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Carbonic anhydrase inhibitors: inhibition of the tumor-associated isozyme IX with fluorine-containing sulfonamides. The first subnanomolar CA IX inhibitor discovered

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Abstract—Polyfluorinated CAIs show very good inhibitory properties against different carbonic anhydrase (CA) isozymes, such as CA I, II, and IV, but such compounds have not been tested for their interaction with the transmembrane, tumor-associated isozyme CA IX. Thus, a series of such compounds has been obtained by attaching 2,3,5,6-tetrafluorobenzoyl- and 2,3,5,6-tetrafluorophenylsulfonyl- moieties to aromatic/heterocyclic sulfonamides possessing derivatizable amino moieties. Some of these compounds showed excellent CA IX inhibitory properties and also selectivity ratios favorable to CA IX over CA II, the other physiologically relevant isozyme with high affinity for sulfonamide inhibitors. The first subnanomolar and rather selective CA IX inhibitor has been discovered, as the 2,3,5,6-tetrafluorobenzoyl derivative of metanilamide showed an inhibition constant of 0.8 nM against hCA IX, and a selectivity ratio of 26.25 against CA IX over CA II. Several other low nanomolar CA IX inhibitors were detected among the new derivatives reported here. The reported derivatives constitute valuable candidates for the development of novel antitumor therapies based on the selective inhibition of tumor-associated CA isozymes.

1. Introduction

Inhibitors of the metallo-enzyme carbonic anhydrase (CA, EC 4.2.1.1) are clinically used mostly as antiglaucoma agents, as they are highly effective in reducing elevated intraocular pressure, after systemic administration of drugs such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** or dichlorophenamide **DCP**, or after topically administered sulfonamides, such as dorzolamide **DZA**, or brinzolamide **BRZ**.[1–4] CA inhibitors (CAIs) are also useful for the treatment or prevention of other diseases, since different CA isozymes are widely distributed in higher vertebrates.[1–4] In these organisms, including humans, the physiological functions of CAs have widely been investigated over the last years. Thus, isozymes CA I, II, and IV are involved in respiration and regulation of the acid/base homeostasy. These complex processes involve both the transport of CO_2/bicarbonate between metabolizing tissues and excretion sites (lungs, kidneys), facilitated CO_2 elimination in capillaries, and pulmonary microvasculature, elimination of H^+ ions in the renal tubules, and collecting ducts, as well as reabsorption of bicarbonate in the brush border, and thick ascending Henle loop in kidneys.[1–4] CA II is also involved in the bone development and function, such as the differentiation of osteoclasts, or the provision of acid for bone resorption in osteoclasts.[1–4] Different CAs are involved in the secretion of electrolytes in many other tissues/organs, such as: cerebrospinal fluid formation, by providing bicarbonate and regulating the pH in the choroid plexus; gastric acid production in the stomach parietal cells; bile production, pancreatic juice secretion, intestinal ion transport, etc.[1–4] Some isozymes, such as CA V are involved in molecular signalling processes, such as insulin secretion signalling in pancreas β-cells, whereas CA II and V are involved in important metabolic processes, as they provide bicarbonate for gluconeogenesis, fatty acids de novo biosynthesis or pyrimidine base synthesis.[5] Finally, some isozymes (such as CA IX, CA XII, CARP VIII) are highly abundant in tumors, being involved in oncogenesis and tumor progression, and

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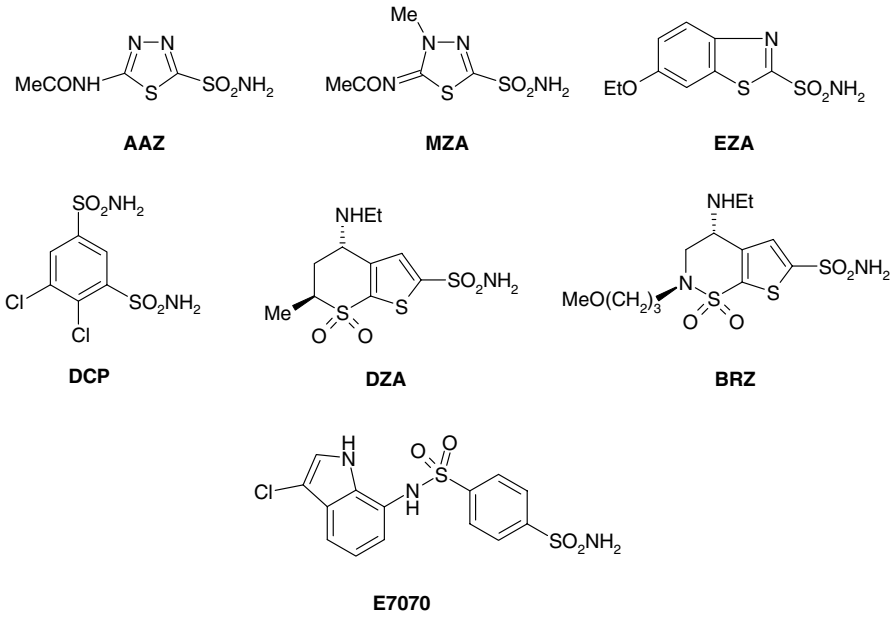


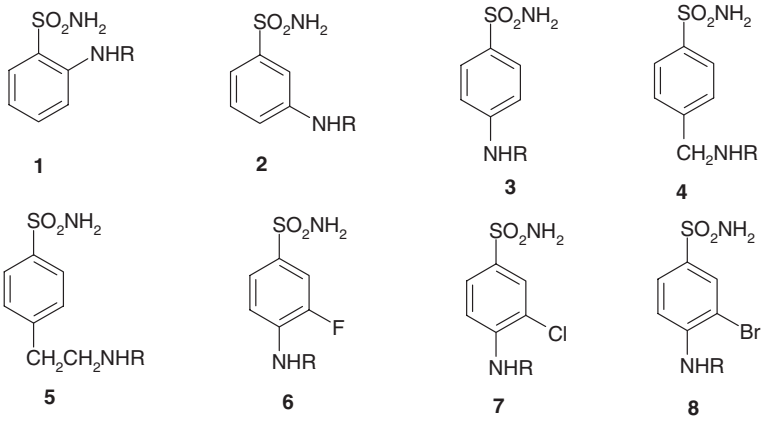
generally lacking from the corresponding non-cancerous cells.[2–7] Thus, it is not at all surprising that many CAIs were recently shown to possess potent antitumor properties in vitro and in vivo[8–10] with one sulfonamide of this type, E7070 in Phase II clinical trials as antitumor agent.[11–13]

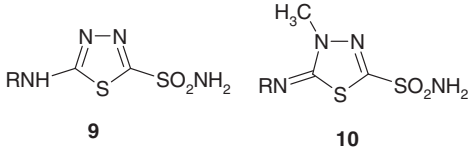
We have recently reported the first inhibition studies of the transmembrane, tumor-associated isozyme IX, with both sulfonamide and sulfamates, and detected several low nanomolar inhibitors, potentially useful for the development of novel antitumor therapies.[14–18] Here we continue these investigations, reporting the synthesis and CA IX inhibitory properties of a series of fluorinated sulfonamides. We decided to investigate this type of compounds due to the fact that in a previous contribution from our laboratory,[19] it was shown that by attaching perfluoroalkyl/aryl-carboxamido/sulfonamido tails, such as for example perfluorobutylsulfonyl, perfluorooctylcarboxamido, perfluorophenylcarboxamido or perfluorophenylsulfonyl, to the aromatic/heterocyclic sulfonamides also incorporating derivatizable amino moieties, of types **1**–**13**, very effective CAIs can be obtained, which also showed good water solubility and efficacy as topically acting antiglaucoma agents in an animal model of the disease. The best CAIs in that series of derivatives were those incorporating the perfluorinated aromatic moieties (such as C_6F_5CO and $C_6F_5SO_2$), a fact that was then explained after the report of the high resolution X-ray crystal structure of isozyme CA II with one of these agents, the perfluorobenzoylated analog of methazolamide.[20] This compound is almost 10 times a more effective CA II inhibitor (K_I of 1.5 nM) as compared to methazolamide (K_I of 14 nM). Its binding to the enzyme active site was shown to be similar to that of other sulfonamide inhibitors, considering the interactions of the sulfonamide zinc anchoring group and thiadiazoline ring contacts,[21] but differs considerably when the perfluorobenzoylimino fragment of the molecule has been analyzed. Thus, several unprecedented strong hydrogen bonds involving the imino nitrogen, carbonyl oxygen, a fluorine atom in meta belonging to the inhibitor, and two water molecules, as well as Gln 92 of the enzyme active site were evidenced. A stacking interaction of the perfluorophenyl ring of the inhibitor and the aromatic ring of Phe 131 was also observed for the first time in a CA—sulfonamide adduct.[20] All these findings proved that the polyfluorophenyl tails of such CAIs are indeed beneficial for obtaining very potent and bioavailable CAIs.

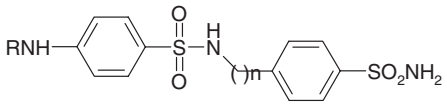
2. Chemistry

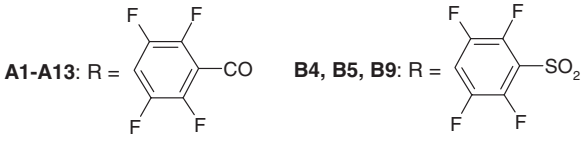
A problem related to the pentafluorophenyl-containing CAIs previously reported by this group,[19] was the high reactivity of the fluorine atom in *para* to the sulfonamido/carboxamido moiety, which was shown to covalently bind to thiol reagents, such as Cys 239 of β-tubulin, glutathione, cysteine itself, etc., by means of nucleophilic aromatic substitution reactions, in which the most reactive fluorine atom of the sulfonamide reacted with the thiol group of the Cys residue, leading to modification of the thiol reagent/protein.[22–25] In order to prepare fluorine-containing CAIs devoid of enhanced reactivity, we report here derivatives lacking the *para*-fluorine reactive group, with two types of such derivatives being prepared: the 2,3,5,6-tetrafluorophenyl-carboxamides **A1**–**A13**, and the 2,3,5,6-tetrafluorophenyl-sulfonamides **B3**, **B5**, and **B9**, respectively (Scheme 1).

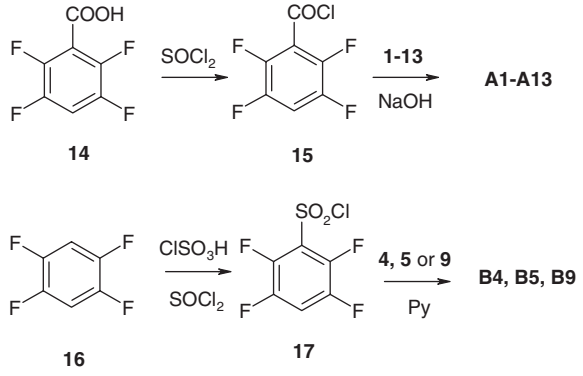
The carboxamides **A1**–**A13** were easily prepared from the commercially available 2,3,5,6-tetrafluorobenzoic acid **14**, which has been converted to the corresponding acyl chloride **15** by treatment with thionyl chloride,











Scheme 1.

followed by reaction with the amino-sulfonamides **1**–**13**, as previously reported.[19,26] For the preparation of the sulfonamides **B3**, **B5**, and **B9**, 1,2,4,5-tetrafluorobenzene **16** was transformed to the monosulfonyl chloride by treatment with chlorosulfonic acid/thionyl chloride, followed by reaction with the amino-sulfonamides **4**, **5** or **9**, as previously reported for structurally-related compounds.[19,27]

3. CA inhibition

CA inhibition data against three human isozymes, hCA I, hCA II, and hCA IX for the prepared compounds and standard inhibitors are shown in Table 1. All the new compounds reported here act as better CAIs than the parent sulfonamides from which they were obtained (data not shown).[15] The following SAR can be drawn from the data of Table 1: (i) with few exceptions (compounds **A6**, **A9**, **A10**, and **B9**), the CAIs investigated here are more potent CA IX inhibitors than CA II (or CA I) inhibitors, a fact rarely observed up to now for other investigated sulfonamides/sulfamates.[14–18] Indeed, the selectivity ratio toward CA IX as compared to CA II is less than 1 for these four compounds, as well as for the clinically used derivatives, meaning that all of them possess a higher affinity for CA II, and not for CA IX, the target enzyme for developing novel antitumor therapies. On the contrary, the remaining 12 derivatives reported here showed selectivity ratios higher than unity, with several such derivatives (**A2**, **A7**, **A8**, and **A11**) possessing selectivity ratios in the range of 4.75–26.25, whereas the remaining ones present this parameter around 1.05–2.72. A first conclusion may be that the heterocyclic derivatives (the clinically used sulfonamides

Table 1. CA inhibition data with standard inhibitors and the new sulfonamides reported in the present study, against human isozymes I, II, and IX

Inhibitor	K_I[c] (nM)			Selectivity ratio[b]
	hCA I[a]	hCA II[a]	hCA IX[a]	K_I (hCA II)/K_I (hCA IX)
Acetazolamide	900	12	25	0.48
Methazolamide	780	14	27	0.51
Ethoxzolamide	25	8	34	0.23
Dichlorophenamide	1200	38	50	0.76
Dorzolamide	50,000	9	52	0.17
Brinzolamide	—	3	37	0.08
E7070	31	15	24	0.62
A1	1500	38	9.5	4.0
A2	1700	21	0.8	26.25
A3	975	16	15.2	1.05
A4	980	13	9.5	1.36
A5	900	12	7.3	1.64
A6	760	1.5	24	0.06
A7	1080	76	16	4.75
A8	1870	84	4.8	17.5
A9	250	2.1	12.6	0.16
A10	270	1.4	10.5	0.13
A11	6300	19	3.2	5.93
A12	6100	15	5.5	2.72
A13	6000	13	7.6	1.71
B4	860	12	8.5	1.41
B5	750	9	7.0	1.28
B9	13	0.7	4.3	0.16

[a] Human (cloned) isozymes,[28] by the CO_2 hydration method for CA IX,[29] and esterase method for CA I and II.[30]
[b] Selectivity ratio toward hCA IX as compared to hCA II.
[c] Errors in the range of 5–10% of the reported value (from three different assays).

and E7070) as well as **A9**, **A10**, and **B9**, and the fluorine-sulfanilamide derivative **A6**, show higher affinity for the cytosolic isozyme CA II, whereas the remaining aromatic derivatives investigated here show higher affinity for the transmembrane, tumor-associated isozyme CA IX; (ii) the affinity of the new sulfonamides reported here for hCA I was medium-low, except for one derivative, **B9**, which behaved as a very potent CA I inhibitor (K_I of 13 nM). Indeed, the other fluorinated sulfonamides showed K_I values in the range of 250–6100 nM, being as inhibitory (or slightly less) as the clinically used compounds (except dorzolamide, which is a very weak CA I inhibitor); (iii) against hCA II, the new compounds reported here generally behave as very potent inhibitors, with K_I values in the range of 0.7–16 nM, except for compounds **A1**, **A2**, **A7**, **A8**, and **A9**, which were slightly less effective inhibitors, with K_I values in the range of 19–84 nM; (iv) against hCA IX, some of these compounds are the most effective inhibitors ever reported up to now,[14–18] with the first subnanomolar inhibitor detected (compound **A2**, with a K_I of 0.8 nM, and an excellent selectivity ratio toward CA IX as compared to CA II, of 26.25). Other derivatives, such as **A1**, **A4**, **A5**, **A8**, **A11**–**A13**, **B4**, **B5**, and **B9**, showed K_I values in the range of 3.2–9.5 nM, being very potent CA IX inhibitors. The less effective CA IX inhibitors were **A3**, **A6**, **A7**, **A9**, and **A10**, which possess K_I values in the range of 10.5–24 nM, that is, they appreciably inhibit the tumor-associated isozyme, being more potent than the clinically used sulfonamides, and equipotent to the antitumor sulfonamide in clinical trials, E7070 (Table 1). It may be observed that the best and more selective CA IX inhibitors incorporate metanilamide, sulfanilyl-sulfanilamide, and sulfanilyl-homosulfanilamide moieties (compounds **A2**, **A11**, and **A12**) and tetrafluorobenzoyl tails; (v) the compounds incorporating tetrafluorosulfonyl moieties were slightly more effective CAIs as compared to the corresponding derivatives incorporating tetrafluorobenzoyl moieties, but the differences of activity were insignificant.

4. Conclusions

In a series of 2,3,5,6-tetrafluorophenyl-carboxamido/sulfonamido aromatic/heterocyclic sulfonamides, very potent inhibitors against the transmembrane, tumor-associated isozyme CA IX were detected, some of them showing also selectivity ratios favorable to CA IX over CA II, the other physiologically relevant isozyme with high affinity for sulfonamide inhibitors. The first subnanomolar and rather selective CA IX inhibitor has also been discovered, as the 2,3,5,6-tetrafluorobenzoyl derivative of metanilamide showed an inhibition constant of 0.8 nM against hCA IX, and a selectivity ratio of 26.25 against CA IX over CA II. Several other low nanomolar CA IX inhibitors were detected among the new derivatives reported here. Correlated with the easy preparation of such compounds, the present derivatives constitute valuable candidates for the development of novel antitumor therapies based on the selective inhibition of tumor-associated CA isozymes.

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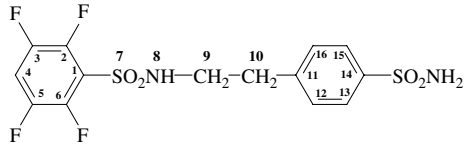
27. Synthesis of 2,3,5,6-tetrafluorobenzenesulfonyl chloride: Chlorosulfonic acid of 5 equiv (97 g; 55.36 mL) were added to 25 g of 1,2,4,5-tetrafluorobenzene and the obtained solution was heated at 150 °C. After 2 h, the heating was halted in order to allow the solution to reach room temperature and 2 equiv of thionyl chloride (39.96 g; 24.30 mL) were added. The obtained mixture was heated for 3 h at 150 °C. The brown solution obtained was then added dropwise under stirring to a mixture of 250 g ice and 100 mL water. The obtained suspension was extracted three times with 50 mL of ethyl acetate. The organic fractions were collected and dried by solvent evaporation under depression. The obtained brown oil was collected and stored at −20 °C. The purity of the final compound was verified by TLC ($MeOH/CHCl_3$: 3/7).
Acylation/sulfonylation of amino-sulfonamides: Sulfonamide **1**–**13** of 5 mmol were dissolved in 15 mL solution of 2.5 M NaOH and cooled to 2–5 °C in a salt–ice bath. Sulfonyl/acyl chloride **15**, **17** of 5 mmol were added in small portions, concomitantly with 10 mL of a 2 M NaOH solution, maintaining the temperature under 10 °C. The reaction mixture was then stirred at room temperature for 5–10 h (TLC control), then the pH was adjusted to 2 with 5 N HCl, and the precipitated sulfonamides were filtered, and recrystallized from aqueous ethanol. Alternative method: Amino sulfonamide **1**–**15** of 1 g was dissolved in

10 mL of *N*,*N*-dimethylacetamide and 2 equiv of 2,3,5,6-tetrafluorobenzenesulfonyl chloride, and 1 equiv of pyridine were added to the solution which was left on ice under stirring. After 60 min of stirring, 50 mL of water were added to the solution, which was then extracted three times with 25 mL of ethyl acetate. The organic fractions were collected and extracted three times with 15 mL of 1 N hydrochloric acid aqueous solution. The organic phase was dried on magnesium sulfate. After charcoal treatment, the organic phase was dried under depression. The purity of the final compound was verified by TLC (MeOH/$CHCl_3$: 3/7).

4-[2,3,5,6-Tetrafluorobenzenesulfonylamidoethyl]-benzenesulfonamide **B5**, colorless crystals, mp 187–188 °C, IR (KBr) cm^{-1}: 1154 and 1163 (SO_2^{sym}), 1350 and 1378 (SO_2^{as}), 3365 (NH, NH_2);



^{1}H NMR (DMSO-d_6, δ ppm, J Hz): 8.79 (t, H-8, 5.5); 8.13 (tt, H-4, 7.5, 10.3); 7.69 (d, H-13-15, 8.4); 7.38 (d, H-12-16, 8.4); 7.30 (s, NH_2); 3.29 (br q, H-9, 5.5, 6.9); 2.83 (t, H-10, 6.9). H-4 is coupled with the 2F from positions 3 and 5, $J = 10.3$ Hz, as well as with the two F from positions 2 and 6, $J = 7.5$ Hz; N–H is coupled with CH_2-9 ($J = 5.5$ Hz), whereas CH_2-9 appears as a broad quartet because it is also coupled with CH_2-10, which appears as a triplet ($J(vic) = 6.9$ Hz); ^{13}C NMR (DMSO-d_6, δ ppm, J Hz): 120.87 (t, C-1, $J(^{19}F–^{13}C) = 15.3$); 110.74 (t, C-4, $J(^{19}F–^{13}C) = 23.5$); 145.63 (dddd, C-3, 4.4, 10.8, 15.2, 248.4); 142.85 (ddt, C-2, 3.3, 15.7, 254.5); 129.12 (C-12-16); 125.49 (C-13-15); 142.59 (C-11 or C-14); 142.21 (C-14 or C-11); 43.36 (C-9); 34.69 (C-10). C-1 = 128.5 + 15.5 − 2×13.0 + 2×1.6 = 121.2; C-2 = 128.5 + 34.8 − 13.0 + 1.6 − 4.4 = 147.5; C-3 = 128.5 + 34.8 − 13.0 + 0.3 + 1.6 − 4.4 = 152.2; C-4 = 128.5 − 2×13.0 + 2×1.6 + 3.1 = 108.8 ppm (the detailed spectrum is presented as this type of compounds show a very complicated behavior due to the multiple couplings between the protons, fluorine and carbon atoms, and no literature data for similar compounds are available as far as we know). Anal. ($C_{14}H_{12}F_4N_2O_4S_2$) C, H, N.

28. The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[6a]) was amplified by using PCR and specific primers for the vector pCAL-n-FLAG (from Stratagene). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *Escherichia coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lyzed and homogenated in a buffered solution (pH 8) of 4 M urea and 2% Triton X-100, as described by Wingo et al.[31] The homogenate thus obtained was extensively centrifuged (11,000 g) in order to remove soluble and membrane associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenation and centrifugation in water, in order to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM $ZnCl_2$, 2 mM EDTA, 2 mM reduced glutathione, 1 mM oxidized glutathione. Active hCA IX was extensively dialyzed into a solution of 10 mM Hepes (pH 7.5), 10 mM tris HCl, 100 mM Na_2SO_4, and 1 mM $ZnCl_2$. The protein was further purified by sulfonamide affinity chromatography,[29] the amount of enzyme was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate.[29] The activity of this preparation was identical to that reported by Wingo et al., with k_{cat}/K_M of 55 $\mu M^{-1}s^{-1}$.

29. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561–2573, An SX.18MV-R Applied Photophysics stopped-flow instrument has been used. Phenol red (at a concn 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. Stock solutions of inhibitors were prepared at a concn of 1–3 mM (in DMSO/water 1:1, v/v) and dilutions up to 0.1 nM done with the assay buffer mentioned above. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results.
30. A stopped-flow variant of the Poker and Stone spectrophotometric method (Pocker, Y.; Stone, J.T. *Biochemistry* **1967**, *6*, 668–678) has been employed, using an SX.18MV-R Applied Photophysics stopped-flow instrument, as described previously.[21]
31. Wingo, T.; Tu, C.; Laipis, P. J.; Silverman, D. N. *Biochem. Biophys. Res. Commun.* **2001**, *288*, 666–669.