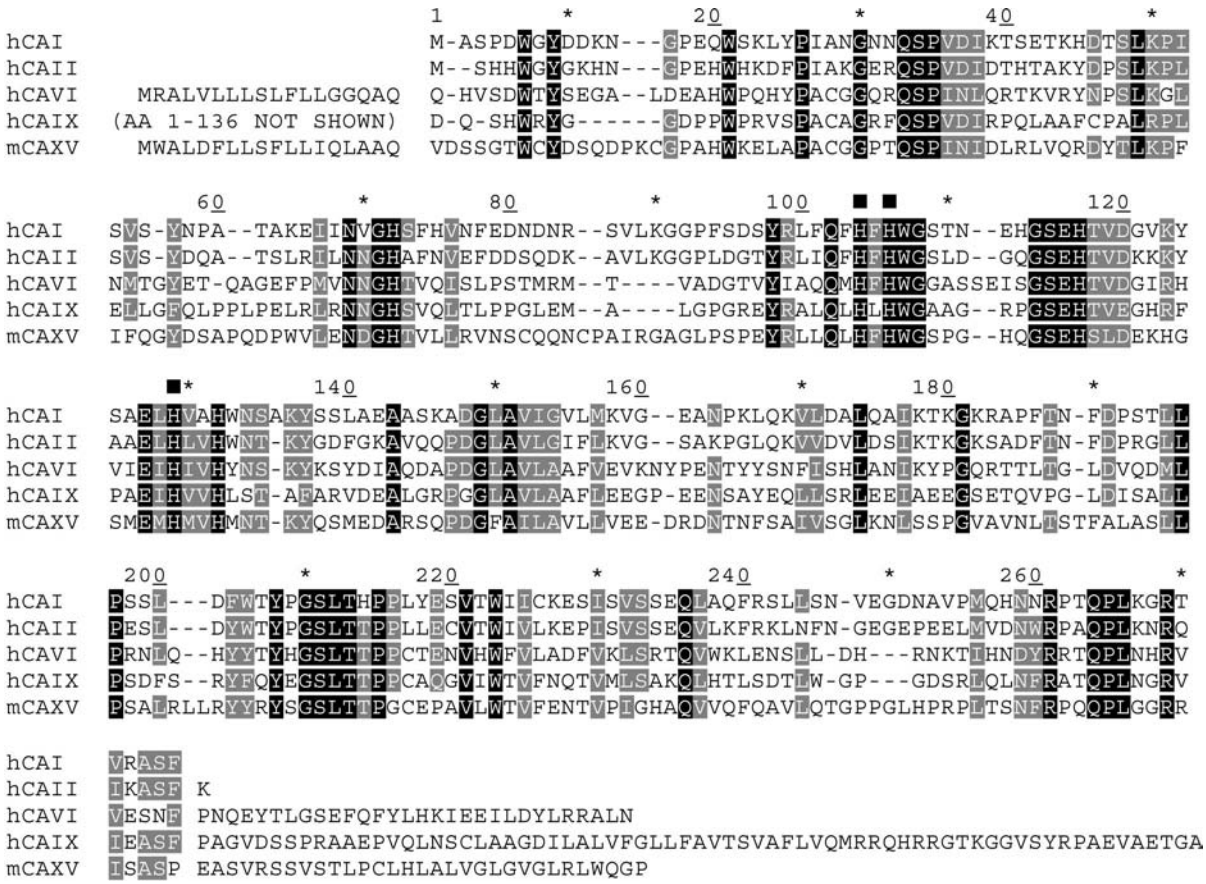


Figure 4. Alignment of human CAs I, II, VI, and IX and mouse CA XV. The alignment for the sequence of the CA domains was performed with TCoffee with regular settings,[26] and the alignment was visualized with Genedoc 2.6.02. For clarity, the signal sequence and the proteoglycan domain of CA IX have been excluded from the figure. The numbering is according to mouse CA XV sequence of Figure 1. The positions shaded with black color have identical residues in all five isozymes, and those positions that have similar residues at least in four isozymes are shaded with gray. The histidine residues that bind zinc ion and are crucial for the CA catalytic activity are pointed out by the ■ symbol.

since the X-ray crystal structures of human (or mouse) CA IV as well as that of mCA XV are not resolved yet.

How can we explain the inhibition data shown in Table 2 and the differences observed between mCA XV and other CA isoforms, such as, for example, the cytosolic hCA II (that has high affinity for sulfonamides), the cytosolic hCA I (that has low affinity for sulfonamides), the secreted isozyme CA VI, as well as the transmembrane one CA IX? As the mCA XV X-ray crystal structure (and also those of hCA VI and IX) is not available yet, we shall only consider an alignment of the amino acid sequence of these five isozymes (Figure 4), as well as the extensive X-ray crystal data of many adducts of sulfonamides and sulfamates with isozyme hCA II.[1,22−25] Thus, from Figure 4 it may be observed that the amino acid residues, which are critical in the CA catalytic cycle, are conserved in all these isozymes: (i) the three zinc ligands, His105, His107, and His120 (corresponding to His94, 96, and 119 in the hCA II numbering system);[1] (ii) the "gate-keeping" residues Thr213 (corresponding to Thr199 in the hCA II numbering system) and Glu117 (corresponding to Glu106 in the hCA II numbering system), which orient the substrate in the right position to be attacked by the zinc-bound hydroxide ion; and (iii) His72 (corresponding to His64 in the hCA II numbering system), the proton shuttle residue, which transfers protons from the zinc bound water molecule toward the external medium, leading to the generation of the active form of the enzyme with hydroxide as the fourth zinc ligand.[1−4] Thus, mCA XV has all the requisites to show a catalytic activity comparable to that of isozymes I, IV, VI, or XII investigated in great detail earlier,[1] as mentioned above. There are, however, several amino acid residues that are characteristic only to CA XV among the α-CA isozymes, such as those in position 75 (corresponding to 67 in the hCA II numbering system) and 140 (corresponding to 131 in the hCA II numbering system), which have been shown earlier to be critically important for the binding of inhibitors and activators to several CA isozyme active sites.[25,27] In fact, in hCA II, the best investigated isoforms from the crystallographic point of view,[25,27] the residue 75 is an Asn and the residue 141 is a Phe, and they were shown to participate in strong interactions (H-bonds for Asn67 and π-stacking for Phe131) with many inhibitors/activators for which the X-ray crystal structures in adducts with this isozyme have been resolved.[22−25,27] From data of Figure 4, it may be observed that in mCA XV there is a leucine in position 75, whereas in the other investigated isozymes the corresponding amino acid is not hydrophobic, namely, His (CA I), Asn (CA II), and Gln (CA VI and IX). Also, for the amino acid in the position 141, which is a methionine in mCA XV, there is a great variability for the other isoforms (Leu in CA I, Phe in CA II, Tyr in CA VI, and Val in CA IX, see Figure 4). It may be hypothesized that, also for mCA XV, these two amino acids may play an important role for the binding of inhibitors, and this may explain the completely different inhibition profile observed with the investigated sulfonamides/sulfamate (Table 2). We do not think that only these two amino acid residues are involved in the binding of inhibitors to the CA active site but, as they show a rather great

variability between the different isozymes, their contribution to the selectivity profiles of inhibitors may be quite relevant.

Conclusions

We have cloned and purified to homogeneity the latest member of the mammalian α-CA family, the mouse CA XV protein. An investigation of the post-translational modifications of this enzyme has also been performed. The enzyme shows a moderate catalytic activity for the physiologic reaction, similarly to CA I, IV, VI, XII, and XIV, and it is susceptible to inhibition by sulfonamides and sulfamates. Best mCA XV inhibitors were celecoxib, sulfanilyl-sulfonamides, methazolamide, ethoxzolamide, dorzolamide, brinzolamide, and sulthiame, with K_Is in the range of 45−65 nM. Due to the presence of this enzyme in rather high amounts in the rodent kidneys, all animal models investigating CA inhibitors in such systems should take into account the contribution of this isoform to the overall response to the drugs.

Experimental Section

Chemistry. Compounds **1**−**24** and **AAZ**−**SLT** are either commercially available (Sigma-Aldrich) or were prepared as described earlier.[18,19,21−23]

Construction of Recombinant Baculoviruses. The recombinant mouse CA XV constructed for the study is illustrated in Figure 1B. The protein was designed to contain a CA XV signal sequence whose codon usage was optimized for *Spodoptera frugiperda*, eight histidine residues for protein purification, and a thrombin cleavage site for removing the histidine tag. The recombinant protein was encoded for residues 1−281 of the mature mouse CA XV enzyme (residues 19−299 of SwissProt entry Q99N23), and it was designed to lack the C-terminal GPI signal sequence to get the protein secreted into the cell culture medium. The cDNA sequences were made by stepwise elongation of sequence-PCR (SES-PCR). In the first PCR reaction, the coding sequence of *Car15* was amplified in a reaction that had a full-length *Car15* cDNA in pME18S-FL3 vector (IMAGE Clone 1908347, Geneservice Ltd.) as a template. The PCR reaction consisted of a 98 °C denaturation step for 2 min, followed by 33 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. The primers in the first reaction were 5′-ATG TGG GCC CTG GAC TTC TT-3′ (F1) and 5′-CTA GGG ACC CTG CCA GAG TC-3′ (R1). For the rest of the PCR reactions, the PCR protocol consisted of a 98 °C denaturation step for 4 min, followed by 33 cycles of denaturation at 98 °C for 10 s, annealing at 45 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 8 min. The primers in the second reaction were 5′-CAC CAC CAT CAC CAC CAT CAC CAC CTG GTG CCC CGT GGT TCC CAG GTG GAC TCC AGT GGT ACC TGG TGC-3′ (F2) and 5′-TTC GCC AAG CTT TTA TGA TCG GAC GGA TGC TTC GGG AGA GGC TGA TAT C-3′(R2), in the third reaction 5′-TTC CTG CTG ATC CAG CTG GCT GCT CAG GTC GAC TCC TCC GGT ACC CAC CAC CAT CAC CAC CAT CAC-3′ (F3) and (R2), and finally in the fourth reaction 5′-GGC CAG ATC TAT GTG GGC TCT GGA CTT CCT GCT GTC CTT CCT GCT GAT CCA GCT GGC T-3′ (F4) and (R2). The PCR reactions were performed using Phusion polymerase (Finnzymes). The primers F4 and R2 contained restriction sites for *Bgl*II and *Hin*dIII (underlined), respectively. The baculoviral genome encoding for the recombinant CA XV protein was generated with Bac-to-Bac Baculovirus Expression System (Invitrogen). Cloning into pFastBac1 vector, sequencing protocol, and site-directed transposition were performed as described for another CA isozyme.[15]

Production, Purification, and Preparation of Recombinant CA XV. The recombinant protein was produced in Sf-9 insect cells and purified with the Probond Purification System (Invitrogen) as has been described previously for CA IX.[15] The purified protein was changed to a buffer of 50 mM Tris-HCl, pH 7.5 (Sigma-Aldrich, St. Louis, MO), using an Amicon Ultra 10 kDa cutoff centrifugal filter device. For His-tag removal, the recombinant protein was treated with 460 μL of 50% thrombin slurry (Thrombin CleanCleave KIT, Sigma-Aldrich) per 1 mg of protein with gentle shaking at 25 °C for 27 h. The thrombin slurry was removed from the protein solution by filtration and the His-tag by washing the protein using the centrifugal filter device. Protein concentration was measured with two different methods and three different dilutions, as described in ref 15. The purified recombinant protein was analyzed by 10% SDS-PAGE under reducing[16] and nonreducing conditions, followed by treatment with the Colloidal Blue Staining Kit (Invitrogen). Western blotting, which was performed as described previously,[15] was used to confirm the removal of the His-tag from the N-terminus: the primary antibody (diluted in 1:1000) in the Western blot were His-tag antibody (Novus Biologicals).

Protein Digestion and Peptide Separation. CA XV was excised from the gel after SDS-PAGE, triturated, *in-gel* reduced, S-alkylated, and digested with trypsin, as previously reported.[15] Gel particles were extracted with 25 mM NH_4HCO_3/acetonitrile (ACN; 1:1 v/v) by sonication, and digests were concentrated. Peptide mixtures were either desalted using μZipTipC_{18} pipet tips (Millipore) before direct MALDI-TOF-MS analysis or resolved on a capillary Hypersil-Keystone Aquasil C_{18} Kappa column (100 × 0.32 mm, 5 μm particle size) (ThermoElectron) using a linear gradient from 10 to 60% of ACN in 0.1% formic acid, at a flow rate of 5 μL/min for 80 min. Collected fractions were concentrated and similarly analyzed by MALDI-TOF-MS.

PNGase F Treatment. Glycopeptides were deglycosylated by reacting with 0.2 U of PNGase F (Roche) in 50 mM NH4HCO3, pH 8, at 37 °C for 12 h. Then, 3 μL of 10% v/v formic acid was added to reaction mixtures, which were desalted on μZipTipC_{18} pipet tips (Millipore) before MALDI-TOF-MS analysis.

MS Analysis. Whole protein digests or selected peptide fractions were loaded on the MALDI target together with 2,5-dihydroxybenzoic acid (10 mg/mL in 70% v/v ACN, 0.06% v/v TFA) as matrix, using the dried droplet technique. Samples were analyzed with a Voyager-DE PRO spectrometer (Applera) operating with a 337 nm laser.[15] Mass spectra were acquired in positive polarity, using the instrument either in reflectron or linear mode. Internal mass calibration was performed with peptides from trypsin autoproteolysis or added molecular markers (Applera). Postsource decay (PSD) fragment ion spectra were acquired after isolation of the appropriate precursor, as previously reported.[15] In both cases, data were elaborated using the DataExplorer 5.1 software (Applera). Observed MALDI-TOF mass values were assigned to peptides or glycopeptides using the GPMAW 4.23 software (Lighthouse Data). This software generated a mass/fragment database output based on protein sequence, protease selectivity, nature of the amino acids susceptible to eventual glycosylation and the molecular mass of the modifying groups. Mass values were matched to protein regions using a 0.02% mass tolerance value. Glycosylation assignments were always confirmed by additional MS experiments on modified peptides as treated above. All masses are reported as average values. Peptide numbering refers to mature mouse CA XV.

CA Inhibition Assay. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[17] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10−20 mM Hepes (pH 7.5) or Tris (pH 8.3) as buffers, and 20 mM Na_2SO_4 or 20 mM $NaClO_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10−100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and 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. Stock solutions of inhibitor (10 mM) were prepared in distilled−deionized water and dilutions up to 0.01 nM were done thereafter with distilled−deionized water. Inhibitor and enzyme

solutions were preincubated together for 15 min at room temperature prior to assay, to allow for the formation of the E-I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, as reported earlier,[20−23] and represent the mean from at least three different determinations.

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