Original article

Structure–activity relationships of novel P2-receptor antagonists structurally related to Reactive Blue 2

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In memoriam of the late Professor Dr. Felix Zymalkowski

Abstract

P2 membrane receptors for nucleotides represent significant targets for experimental pharmacology and drug research. In earlier publications, we have shown that Reactive Blue 2 (RB 2), one of the most widely used P2-receptor antagonists, displays only moderate affinity and does not discriminate between native P2X- and P2Y-receptor subtypes. In the present study we have pharmacologically evaluated a series of 15 synthesized and re-evaluated four commercially obtained and chromatographically purified RB 2 type anthraquinone derivatives on contractions of the rat vas deferens (RVD) elicited by α,β-methylene ATP (α,β-meATP), mediated by $P2X_1$-receptors, and relaxations of the carbachol-precontracted guinea-pig taenia coli (GPTC) elicited by adenosine 5′-O-(2-thiodiphosphate) (ADPβS), mediated by $P2Y_1$-like receptors. Based on the structure–activity relationships (SAR) it is concluded that hydrophobic interactions of aromatic π-electron systems, hydrogen bonds with nitrogen as donor and acceptor atoms, and, particularly, position, conformational distance and number of anionic sulfonate groups are of great importance for the blockade of the two native P2-receptor subtypes. We have also identified novel, for the most part reversible antagonists that bind with higher affinity and improved subtype selectivity in comparison to RB 2. In particular, 1-amino-4-{4-[4-chloro-6-(2-sulfonatophenylamino)-[1,3,5]triazine-2-ylamino]-2-sulfonatophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid trisodium salt (MG 50-3-1) is the most potent antagonist at the $P2Y_1$-like-receptors of the GPTC reported so far (IC_{50} = 4.6 nM). It is significantly less potent as reversible antagonist at the $P2X_1$-receptors of the RVD (IC_{50} = 2.8 μM). Thus, MG 50-3-1 represents a selective pharmacological tool and may be a lead compound for future investigations.
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1. Introduction

Plasma membrane nucleotide P2-receptors subdivided in ligand gated ion channels (P2X) and G-protein coupled receptors (P2Y) are interesting targets not only in experimental pharmacology but also in drug research [1–5]. To date, seven mammalian P2X-receptors termed $P2X_1$ to $P2X_7$ and eight P2Y-receptors termed $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ have been identified by molecular cloning [6–10]. However, experimental and therapeutic progress in the P2-receptor field is dependent on the availability of potent and selective antagonists. For instance, novel suramin analogues have been reported as potent $P2X_1$-selective antagonists [11–14], and 3′,5′-bisphosphate nucleotides have been developed as selective high affinity antagonists of the $P2Y_1$-receptor [15–18]. Nevertheless, the identification of antagonists within other chemical classes remains highly desirable.

The anthraquinone dye Reactive Blue 2 (RB 2) (1-amino-4-{4-[4-chloro-6-(3-/4-sulfonatophenylamino)- [1,3,5]triazin-2-ylamino]-3-sulfonato-phenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid) (1) (Fig. 1) [20,21] is still one of the most widely used "classical" P2-receptor antagonists. A former study of a series of commercially obtained and chromatographically purified compounds [19], all structurally related to RB 2, identified P2-receptor ligands with higher affinity and improved subtype selectivity. Therefore, we have further investigated this chemical class of P2-receptor antagonists.

Unfortunately there has been some confusion concerning the identity and purity of commercially available RB 2 (1) [22–29]. Furthermore, it has been claimed to be P2Y-selective [30–32]. According to the Color Index (C.I.) of the Society of Dyers and Colorists, RB 2 (1) is defined as a mixture of two constitutional isomers with the sulfonate group at ring F either in *meta* (1a) or *para* (1b) position [33]. In fact, we have recently shown that the commercially available product is a 1:2 ring F *meta*/*para* sulfonate mixture and—more important—does not discriminate between native $P2X_1$-receptors of the rat vas deferens (RVD) and $P2Y_1$-like receptors of the guinea-pig taenia coli (GPTC) [34]. In contrast, the synthesized pure ring F *para* sulfonate isomer (1b) turned out to be a moderately selective antagonist at the $P2Y_1$-like versus the $P2X_1$-receptor.

In the present structure–activity relationships (SAR) study within this class of antagonists, the lead compound RB 2 (1) (Fig. 1) was structurally simplified to ascertain the minimal requirements of the anthraquinone core (rings ABC) and the (hetero)aromatic side chain (rings DEF) for P2-receptor subtype blockade. The concept of structural variations to identify molecules binding with higher affinity and improved subtype selectivity is shown in the general formula **A** (Fig. 1). Structures of all novel 13 synthesized anthraquinone derivatives **2**–**4** (ABC), **6**–**9**, **11**(ABCD), **12**, **15**, **16** (ABCDE), **18**, and **19** (ABCDEF) are listed together with two re-synthesized compounds **10** (ABCD), and **13** (ABCDE) [34] in ascending order of complexity (Schemes 1–5). These compounds were tested pharmacologically together with a comparative re-evaluation [19,34,35] of the four commercially obtained dyes Acid Blue 25 (AB 25) (**5**) (ABCD), Reactive Blue 4 (RB 4) (**14**) (ABCDE), Cibacron Blue 3GA (CB 3GA) (**17**), and Reactive Blue 5 (RB 5) (**20**) (ABCDEF) (Schemes 1,3 and 5).

As in our previous studies, both the synthesized and the commercially obtained compounds were purified by reversed phase flash column chromatography (RP-FCC) before use, since the dye content of the purchased dye stuffs was only about 35–65% and organic impurities and inorganic salts may have caused non-reliable pharmacological results in the literature. The chemical structures and purities were confirmed by ^{1}H- and ^{13}C-NMR, ESI-MS and RP-TLC.

Pharmacological effects of the pure compounds were studied on contractions of the RVD elicited by α,β-meATP, mediated by the $P2X_1$-receptor, which has been cloned [37–44], and relaxations of the carbachol-precontracted GPTC elicited by ADPβS, mediated by a P2Y-receptor which has not so far been cloned but displays close similarities with the cloned $P2Y_1$-receptor [19,45–50].

2. Chemistry

2.1. Chemical synthesis

2.1.1. Scheme 1

1-Amino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid monosodium salt (**2**), the functionalized ABC anthraquinone core of the RB 2 isomer CB 3GA (1-amino-4-
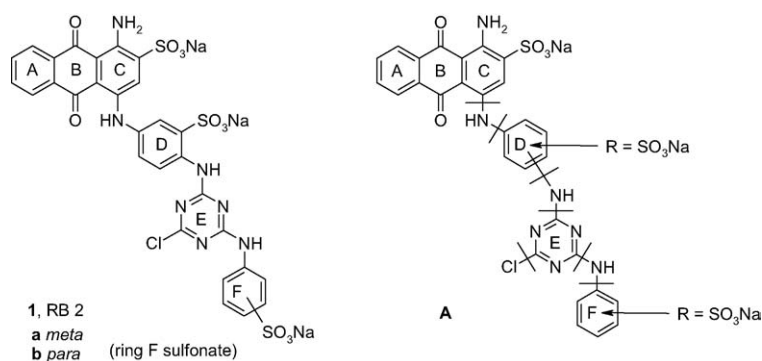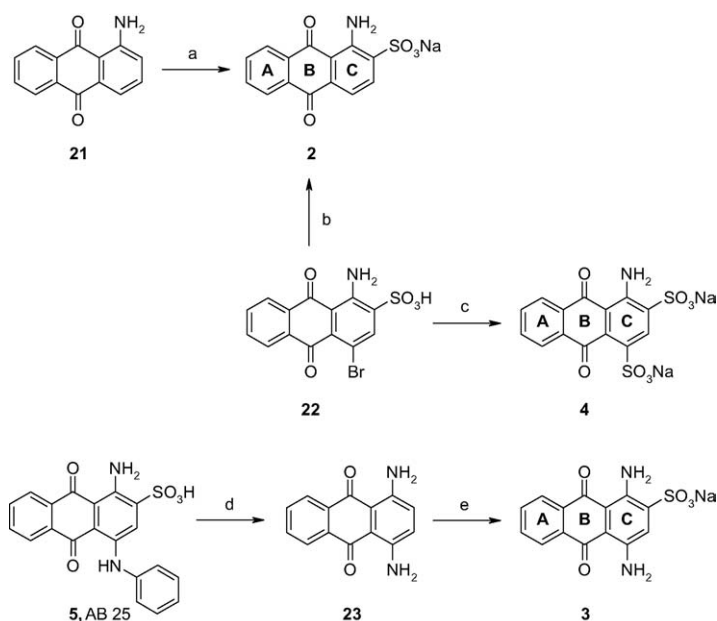

Fig. 1. Chemical structure of the lead compound RB 2 (**1**) as ring F *meta* (**1a**)/*para* (**1b**) constitutional mixture and general formula **A** with synthetic variations.

Scheme 1. Synthesis of the anthraquinone ABC-core derivatives **2–4**[a].
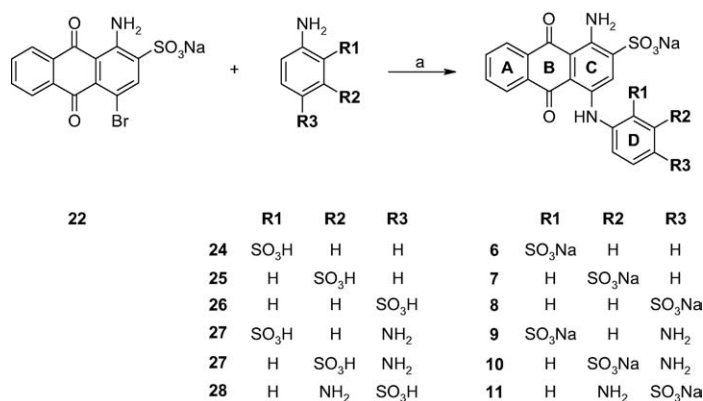and structure formula of AB 25 (**5**).

[a] Reaction conditions: (a) ClSO$_3$H, nitrobenzene, 130 °C, 3 h, Na$_2$CO$_3$; (b) Fe, acetic acid, reflux, 6 h, Na$_2$CO$_3$; (c) Na$_2$S$_2$O$_4$, DMF/H$_2$O 1:1, 90 °C, 8 h, N$_2$; (d) SnCl$_2$, HCl (2 M), acetic acid, reflux, 3 h; (e) ClSO$_3$H, nitrobenzene, 130 °C, 3 h, Na$_2$CO$_3$.

{4-[4-chloro-6-(2-sulfonatophenylamino)-[1,3,5]triazin-2-ylamino]-3-sulfonatophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid trisodium salt) (**17**), was obtained either by sulfonation of 1-aminoanthraquinone (**21**) or by debromination of bromaminic acid (1-amino-4-bromo-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid) (**22**). In the first case, treatment with ClSO$_3$H in nitrobenzene at 130 °C afforded compound **2** in 96% yield, whereas the reaction of **22** with iron powder in acetic acid under reflux led to compound **2** in a yield of only 71%. The reaction of **22** with sodium dithionite in DMF and water at 90 °C under nitrogen atmosphere resulted in 1-amino-9,10-dioxo-9,10-dihydroanthracene-2,4-disulfonic acid disodium salt (**4**) in 69% yield. 1,4-Diamino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid monosodium salt (**3**) was synthesized from AB 25 (1-amino-4-phenylamino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid monosodium salt) (**5**). In the first

step, treatment of **5** with SnCl$_2$ in HCl and acetic acid evoked a reductive cleavage of the sulfonic acid group under reflux conditions. In addition, a partial hydrogenation of the ring D aromatic system led via enamine formation and subsequent hydrolysis to 1,4-diamino-9,10-dioxo-9,10-dihydroanthracene (**23**) and cyclohexanone. Compound **23** was then sulfonated with ClSO$_3$H in nitrobenzene at 130 °C to give compound **3** in 51% yield.

2.1.2. Scheme 2

The synthetic approach for ring D substitution pattern modifications utilized bromaminic acid (**22**) as the starting compound. According to a general procedure previously described [22,34,51], compounds **6–11** were obtained by an Ullmann coupling reaction of **22** with (di)aminosubstituted benzenesulfonic acids **24–28** (cf. Section 6) as the respective building blocks. In general, **22** was reacted with compounds

		R1	R2	R3		R1	R2	R3
	24	SO$_3$H	H	H	6	SO$_3$Na	H	H
	25	H	SO$_3$H	H	7	H	SO$_3$Na	H
	26	H	H	SO$_3$H	8	H	H	SO$_3$Na
	27	SO$_3$H	H	NH$_2$	9	SO$_3$Na	H	NH$_2$
	27	H	SO$_3$H	NH$_2$	10	H	SO$_3$Na	NH$_2$
	28	H	NH$_2$	SO$_3$H	11	H	NH$_2$	SO$_3$Na

Scheme 2. Synthesis of the ABCD anthraquinone derivatives **6–11**[a].

[a] Reaction conditions: (a) Na$_2$CO$_3$ (except for **9**), Na$_2$SO$_3$, CuCl, H$_2$O, r.t. (60 °C for **6** and **9**), 8 h.

|	R1	R2		R3	X		R1	R2	R3	X	
9	SO₃Na	H	29	Cl	N	12	SO₃Na	H	Cl	N	MG 40-3
10	H	SO₃Na	29	Cl	N	13	H	SO₃Na	Cl	N	MG 40-1
10	H	SO₃Na	30	NH₂	N	15	H	SO₃Na	NH₂	N	MG 42-1
10	H	SO₃Na	31	H	C	16	H	SO₃Na	H	C	

14, RB 4
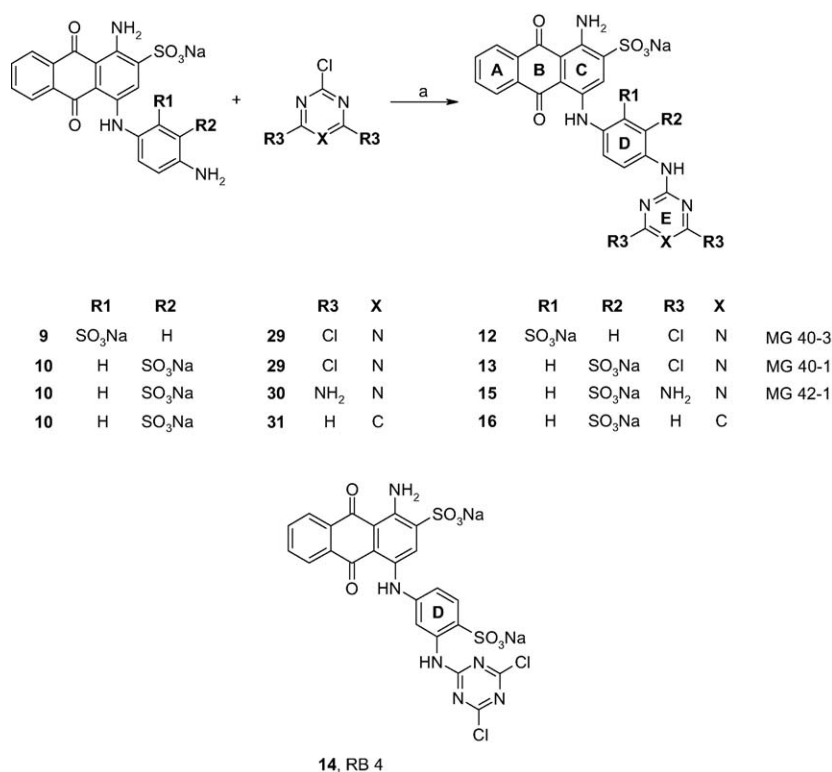
Scheme 3. Synthesis of the ABCDE anthraquinone derivatives **12**, **13**, **15** and **16**[a] and structure formula of RB 4 (**14**).
a Reaction conditions: (a) Na₂CO₃, acetone/H₂O, 0–5 °C, 1 h (plus 2 h: 60 °C for **15** and 90 °C for **16**).

24–**28** and copper(I) chloride as a catalyst in an aqueous solution containing sodium carbonate and sodium sulfite. After stirring at r.t. for 8 h, 1-amino-4-(3-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (**7**), 1-amino-4-(4-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (**8**), 1-amino-4-(4-amino-3-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (**10**), and 1-amino-4-(3-amino-4-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (**11**), respectively, were obtained each in about 50% yield. The synthesis of 1-amino-4-(2-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (**6**) required an increased reaction temperature of 60 °C, which led to a lower yield of 38%, due to an enlarged formation of by-products. Reaction of **22** with 2,5-diaminobenzenesulfonic acid (**27**) afforded 1-amino-4-(4-amino-2-

sulfonatophenyl-amino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (**9**) only in unacceptable low yields, because the steric influence of the sulfonic acid group rendered the 5-amino group more sensitive to the coupling reaction, leading favorably to the constitutional isomer **10** [34]. The best yield of **9** was obtained by dropwise addition of an aqueous solution of **27** to a sodium carbonate free solution of **22**, sodium sulfite and copper(I) chloride in water at 60 °C. Stirring at this higher temperature for 8 h led to a 10% yield of compound **9**.

2.1.3. Scheme 3

As shown in Scheme 3, reaction of the ABCD compounds **9** or **10** [34] with cyanuric chloride (2,4,6-trichloro- [1,3,5]-triazine) (**29**) as ring E equivalent in aqueous acetone (2:1) at 0–5 °C and pH 5–7 for 1 h afforded 1-amino-4-[4-(4,6-dichloro-[1,3,5]triazine-2-ylamino)-2-sulfonatophenyl-
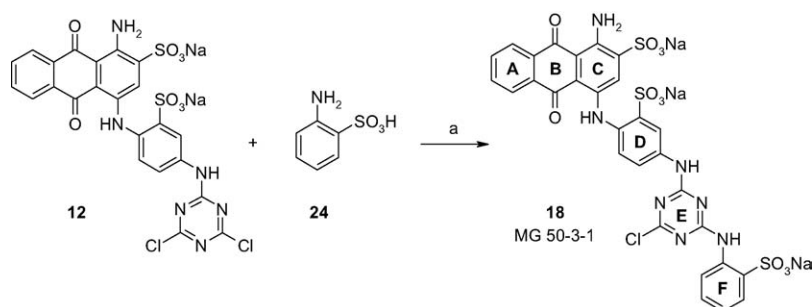
Scheme 4. Synthesis of **18**, the structural ring D *ortho* isomer of CB 3GA (**17**) (see Scheme 5)[a].
a Reaction conditions: (a) Na₂CO₃, acetone/H₂O, 40–60 °C, 4 h.
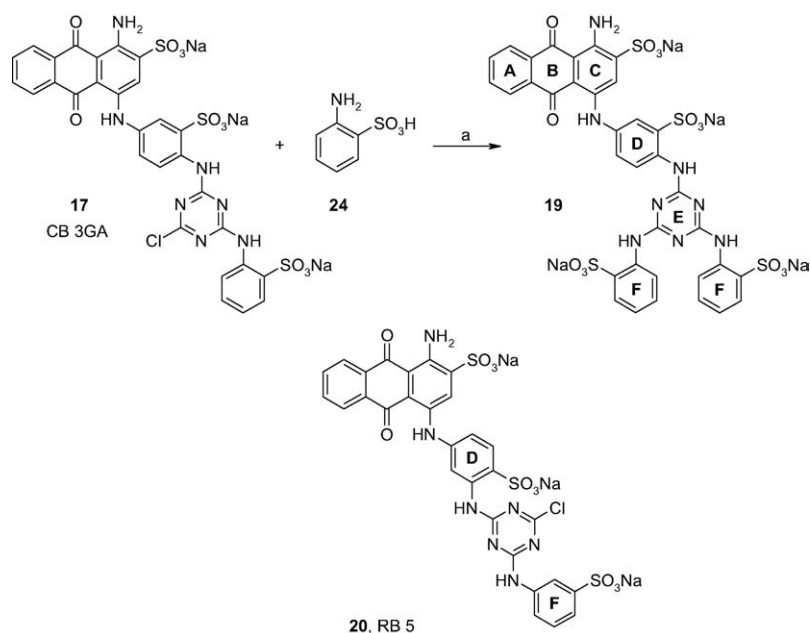
Scheme 5. Synthesis of the ABCDE double ring F anthraquinone derivative **19**[a] and structure formula of RB 5 (**20**).
a Reaction conditions: (a) Na_2CO_3, H_2O, 90 °C, 5 h.

amino]-9,10-dioxo-9,10-dihydro-anthracene-2-sulfonic acid disodium salt (**12**) or 1-amino-4-[4-(4,6-dichloro-[1,3,5] triazine-2-ylamino)-3-sulfonatophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (**13**) [34] in about 70% yield. Alternative treatment of **10** with 2-chloro-4,6-diamino- [1,3,5]-triazine (**30**) or 2-chloro-pyrimidine (**31**) led to 63% of the ring E diaminotriazinyl analogue 1-amino-4-[4-(4,6-diamino-[1,3,5]triazine-2-yl-amino)-3-sulfonatophenylamino]-9,10-dioxo-9,10-dihydro-anthracene-2-sulfonic acid disodium salt (**15**) or 47% of the ring E diazinyl analogue 1-amino-4-[4-[1,3]diazine-2-ylamino)-3-sulfonatophenylamino]-9,10-dioxo-9,10-dihy-droanthracene-2-sulfonic acid disodium salt (**16**), using the same reaction conditions described for the ring E dichloro-triazinyl compounds **12** and **13**, except an additional heating for 2 h at 60 and 90 °C, respectively.

2.1.4. Scheme 4

The ABCDE compound **12** was reacted with the ring F equivalent 2-aminobenzenesulfonic acid (**24**) in aqueous acetone (3:1) at 40–60 °C and pH 5–7 for 4 h according to a previously reported procedure [22,34] to give the CB 3GA analogue 1-amino-4-{4-[4-chloro-6-(2-sulfonatophenyl-amino)-[1,3,5]triazine-2-ylamino]-2-sulfonatophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid triso-dium salt (**18**) in 81% yield.

2.1.5. Scheme 5

The ABCDE double F derivative 1-amino-4-{4-[4,6-bis(2-sulfonatophenylamino)-[1,3,5]triazine-2-ylamino]-3-sulfo-natophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid tetrasodium salt (**19**) was obtained in 88% yield by stirring of CB 3GA (**17**) and a further ring F equivalent **24** at 90 °C for 5 h in an aqueous solution.

2.2. Structure analysis

Chemical structures of all synthesized as well as of the commercially obtained and purified compounds were eluci-dated by ^{1}H- and ^{13}C-NMR. The unambiguous signal identi-fication and the unequivocal differentiation of constitutional isomers required 1D- and 2D-homo- and heteronuclear spec-tra (see Section 6) as well as the application of respective chemical shift additivity rules. The spectra were recorded in DMSO-d_6 of the sodium sulfonate salts at the elevated tem-perature of 50 °C. The elementary composition could only be given evidence by means of electrospray ionization (ESI) mass spectra. Elemental combustion analysis could not be per-formed with sufficient accuracy because of the amorphous material with irreversible inclusion of traces of chromato-graphic stationary phases and solvents.

3. Pharmacology

As mentioned in Section 1, compounds were examined for antagonism at the native $P2X_1$-receptor of the RVD and at the native $P2Y_1$-like receptor of the GPTC. All results are summarized in Table 1. In Fig. 2, concentration response curves are exemplified for MG 50-3-1 (**18**), the most potent and a highly selective $P2Y_1$-like receptor antagonist in the present study.

3.1. Contraction of rat vas deferens

α,β-meATP (3.2 μM), when added every 60 min (see Sec-tion 6), elicited rapid, transient contraction of the RVD. Increasing concentrations of all compounds progressively inhibited the contraction, except **2** and **8**, which caused mini-

Table 1

Antagonist IC_{50} values at $P2X_1$-receptors (rat vas deferens) and $P2Y_1$-like receptors (GPTC); expressed as arithmetic mean ± S.E.M. from $n = 4$–12 experiments

Level	Compound		Ring D	IC_{50} (μM)	
			sulfonate position	$P2X_1$ (RVD)	$P2Y_1$-like (GPTC)
ABC	**2**		–	> 100[a]	> 100[b]
	3		–	49.2 ± 4.8	> 100[c]
	4		–	43.4 ± 4.3	100
ABCD	**5**[e]	AB 25	–	58.9 ± 2.6[f]	4.0 ± 0.8[f]
	6		ortho	16.4 ± 2.6	> 100[d]
	7		meta	74.2 ± 6.6	37.7 ± 3.1[g]
	8		para	> 100[b]	20.5 ± 2.3[h]
	9		ortho	11.9 ± 2.3	41.0 ± 6.8[i]
	10		meta	27.9 ± 5.7	n.d.[m]
	11		para	69.5 ± 8.9	12.9 ± 3.4[k]
ABCDE	**12**[n]	MG 40-3	ortho	2.4 ± 0.5[p]	< 1[q]
	13[n]	MG 40-1	meta	8.7 ± 0.8[p]	> 100[b]
	14[e]	RB 4	para	73.2 ± 5.2	72.9 ± 15.4
	15	MG 42-1	meta	16.2 ± 2.1	0.37 ± 0.02
	16		meta	34.1 ± 5.8	74.2 ± 14.6
ABCDEF	**17**[e]	CB 3GA	meta	9.1 ± 0.8[r]	17.4 ± 4.7[r]
	18	MG 50-3-1	ortho	2.8 ± 0.1	<< 1[s]
	19		meta	4.9 ± 0.4	2.9 ± 0.6
	20[e]	RB 5	para	66.5 ± 4.1	> 100[d]
	1a	RB 2 meta	meta	19.7 ± 1.1[r]	12.0 ± 4.3[r]
	1b	RB 2 para	meta	35.5 ± 5.4[r]	2.6 ± 0.4[r]

[a] 34%.

[b] None.

[c] 36%.

[d] 42% inhibition at 100 μM conc.

[e] Also studied in Tuluc et al. [19].

[f] Data taken from Glänzel et al. [35].

[g] 1 of 5.

[h] 1 of 6.

[i] 3 of 10.

[k] One of seven experiments were not considered for IC_{50} calculation since 50% inhibition was not reached.

[m] Not determinable (U-shaped concentration–response curve).

[n] Also studied in Glänzel et al. [36].

[p] Only partly reversible antagonist.

[q] 71% inhibition at 1 μM conc.

[r] Data taken from Glänzel et al. [34].

[s] 4.6 ± 1.2 nM.

mal no change at concentrations of up to 100 μM. The most potent antagonists were MG 40-3 (**12**), MG 50-3-1 (**18**), compound **19**, MG 40-1 (**13**), and CB 3GA (**17**) with calculated IC_{50} values ranging from 2.4 ± 0.5 to 9.1 ± 0.8 μM (Table 1).

The reversibility of the effects at the $P2X_1$-receptor of CB 3GA (**17**), MG 50-3-1 (**18**), RB4 (1-amino-4-[3-(4,6-dichloro-[1,3,5]triazin-2-ylamino)-4-sulfonatophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt) (**14**) (Scheme 3), MG 40-3 (**12**), MG 40-1 (**13**), MG 42-1 (**15**), and compound **16** was examined as follows (results for dichlorotriazinyl derivatives **12**–**14** are exemplarily shown in Fig. 3): α,β-meATP (3.2 μM) was administered every 30 min. Solvent or antagonist was added from immediately after the first till the fifth response to α,β-meATP, i.e. for 120 min, and then washed out. CB 3GA (**17**) (10 μM), MG 50-3-1 (**18**) (3.2 μM), RB4 (**14**) (100 μM), MG 40-3 (**12**) (3.2 μM), MG 40-1 (**13**) (10 μM), MG 42-1 (**15**) (32 μM) and compound **16** (32 μM), while present in the medium, reduced the contrac-

tion by 79 ± 3, 47 ± 8, 80 ± 8, 77 ± 7, 62 ± 7, 61 ± 8 and 55 ± 8%, respectively (fifth response to α,β-meATP). The antagonism by MG 40-3 (**12**) and MG 40-1 (**13**) was only partly reversible: 55 ± 9, 55 ± 9, 53 ± 8 and 51 ± 9% reduction by **12**; 33 ± 6, 33 ± 7, 30 ± 6 and 36 ± 5% reduction by **13** remained after washout for 120, 180, 240 and 300 min, respectively ($n = 4$ each; all corrected for the solvent controls). All other tested compounds were completely reversible antagonists.

3.2. Relaxation of guinea-pig taenia col

ADPβS (0.1 μM), when added every 60 min during the plateau of a contraction elicited by carbachol (50–90 nM; see Section 6), elicited rapid, transient relaxation of the GPTC. All compounds inhibited the relaxation, except compound **2** and MG 40-1 (**13**), which caused no change at concentra-
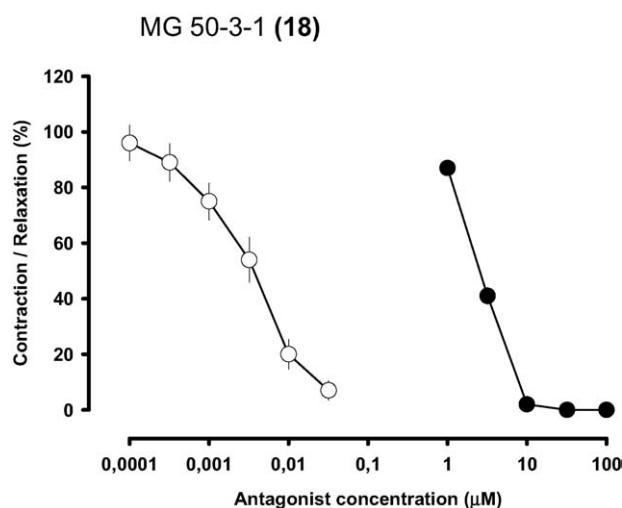
Fig. 2. Effects of MG 50-3-1 (**18**) on the contraction of the RVD elicited by 3.2 μM α,β-meATP (●) and on the relaxation of the carbachol-precontracted GPTC elicited by 0.1 μM ADPβS (○); arithmetic mean ± S.E.M. from $n = 5$ and $n = 8$ experiments, respectively.

tions of up to 100 μM. The most potent $P2Y_1$-like receptor antagonist was MG 50-3-1 (**18**) with an IC_{50} value of 4.6 ± 1.2 nM. Further potent antagonists were MG 40-3 (**12**) (71% inhibition at 1 μM, the lowest concentration applied in the present study) and MG 42-1 (**15**) (IC_{50} value of 0.37 ± 0.02 μM). Compound **19** and AB 25 (**5**) displayed calculated IC_{50} values of 2.9 ± 0.6 and 4.0 ± 0.8 μM, respectively (Table 1).

In contrast to the studies at the RVD, we did not investigate the reversibility of inhibitory effects in GPTC experiments, because effects of "classical" reversible antagonists like Suramin or RB 2 cannot be washed out after hours [52].

4. Discussion

All 19 compounds, 15 of which for the first time, investigated in the present study antagonized P2-receptor mediated responses, either in RVD or in GPTC or in both whole tissue preparations.

4.1. $P2X_1$-receptor

Contraction of the RVD elicited by the ecto-nucleotidase resistant agonist α,β-meATP is mediated by the cloned $P2X_1$-
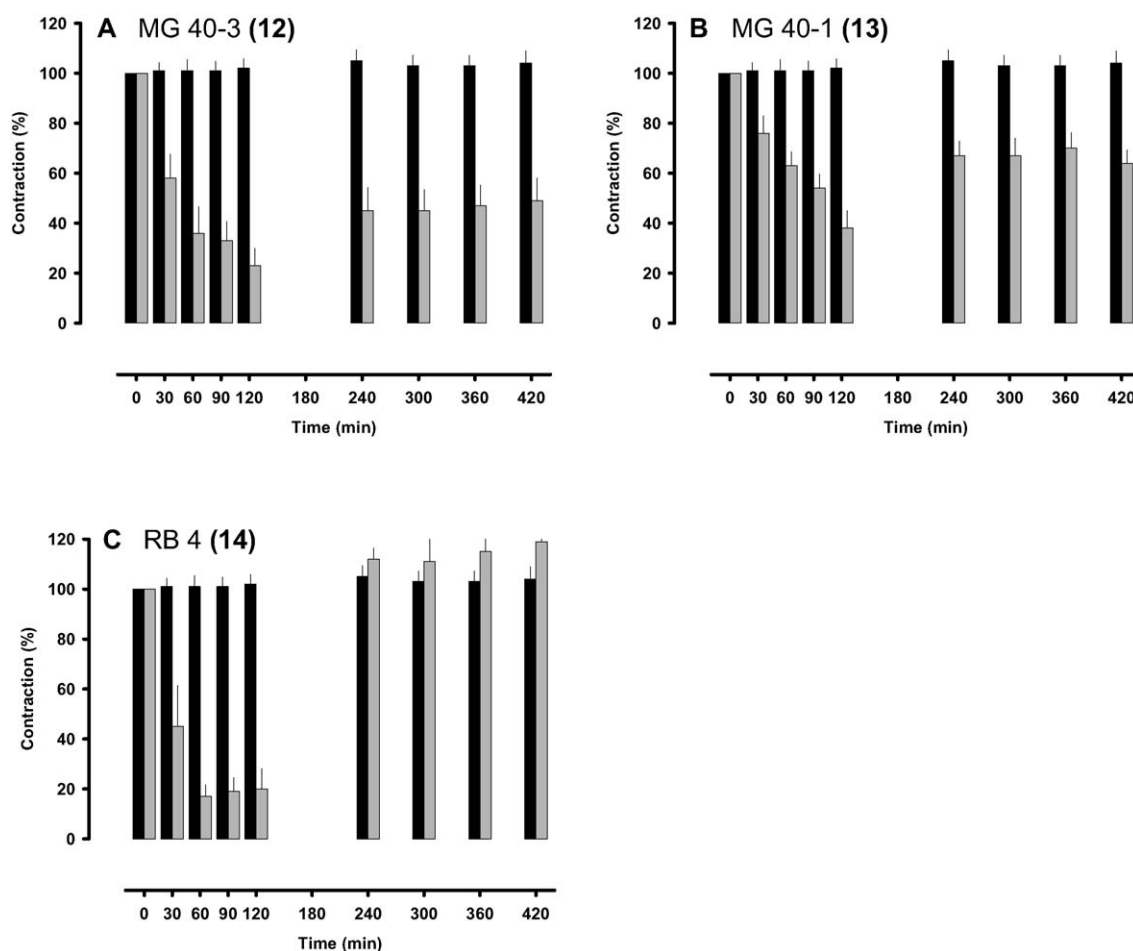
Fig. 3. Reversibility of the effects of antagonists on the contraction of the RVD elicited by 3.2 μM α,β-meATP: washout experiments for **a** MG 40-3 (**12**), **b** MG 40-1 (**13**), and **c** RB 4 (**14**); arithmetic mean ± S.E.M. from $n = 4$ experiments in each case. Immediately after the first response to α,β-meATP, the tissue was incubated with either antagonist (gray columns) or solvent (black columns) for the first 120 min, followed by a washout period of 300 min.

receptor [37–44]. Eigh of the 19 tested anthraquinone derivatives administered in concentrations of up to 100 μM antagonized the effect of α,β-meATP (Table 1). Only the ring D *para* sulfonate ABCD derivative **8** did not act as an antagonist under these conditions. In case of MG 40-3 (**12**) and MG 40-1 (**13**), each compound with an activated dichlorosubstituted ring E triazinyl residue, the observed blockade of the P2X$_1$-receptor was only partly reversible. In contrast, CB 3GA (**17**), MG 50-3-1 (**18**), RB 4 (**14**), MG 42-1 (**15**) and compound **16** were completely reversible antagonists, clearly demonstrating that monochlorotriazine or even dichlorotriazine moieties (in **14**) are not per se chemically reactive towards nucleophiles contained in receptor proteins.

The IC$_{50}$ values of the tested compounds against α,β-meATP suggest structure dependent activity.

First, the isolated ABC anthraquinone core (compound **2**) of the lead compound RB 2 (**1**) is obviously not sufficient for effective P2X$_1$-receptor blockade. Only further substitution of the ABC anthraquinone core in position 4 led to an increase in affinity which was independent of the chemical character of the substituent: ABC derivatives with a primary 4-amino group (compound **3**) an anionic 4-sulfonate group (compound **4**) as well as with a lipophilic 4-phenylamino group (AB 25, **5**) blocked the receptor with comparable, but still relatively low affinity.

Second, on the ABCD level, the position of the sulfonate residue in ring D has a distinct impact on P2X$_1$-receptor affinity. The ring D *ortho* sulfonate group in **6** remarkably increased affinity as compared to the ring D unsubstituted compound AB 25 (**5**). In contrast, introduction of a *meta* sulfonate group led to a considerably lower affinity of **7**, and the sulfonate substituent in ring D *para* position entirely prevented compound **8** from inhibiting the P2X$_1$-receptor mediated contraction at up to 100 μM. Additional ring D amino substitution improved the affinity in both the ring D *ortho* (**9**) and the ring D *meta* sulfonated (**10**) series, yet the affinity of the ring D *para* sulfonate **11** was still lower than that of AB 25 (**5**). The ring D aminosulfonates **9**–**11** displayed the same rank order of potency (*ortho* sulfonate > *meta* sulfonate > *para* sulfonate) as the ring D sulfonates **6**–**8**. This did not even change when the side chain was extended by further (hetero)aromatic rings E and F (see below).

Apparently, the ring D *para* sulfonate group impedes binding to the P2X$_1$-receptor. This observation is supported by data obtained from structurally related P2-receptor antagonists in former studies: 4-[4-(N-acetyl-N-methylamino)-phenylamino]-1-amino-9,10-dioxo-9,10-dihydoanthracene-2-sulfonic acid (Acid Blue 41) with its ring D *para* N-methylacetamido group did not show any effect at the P2X$_1$-receptor at up to 100 μM [19]. The same holds true for 1-amino-4-(4-methylphenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid monosodium salt (MG 32) [35] the ring D *para* methyl group of which prevents an interaction with the P2X$_1$-receptor.

Third, on the ABCDE level, P2X$_1$-receptor affinity was enhanced by introduction of a dichlorotriazinyl ring E in both

the ring D *ortho* and *meta* sulfonate series to give MG 40-3 (**12**), and MG 40-1 (**13**), respectively. Both isomers are relatively potent but only partly reversible antagonists. Obviously, the reactive ring E chloro substituents enable covalent binding of these two compounds to the receptor protein. In contrast, the dichlorotriazinyl ring E did *not* improve affinity in the ring D *para* sulfonate series as shown for RB 4 (**14**), which is a weak but fully reversible antagonist. Its ring D *para* sulfonate group apparently hinders an approach of the reactive ring E chloro substituents to the respective receptor binding area. Substitution of both ring E chloro substituents in MG 40-1 (**13**) by amino groups led to the fully reversible antagonist MG 42-1 (**15**), but reduced its receptor affinity to a certain extent. The ring E pyrimidine analogue **16** was also reversibly binding but of lower affinity. Reversible antagonism was already achieved by substituting only one of the two ring E chloro substituents by a sulfonatophenylamino residue, lowering the reactivity of the remaining chloro substituent in ABCDEF compounds to a major extent.

Fourth, on the final ABCDEF level, the three possible ring D sulfonate isomers were characterized by the same rank order of potency (*ortho* > *meta* > *para* sulfonate) as their respective precursors on the lower levels. In fact, MG 50-3-1 (**18**), CB 3GA (**17**), and RB 5 (1-amino-4-{3-[4-chloro-6-(3-sulfonatophenylamino)-[1,3,5]triazin-2-ylamino]-4-sulfonatophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid trisodium salt) (**20**) (Scheme 5) were equipotent to their corresponding ABCDE dichlorotriazinyl derivatives MG 40-3 (**12**), MG 40-1 (**13**), and RB 4 (**14**).

A former study demonstrated also a distinct impact of the ring F sulfonate position in RB 2 constitutional isomers on the P2X$_1$-receptor affinity. The ring F *meta* sulfonate isomer RB 2 *meta* (**1a**) was more potent than the ring F *para* sulfonate isomer RB 2 *para* (**1b**) whereas the highest affinity was observed for the third ring F *ortho* sulfonate constitutional isomer CB 3GA (**17**) [34].

In the present study, the remaining ring E chloro substituent in CB 3GA (**17**) was replaced by a further 2-sulfonatophenylamino residue to give the partly symmetrical ABCDE double ring F compound **19** with slightly higher P2X$_1$-receptor affinity, presumably due to the additional *ortho* sulfonate group.

Thus, the position, conformational distance and the number of anionic sulfonate groups seem to be of importance for the P2X$_1$-receptor blockade.

Comparing the three series of constitutional isomers, it turned out that in both the ring D *ortho* sulfonate and the ring D *meta* sulfonate series this blockade was improved by extension of the functionalized (hetero)aromatic side chain with rings E and F, presumably due to additional hydrogen bonds and, particularly, hydrophobic interactions via aromatic π-electron systems causing extra ligand–receptor interactions.

In contrast, in the ring D *para* sulfonate series, potency hardly increased with extension of the DEF side chain.

Summarizing, the ring E dichlorotriazinyl derivative MG 40-3 (**12**), MG 50-3-1 (**18**) as the ring D *ortho* sulfonate iso-

mer of CB 3GA (**17**), and the ABCDE double ring F derivative **19** were the most potent antagonists at the P2X$_1$-receptor of the RVD. This finding is independent of whether they are binding reversibly (**18**, **19**) or only in part reversibly (**12**).

4.2. P2Y$_1$-like receptor

Relaxation of the GPTC caused by the ecto-nucleotidase resistant agonist ADPβS is mediated by a P2Y$_1$-like receptor which has not so far been cloned but displays close similarities to the recombinant P2Y$_1$-receptor [19,45–50]. Seventeen of the 19 tested anthraquinone derivatives added at concentrations of up to 100 μM antagonized the effect of ADPβS. Only 1-aminoanthraquinone-2-sulfonate (**2**) and MG 40-1 (**13**) did not block the P2Y$_1$-like receptor under these conditions.

The IC$_{50}$ values of the tested compounds against ADPβS again suggest structure dependent activity.

First, as found for the P2X$_1$-receptor, the ABC anthraquinone core (compound **2**) of the lead compound RB 2 (**1**) is not sufficient for an effective blockade of the P2Y$_1$-like receptor. Adding an additional amino or sulfonate group in 4-position of the ABC anthraquinone core led to compound **3** or **4**, which inhibited the contraction by only 36% and 50%, respectively, at 100 μM.

Second, on the ABCD level, AB 25 (**5**) with an additional unsubstituted aromatic ring D as compared to **3** is a comparatively potent antagonist. This receptor affinity was remarkably attenuated by both ring D sulfonate and aminosulfonate substitution: the ring D *ortho* sulfonate derivative **6** showed only a 42% inhibition at 100 μM whereas the *meta* isomer **7** and the *para* isomer **8** displayed IC$_{50}$ values of 37.7 ± 3.1 and 20.5 ± 2.3 μM, respectively. This ring D *para > meta > ortho* sulfonate rank order of potency is reverse to that observed at the RVD. Additional ring D amino substitution slightly improved P2Y$_1$-like receptor affinity of the ring D *ortho* and the ring D *para* sulfonate analogues **9** and **11**. Nevertheless, affinities of **9** and **11** were still lower compared to AB 25 (**5**).

Third, on the ABCDE level, the ring D *ortho* sulfonated ring E dichlorotriazinyl derivative MG 40-3 (**12**) potently inhibited the relaxation of GPTC (by 71% at 1 μM, the lowest concentration applied in the present study). In contrast, the ring D *meta* sulfonate isomer MG 40-1 (**13**) did not affect the relaxation at up to 100 μM, while the ring D *para* sulfonate isomer RB4 (**14**) was a weak antagonist.

Formal substitution of the two ring E chloro substituents in the inactive dichlorotriazinyl derivative MG 40-1 (**13**) by amino groups led to the nanomolar P2Y$_1$-like receptor antagonist **15**. It probably interacts via additional hydrogen bonds. Its ring E pyrimidino analogue **16** was much less potent.

Fourth, on the ABCDEF level, the ring D *ortho* sulfonate isomer MG 50-3-1 (**18**) (IC$_{50}$ value of 4.6 ± 1.2 nM) was exceedingly more potent than its corresponding ring D *meta* sulfonate isomer CB 3GA (**17**) and about 870 times more potent than AB 25 (**5**). The ring D *para* sulfonate isomer RB 5 (**20**) inhibited the relaxation by only 42% at 100 μM.

In a former study, importance of the sulfonate position at ring F has also been demonstrated. Thus, compared to the ring F *ortho* sulfonate substituted CB 3GA (**17**) and its ring F *meta* sulfonate isomer RB 2 *meta* (**1a**), the corresponding constitutional *para* isomer RB 2 (**1b**) was significantly more potent [34].

In the present study, replacement of the remaining ring E chloro substituent of CB 3GA (**17**) by a second 2-sulfonatophenylamino residue to give the partly symmetrical ABCDE double ring F derivative **19** caused a remarkable increase in P2Y$_1$-like receptor affinity, presumably due to the additional ring F bearing a further *ortho* sulfonate group.

It is obvious that interpretation of SAR at the taenia coli P2Y$_1$-like receptor is rather complex. However, a key finding is the great impact of the anionic ring D *ortho* sulfonate group for P2Y$_1$-like receptor blockade: its combination with additional hydrogen bonds and, particularly, hydrophobic interactions via aromatic π-electron systems of the rings E and F results in high affinity compounds within the ring D *ortho* sulfonate series. Thus, MG 50-3-1 (**18**) is the most potent antagonist tested so far at the P2Y$_1$-like receptor of the GPTC.

4.3. Selectivity

Ratios of affinity estimates are a measure for selectivities of receptor antagonists. For the sake of clarity we have defined a ratio "IC$_{50}$ value at P2Y$_1$-like receptors/IC$_{50}$ value at P2X$_1$-receptors" > 10 of a P2-receptor antagonist as selectivity for the P2X$_1$ versus the P2Y$_1$-like receptor and a ratio "IC$_{50}$ value at P2X$_1$-receptors/IC$_{50}$ value at P2Y$_1$-like receptors" > 10 as selectivity for the P2Y$_1$-like- versus the P2X$_1$-receptor.

In terms of these definitions applied to the 19 compounds tested CB 3GA (**17**), the constitutional isomer of RB 2 (**1**), as an example displays little P2-receptor subtype selectivity, as already shown in previous studies [19,34]. The same holds true for most of the other compounds.

On the other hand, MG 40-1 (**13**) with a selectivity ratio > 11 is the only P2X$_1$ versus P2Y$_1$-like selective antagonist in the present study. However, it has to be considered that the blockade of P2X$_1$-receptors by **13** is only partly reversible.

More promising results were achieved for P2Y$_1$-like versus P2X1-selective antagonists. MG 42-1 (**15**) displays a selectivity ratio of > 16 in the range of that of AB 25 (**5**) [15]. The selectivity of the novel ring D *ortho* sulfonated CB 3GA isomer MG 50-3-1 (**18**), however, is considerably higher (610), even compared to AB 129 (> 25) and MG 32 (> 27) [19,35], making MG 50-3-1 (**18**) the most potent, reversible, and selective antagonist at the P2Y$_1$-like receptors of the GPTC reported so far.

5. Conclusions

It is clearly demonstrated with this SAR study of 19 structural modifications of RB 2 that MG 50-3-1 (**18**) as the ring

D *ortho* sulfonate isomer of CB 3GA (**17**) is the most potent antagonist tested so far at the P2Y$_1$-like receptor of the GPTC and discriminates it from the P2X$_1$-receptor of RVD by the factor 610. Both, the high potency and selectivity are associated with the ring D *ortho* position of the negatively charged sulfonate group.

Another important finding associated with the structure–reversibility relationships is the fact that all of the ring E monochloro derivatives (**1a**, **1b**, **17**, **18**, **20**) behave as fully reversible antagonists at the P2X$_1$-receptor of RVD. Only two (**12**, **13**) of the three ring E dichloro derivatives (**12–14**) are partly reversible antagonists at the P2X$_1$-receptor. In addition, **13** as a ring E dichloro derivative does not bind at all at the P2Y$_1$-like receptor of the GPTC. From these results it is finally concluded that the nanomolar potency of MG 50-3-1 (**18**) at the P2Y$_1$-like receptor is due to high non-covalent binding affinity rather than to reactivity of this ring E monochloro derivative.

6. Experimental protocols

6.1. Chemistry

6.1.1. Chemicals

Bromaminic acid (1-amino-4-bromo-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid) (**22**) and 2,4-diaminobenzenesulfonic acid (**28**) were supplied by Bayer AG (Leverkusen, Germany). CB 3GA (**17**), RB 4 (**14**), and RB 5 (**20**) were obtained from Sigma (Taufkirchen, Germany) and RB 2 (**1**) from RBI (Biotrend, Köln, Germany). AB 25 (**5**) and all other reagents used for the synthesis were purchased from Aldrich (Taufkirchen, Germany). Solvents were obtained from commercial suppliers and used after distillation.

6.1.2. Analytical studies

^{1}H- and ^{13}C-1D- and -2D-NMR spectra were recorded at 50 °C on a Varian Unity 300 spectrometer operating at 299.5 and 75.4 MHz, respectively. Chemical shift values δ (in ppm) are referenced to the signal of DMSO in DMSO-d$_6$, with $\delta_{TMS} = \delta_{DMSO}$ –2.49 for ^{1}H-NMR and $\delta_{TMS} = \delta_{DMSO}$ –39.7 for ^{13}C-NMR. The chemical shifts of proton multiplets were determined via the corresponding ^{1}H, ^{13}C-HETCOR spectra. ^{1}H-NMR data are listed in the following order: multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet), number of nuclei, assignment. 2D-NMR techniques like ^{1}H, ^{1}H-COSY, NOESY, *J*-resolved ^{1}H and ^{13}C, ^{1}H, ^{13}C-HETCOR, and long-range-^{1}H, ^{13}C- as well as -^{1}H, ^{15}N-HETCOR were used. The ESI mass spectra were obtained on a TSQ 7000 model (data system ICIS-II).

FCC was carried out on Silica gel RP C18 (32–63 μm) (ICN Biomedicals, Eschwege, Germany). RP-TLC was performed on Silica gel aluminum sheets RP-18 F$_{254 s}$ (E. Merck, Darmstadt, Germany). Non-colored spots were visualized under UV light ($\lambda = 254$ nm). All chemical yields are unoptimized and generally represent the result of only one or few experiments.

6.1.3. Commercially obtained compounds

General procedure for the purification. The commercially obtained compounds AB 25 (**5**), RB 4 (**14**), CB 3GA (**16**), and RB 5 (**17**) were separated from impurities by FCC on RP-18 silica gel. In each case, the combined fractions were filtrated, evaporated, and the blue product was dried under vacuum.

1-Amino-4-phenylamino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid mono-sodium salt (AB 25 (5)). RP-FCC was carried out with a methanol/water (1:1) eluent ($R_f = 0.23$). ^{1}H-NMR (DMSO-d$_6$) δ 7.17 (t, 1H, 4′-H), 7.26 (d, 2H, 2′-H, 6′-H), 7.41 (t, 2H, 3′-H, 5′-H), 7.80 (m, 2H, 6-H, 7-H), 8.06 (s, 1H, 3-H), 8.27 (m, 2H, 5-H, 8-H), 11.97 (s, 1H, 4-NH). ^{13}C-NMR (DMSO-d$_6$) δ 109.17 (C-9a), 111.47 (C-4a), 122.68 (C-3, C-2′, C-6′), 124.00 (C-4′), 125.67 (C-5), 125.76 (C-8), 129.35 (C-3′, C-5′), 132.30 (C-6), 132.70 (C-7), 133.45 (C-10a), 134.02 (C-8a), 139.25 (C-1′), 140.57 (C-4), 142.50 (C-2), 144.21 (C-1), 181.70 (C-9), 182.38 (C-10).

1-Amino-4-[3-(4,6-dichloro- [1,3,5]triazin-2-ylamino)-4-sulfonatophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (RB 4 (14)). RP-FCC was carried out with a methanol/water (1:3) eluent ($R_f = 0.51$). ^{1}H-NMR (DMSO-d$_6$) δ 6.83 (dd, 1H, 6′-H), 6.88 (d, 1H, 2′-H), 7.64 (d, 1H, 5′-H), 7.82 (m, 2H, 6-H, 7-H), 8.06 (s, 1H, 3-H), 8.24 (m, 2H, 5-H, 8-H), 10.82 (br, 1H, 3′-NH), 9-11 (br, 2H, 1-NH$_2$), 11.90 (br, 1H, 4-NH). ^{13}C-NMR (DMSO-d$_6$) δ 109.33 (C-9a), 112,13 (C-4a), 112.82 (C-6′), 113.92 (C-2′), 123.33 (C-3), 125.74 (C-5, C-8), 128.67 (C-5′), 132.33 (C-4′), 132.47 (C-6), 132.77 (C-7), 133.30 (C-10a), 133.95 (C-8a), 134.41 (C-3′), 139.41 (C-1′), 140.97 (C-4), 142.34 (C-2), 144.38 (C-1), 152.68 (C-2″), 154.51 (C-4″, C-6″), 181.82 (C-9), 182.67 (C-10).

1-Amino-4-{4-[4-chloro-6-(2-sulfonatophenylamino)-[1,3,5]triazin-2-ylamino]-3-sulfonatophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid trisodium salt (CB 3GA (17)). RP-FCC was carried out with a methanol/water (2:3) eluent ($R_f = 0.40$). ^{1}H-NMR (DMSO-d$_6$) [34]. ^{13}C-NMR (DMSO-d$_6$) [34].

1-Amino-4-{3-[4-chloro-6-(3-sulfonatophenylamino)-[1,3,5]triazin-2-ylamino]-4-sulfonatophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid trisodium salt (RB 5 (20)). RP-FCC was carried out with a methanol/water (1:3) eluent ($R_f = 0.45$). ^{1}H-NMR (DMSO-d$_6$) δ 6.95 (dd, 1H, 6′-H), 7.23 (dd, 1H, 5‴-H), 7.30 (dd, 1H, 6‴-H), 7.76 (d, 1H, 5′-H), 7.78 (m, 1H, 2‴-H), 7.80 (d, 1H, 4‴-H), 7.84 (m, 2H, 6-H, 7-H), 8.16 (s, 1H, 3-H), 8.19 (d, 1H, 2′-H), 8.29 (m, 2H, 5-H, 8-H), 7–11 (br, 2H, 1-NH$_2$), 10.12 (s, 1H, 6″-NH), 10.38 (s, 1H, 3′-NH), 11.89 (s, 1H, 4-NH). ^{13}C-NMR (DMSO-d$_6$) δ 109.32 (C-9a), 112,13 (C-4a), 114.34 (C-2′), 115.74 (C-6′), 118.73 (C-2‴), 120.83 (C-6‴), 121.42 (C-4‴), 123.02 (C-3), 125.77 (C-5, C-8), 127.38 (C-5‴), 128.03 (C-5′), 131.86 (C-4′), 132.52 (C-6), 132.94 (C-7), 133.31 (C-10a), 133.95 (C-8a), 135.68 (C-3′), 137.44 (C-1‴), 139.65 (C-4), 140.35 (C-1′), 142.23 (C-2), 144.25 (C-1), 148.30 (C-3‴), 163.16 (C-6″), 163.98 (C-2″), 168.25 (C-4″), 181.83 (C-9), 182.66 (C-10).

6.1.4. Synthesized compounds

1-Amino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid monosodium salt (2). **Method 1:** A solution of 1-aminoanthraquinone **(21)** (100 mg, 0.45 mmol) in nitrobenzene (20 ml) was heated at 120 °C to remove traces of water and then cooled to 80–85 °C. Chlorosulfonic acid (68 mg, 0.58 mmol) was added within 30 min with stirring. The mixture was heated at 130 °C for 3 h, then cooled to r.t. Na_2CO_3 (30 mg) was added, nitrobenzene was removed in vacuo, and the residue was flash chromatographed on RP-18 silica gel (MeOH/H_2O 2:3, R_f = 0.39) to give **2** (141 mg, 96%) as an orange colored solid. **Method 2:** Iron powder (20 mg) was added to a solution of 1-amino-4-bromo-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (bromaminic acid) **(22)** (100 mg, 0.26 mmol) in acetic acid (20 ml). The mixture was stirred under reflux for 6 h and then cooled to r.t. After adjustment to pH 7 by addition of 2 M Na_2CO_3, the mixture was filtered, the solvent was evaporated to dryness and the residue was purified by FCC on RP-18 silica gel (MeOH/H_2O 2:3, R_f = 0.39) to give the orange colored solid **2** (60 mg, 71%). ^{1}H-NMR (DMSO-d_6) δ 6.8–7.2 (br, 2H, 1-NH_2), 7.38 (d, 1H, 4-H), 7.80 (m, 1H, 6-H), 7.87 (m, 1H, 7-H), 7.94 (d, 1H, 3-H), 8.10 (dd, 1H, 5-H), 8.20 (dd, 1H, 8-H). ^{13}C-NMR (DMSO-d_6) δ 112.56 (C-9a), 114.01 (C-4), 126.00 (C-5), 126.30 (C-8), 132.31 (C-10a), 132.63 (C-3), 133.21 (C-6), 134.00 (C-4a), 134.21 (C-7), 134.42 (C-8a), 138.15 (C-2), 148.41 (C-1), 182.49 (C-10), 183.87 (C-9). ESI-MS (m/z): 238 [M-SO_2-Na]$^-$, 302 (100%) [M-Na]$^-$, 627 [2M-Na]$^-$, 952 [3M-Na]$^-$.

1,4-Diamino-9,10-dioxo-9,10-dihydroanthracene (23). A solution of AB 25 **(5)** (200 mg, 0.51 mmol) in acetic acid (20 ml) was stirred under reflux. After a solution of $SnCl_2$ (1.6 g, 8.44 mmol) in HCl (2 M, 3.5 ml) was added dropwise within 3 h, the mixture was cooled to r.t., filtered, and the residue was washed with methanol (3 × 5 ml). The combined filtrates were evaporated and the residue was recrystallized from methanol/water (1:1) to yield **23** (108 mg, 89%) as a brown solid. ^{1}H-NMR (DMSO-d_6) δ 7.41 (d, 2H, 2-H, 3-H), 7.96 (m, 2H, 6-H, 7-H), 8.26 (m, 2H, 5-H, 8-H), 12.64 (s, 4H, 1-NH_2, 4-NH_2). ^{13}C-NMR (DMSO-d_6) δ 112.51 (C-4a, C-9a), 126.45 (C-5, C-8), 129.08 (C-2, C-3), 132.73 (C-8a, C-10a), 134.81 (C-6, C-7), 156.53 (C-1, C-4), 186.44 (C-9, C-10).

1,4-Diamino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid monosodium salt (3). A solution of 1,4-diaminoanthraquinone **(23)** (90 mg, 0.38 mmol) in nitrobenzene (20 ml) was at first heated at 120 °C to remove traces of water and then cooled to 80–85 °C. Chlorosulfonic acid (57 mg, 0.49 mmol) was added within 30 min with stirring. The mixture was heated at 130 °C for 3 h, and then cooled to r.t. Na_2CO_3 (25 mg) was added, nitrobenzene was removed in vacuo, and the residue was flash chromatographed on RP-18 silica gel (MeOH/H_2O 2:3, R_f = 0.37) to give **3** (66 mg, 51%) as a brown solid. ^{1}H-NMR (DMSO-d_6) δ 4.5-5.5 (br, 4H, 1-NH_2, 4-NH_2), 7.67 (s, 1H, 3-H), 7.97 (m, 2H, 6-H, 7-H), 8.28 (m, 2H, 5-H, 8-H). ^{13}C-NMR (DMSO-d_6) δ 113.27 (C-4a) 113.37 (C-9a), 126.23 (C-3), 126.35 (C-5), 126.52

(C-8), 132.65 (C-10a), 133.18 (C-8a), 134.61 (C-6), 134.82 (C-7), 145.92 (C-2), 154.40 (C-1), 155.35 (C-4), 185.93 (C-9), 186.35 (C-10). ESI-MS (m/z): 255 [MH_2-SO_2-Na]$^-$, 319 (100%) [MH_2-Na]$^-$, 661 [$2MH_2$-Na]$^-$.

1-Amino-9,10-dioxo-9,10-dihydroanthracene-2,4-disulfonic acid disodium salt (4). Sodium dithionite (450 mg, 2.6 mmol) was added to a solution of bromaminic acid **(22)** (100 mg, 0.26 mmol) in DMF (20 ml) and water (20 ml) under nitrogen atmosphere. After the mixture was stirred for 8 h at 90 °C it was cooled to r. t., filtered, and the residue was washed with methanol (3 × 10 ml). The combined filtrates were evaporated and the residue was purified by FCC on RP-18 silica gel (MeOH/H_2O 1:2, R_f = 0.27) to give **4** (77 mg, 69%) as a red solid. ^{1}H-NMR (DMSO-d_6) δ 7-8.5 (br, 2H, 1-NH_2), 7.81 (m, 1H, 6-H), 7.83 (m, 1H, 7-H), 8.08 (dd, 1H, 5-H), 8.18 (dd, 1H, 8-H), 8.54 (s, 1H, 3-H). ^{13}C-NMR (DMSO-d_6) δ 112.21 (C-9a), 125.79 (C-8), 125.85 (C-5), 129.00 (C-4a), 129.93 (C-4), 132.64 (C-10a), 132.95 (C-6), 133.43 (C-7), 133.51 (C-8a), 134.42 (C-3), 137.89 (C-2), 147.20 (C-1), 182.47 (C-10), 183.22 (C-9). ESI-MS (m/z): 302 [M-SO_3Na+H-Na]$^-$, 366 (100%) [M+SO_2-SO_3Na+H-Na]$^-$, 446 [M+SO_2-2Na+H]$^-$, 468 [M+SO_2-Na]$^-$, 605 [2M-$2SO_3$Na+2H-2Na]$^-$, 937 [2M+$2SO_2$-2Na+H]$^-$.

General procedure A for Ullmann coupling reactions of bromaminic acid (22) (ABC-core) with an amino substituted benzenesulfonic acid 24–28 as ring D equivalent. To a solution of Na_2CO_3 (125 mg) and Na_2SO_3 (100 mg) in water (20 ml) were added bromaminic acid **(22)** (0.5 mmol) and a respective amino substituted benzenesulfonic acid **24–28** (1.0 mmol) at r.t. with stirring. After addition of copper(I) chloride (15 mg), the mixture was stirred for 8 h at r.t. or at 60 °C, monitoring the product formation by RT-TLC with a mobile phase of methanol/water (2:3). After completion of the reaction the mixture was filtered and the residue was washed with water (3 × 10 ml) and methanol (3 × 10 ml). The combined filtrates were evaporated to dryness. The residue was separated from impurities by FCC on RP-18 silica gel with a methanol/water eluent to give the respective ABCD anthraquinone derivative.

1-Amino-4-(2-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (6). Following the general procedure A with 190 mg (0.5 mmol) of bromaminic acid **(22)**, 107 mg (1.0 mmol) of 2-aminobenzenesulfonic acid **(24)**, 8 h at 60 °C, and RP-FCC performed with a methanol/water (1:3) eluent (R_f = 0.56), **6** (98 mg, 38%) was obtained as a blue solid. ^{1}H-NMR (DMSO-d_6) δ 7.04 (td, 1H, 4′-H), 7.17 (d, 1H, 6′-H), 7.30 (td, 1H, 5′-H), 7.80 (d, 1H, 3′-H), 7.81 (m, 2H, 6-H, 7-H), 8.04 (s, 1H, 3-H), 8.25 (m, 2H, 5-H, 8-H), 11.80 (s, 1H, 4-NH). ^{13}C-NMR (DMSO-d_6) δ 109.49 (C-9a), 113.26 (C-4a), 121.92 (C-4′), 122.23 (C-6′), 124.60 (C-3), 125.69 (C-5), 125.82 (C-8), 127.75 (C-3′), 129.11 (C-5′), 132.41 (C-6), 132.61 (C-7), 133.70 (C-10a), 133.97 (C-8a), 137.17 (C-1′), 138.84 (C-4), 139.18 (C-2′), 141.47 (C-2), 144.37 (C-1), 181.29 (C-10), 181.96 (C-9). ESI-MS (m/z): 302 [M-SO_3Na+H-C_6H_5N-Na]$^-$, 393 [M-SO_3Na+H-Na]$^-$, 473 (100%)

[M-2Na+H]⁻, 495 [M-Na]⁻, 947 [2M-4Na+3H]⁻, 969 [2M-3Na+2H]⁻, 991 [2M-2Na+H]⁻.

1-Amino-4-(3-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (7). Following the general procedure A with 190 mg (0.5 mmol) of bromaminic acid **(22)**, 107 mg (1.0 mmol) of 3-aminobenzenesulfonic acid **(25)**, 8 h at r.t., and RP-FCC performed with a methanol/water (1:3) eluent (R_f = 0.51), **7** (119 mg, 46%) was obtained as a blue solid. ¹H-NMR (DMSO-d₆) δ 7.22 (dd, 1H, 6′-H), 7.38 (t, 1H, 5′-H), 7.43 (dd, 1H, 4′-H), 7.46 (d, 1H, 2′-H), 7.83 (m, 2H, 6-H, 7-H), 8.00 (s, 1H, 3-H), 8.27 (m, 2H, 5-H, 8-H), 12.00 (s, 1H, 4-NH). ¹³C-NMR (DMSO-d₆) δ 109.14 (C-9a), 111.59 (C-4a), 120.09 (C-2′), 121.45 (C-4′), 122.38 (C-6′), 122.67 (C-3), 125.82 (C-5, C-8), 128.72 (C-5′), 132.54 (C-6), 132.95 (C-7), 133.44 (C-10a), 134.02 (C-8a), 138.69 (C-1′), 140.52 (C-4), 142.66 (C-2), 144.27 (C-1), 149.90 (C-3′), 181.74 (C-9), 182.50 (C-10). ESI-MS (m/z): 473 (100%) [M-2Na+H]⁻, 495 [M-Na]⁻, 947 [2M-4Na+3H]⁻, 969 [2M-3Na+2H]⁻, 991 [2M-2Na+H]⁻.

1-Amino-4-(4-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (8). Following the general procedure A with 190 mg (0.5 mmol) of bromaminic acid **(22)**, 107 mg (1.0 mmol) of 4-aminobenzenesulfonic acid **(26)**, 8 h at r.t., and RP-FCC performed with a methanol/water (1:3) eluent (R_f = 0.49), **8** (127 mg, 49%) was obtained as a blue solid. ¹H-NMR (DMSO-d₆) δ 7.20 (d, 2H, 2′-H, 6′-H), 7.66 (d, 2H, 3′-H, 5′-H), 7.84 (m, 2H, 6-H, 7-H), 8.06 (s, 1H, 3-H), 8.27 (m, 2H, 5-H, 8-H), 11.98 (s, 1H, 4-NH). ¹³C-NMR (DMSO-d₆) δ 109.14 (C-9a), 111.73 (C-4a), 121.34 (C-2′, C-6′), 122.74 (C-3), 125.77 (C-5), 125.82 (C-8), 126.86 (C-3′, C-5′), 132.53 (C-6), 132.96 (C-7), 133.40 (C-10a), 134.00 (C-8a), 139.28 (C-1′), 140.10 (C-4), 142.60 (C-2), 144.09 (C-4′), 144.31 (C-1), 181.72 (C-9), 182.48 (C-10). ESI-MS (m/z): 236 [M-2Na]²⁻, 473 (100%) [M-2Na+H]⁻, 495 [M-Na]⁻, 947 [2M-4Na+3H]⁻, 969 [2M-3Na+2H]⁻, 991 [2M-2Na+H]⁻.

1-Amino-4-(4-amino-2-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (9). According to the general procedure A, bromaminic acid **(22)** (190 mg, 0.5 mmol) was added to a solution of Na₂SO₃ (100 mg) in water (20 ml) at r.t. with stirring. The mixture was at first heated to 60 °C, followed by the addition of copper(I) chloride (15 mg). Then a solution of 2,5-diaminobenzenesulfonic acid **(27)** (188 mg, 1.0 mmol) in water (20 ml) was added dropwise within 30 min and the mixture was stirred for approximately 8 h at 60 °C, further on following the general procedure A. RF-CC was performed with a methanol/water (1:3) eluent (R_f = 0.72), and **9** (26 mg, 10%) was obtained as a blue solid. ¹H-NMR (DMSO-d₆) δ 5.05 (s, 2H, 4′-NH₂), 6.55 (dd, 1H, 5′-H), 6.84 (d, 1H, 6′-H), 7.16 (d, 1H, 3′-H), 7.79 (m, 2H, 6-H, 7-H), 7.89 (s, 1H, 3-H), 8.27 (m, 2H, 5-H, 8-H), 9–11 (br, 2H, 1-NH₂), 11.95 (s, 1H, 4-NH). ¹³C-NMR (DMSO-d₆) δ 108.94 (C-9a), 110.41 (C-4a), 113.31 (C-3′), 114.22 (C-5′), 124.33 (C-3), 124.79 (C-1′), 125.39 (C-6′), 125.57 (C-5, C-8), 131.90 (C-7), 132.04 (C-6), 133.94 (C-10a), 134.05 (C-8a), 141.70 (C-2′), 141.90

(C-4), 141.94 (C-2), 143.93 (C-1), 144.88 (C-4′), 179.72 (C-10), 181.35 (C-9). ESI-MS (m/z): 244 [M-2Na]²⁻, 488 (100%) [M-2Na+H]⁻, 510 [M-Na]⁻, 897 [2 M-SO₃Na+H-3Na+2H]⁻, 919 [2M-SO₃Na+H-2Na+H]⁻, 977 [2M-4Na+3H]⁻, 999 [2M-3Na+2H]⁻.

1-Amino-4-(4-amino-3-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (10). Following the general procedure A with 764 mg (2.0 mmol) of bromaminic acid **(22)**, 760 mg (4.0 mmol) of 2,5-diaminobenzenesulfonic acid **(27)**, 8 h at r.t., and RP-FCC performed with a methanol/water (1:3) eluent (R_f = 0.58), **10** (512 mg, 48%) was obtained as a blue solid [34]. ¹H-NMR (DMSO-d₆) [34], ¹³C-NMR (DMSO-d₆) [34]. ESI-MS (m/z): 244 [M-2Na]²⁻, 488 (100%) [M-2Na+H]⁻, 510 [M-Na]⁻, 999 [2M-3Na+2H]⁻.

1-Amino-4-(3-amino-4-sulfonatophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (11). Following the general procedure A with 190 mg (0.5 mmol) of bromaminic acid **(22)**, 188 mg (1.0 mmol) of 2,4-diaminobenzenesulfonic acid **(28)**, 8 h at r.t., and RP-FCC performed with a methanol/water (1:3) eluent (R_f = 0.57), **11** (131 mg, 49%) was obtained as a blue solid. ¹H-NMR (DMSO-d₆) δ 5.71 (s, 2H, 3′-NH₂), 6.34 (dd, 1H, 6′-H), 6.48 (d, 1H, 2′-H), 7.46 (d, 1H, 5′-H), 8.08 (s, 1H, 3-H), 7.82 (m, 2H, 6-H, 7-H), 8.26 (m, 2H, 5-H, 8-H), 9–11 (br, 2H, 1-NH₂), 11.95 (s, 1H, 4-NH). ¹³C-NMR (DMSO-d₆) δ 108.69 (C-2′), 108.75 (C-6′), 108.88 (C-9a), 110.99 (C-4a), 123.26 (C-3), 125.72 (C-5), 125.76 (C-8), 127.06 (C-4′), 128.51 (C-5′), 132.41 (C-6), 132.77 (C-7), 133.47 (C-10a), 134.00 (C-8a), 140.02 (C-1′), 140.89 (C-4), 142.67 (C-2), 144.20 (C-1), 146.22 (C-3′), 181.53 (C-9), 182.05 (C-10). ESI-MS (m/z): 244 [M-2Na]²⁻, 408 [M-SO₃Na+H-Na]⁻, 488 (100%) [M-2Na+H]⁻, 510 [M-Na]⁻, 897 [2 M-SO₃Na+H-3Na+2H]⁻, 919 [2 M-SO₃Na+H-2Na+H]⁻.

General procedure B for the addition of a ring E equivalent. An ice-cooled solution of a ring E equivalent **29**–**31** (0.1 mmol) in water (10 ml) and acetone (10 ml) was added to a stirred solution of the respective ABCD anthraquinone derivative **12**, **13**, **15** or **16** (0.1 mmol) in water (10 ml) at 0–5 °C. The resulting mixture was stirred for 1 h at 0–5 °C, and the pH was kept at 5–7 by dropwise addition of 2 M Na₂CO₃. The formation of product was monitored by RP-TLC using a mobile phase of methanol/water (2:3). After completion of the reaction the solvents were evaporated to dryness and the anhydrous residue was purified by FCC RP-18 silica gel using a methanol/water eluent to obtain the respective ABCDE anthraquinone derivative.

1-Amino-4-[4-(4,6-dichloro- [1,3,5]triazine-2-ylamino)-2-sulfonatophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (12). Following the general procedure B with 17 mg (0.09 mmol) of cyanuric chloride **(29)**, 50 mg (0.09 mmol) of **9** and RP-FCC performed with a methanol/water (1:3) eluent (R_f = 0.67), **12** (43 mg, 68%) was obtained as a blue solid. ¹H-NMR (DMSO-d₆) δ 7.17 (d, 1H, 6′-H), 7.55 (dd, 1H, 5′-H), 7.82 (m, 2H, 6-H, 7-H), 7.87 (d, 1H, 3′-H), 8.03 (s, 1H, 3-H), 8.25 (m, 2H, 5-H, 8-H), 10.65

(br, 1H, 4′-NH), 9–11 (br, 2H, 1-NH$_2$), 12.05 (br, 1H, 4-NH). ^{13}C-NMR (DMSO-d$_6$) δ 109.54 (C-9a), 113,12 (C-4a), 121.99 (C-3′), 123.00 (C-6′), 123.26 (C-5′), 124.55 (C-3), 125.74 (C-5, C-8), 130.65 (C-4′), 132.46 (C-6), 132.62 (C-7), 133.72 (C-10a), 133.98 (C-8a), 134.22 (C-1′), 138.96 (C-4), 139.72 (C-2′), 141.53 (C-2), 144.34 (C-1), 152.07 (C-2″), 153.76 (C-4″, C-6″), 181.27 (C-10), 181.98 (C-9). ESI-MS (m/z): 408 (100%) [M-C$_3$N$_3$Cl$_2$+H-SO$_3$Na+H-Na]$^−$, 488 [M-C$_3$N$_3$Cl$_2$+H-2Na+H]$^−$, 510 [M-C$_3$N$_3$Cl$_2$+H-Na]$^−$.

1-Amino-4-[4-(4,6-dichloro-[1,3,5]triazine-2-ylamino)-3-sulfonatophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (13). Following the general procedure B with 52 mg (0.28 mmol) of cyanuric chloride (29), 150 mg (0.28 mmol) of 10 and RP-FCC performed with a methanol/water (1:3) eluent (R$_f$ = 0.57), 13 (136 mg, 71%) was obtained as a blue solid [34]. ^{1}H-NMR (DMSO-d$_6$) [34], ^{13}C-NMR (DMSO-d$_6$) [34]. ESI-MS (m/z): 408 (100%) [M-C$_3$N$_3$Cl$_2$+H-SO$_3$Na+H-Na]$^−$, 488 [M-C$_3$N$_3$Cl$_2$+H-2Na+H]$^−$, 510 [M-C$_3$N$_3$Cl$_2$+H-Na]$^−$.

1-Amino-4-[4-(4,6-diamino-[1,3,5]triazine-2-ylamino)-3-sulfonatophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (15). According to the general procedure B, the solution of 41 mg (0.28 mmol) of 2-chloro-4,6-diamino- [1,3,5]-triazine (30) and 150 mg (0.28 mmol) of 10 was stirred for 1 h at 0–5 °C and for additional 2 h at 60 °C. RP-FCC was performed with a methanol/water (2:3) eluent (R$_f$ = 0.37), 15 (114 mg, 63%) was obtained as a blue solid. ^{1}H-NMR (DMSO-d$_6$) δ 6.31 (s, 4H, 4″-NH$_2$, 6″-NH$_2$), 7.17 (dd, 1H, 6′-H), 7.51 (d, 1H, 2′-H), 7.83 (m, 2H, 6-H, 7-H), 7.93 (s, 1H, 3-H), 8.29 (m, 2H, 5-H, 8-H), 8.81 (d, 1H, 5′-H), 7–11 (br, 2H, 1-NH$_2$), 9.50 (br, 1H, 4′-NH), 12.11 (br, 1H, 4-NH). ^{13}C-NMR (DMSO-d$_6$) δ 108.89 (C-9a), 110,48 (C-4a), 120.27 (C-5′), 122.35 (C-2′), 122.40 (C-3), 124.02 (C-6′), 125.75 (C-5, C-8), 130.64 (C-1′), 132.36 (C-6), 132.63 (C-7), 133.55 (C-10a), 133.98 (C-4′), 134.06 (C-8a), 135.63 (C-3′), 141.81 (C-4), 142.93 (C-2), 144.07 (C-1), 164.13 (C-2″), 167.07 (C-4″, C-6″), 181.49 (C-9), 181.75 (C-10). ESI-MS (m/z): 473 [M-C$_3$N$_6$H$_4$-2Na+H]$^−$, 488 [M-C$_3$N$_5$H$_3$-2Na+H]$^−$, 597 (100%) [M-2Na+H]$^−$, 619 [M-Na]$^−$.

1-Amino-4-[4-[1,3]diazine-2-ylamino)-3-sulfonatophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid disodium salt (16). According to the general procedure B, the solution of 32 mg (0.28 mmol) of 2-chloropyrimidine (31) and 150 mg (0.28 mmol) of 10 was stirred for 1 h at 0–5 °C and for additional 2 h at 90 °C. RP-FCC was performed with a methanol/water (2:3) eluent (R$_f$ = 0.32), 16 (81 mg, 47%) was obtained as a blue solid. ^{1}H-NMR (DMSO-d$_6$) δ 6.88 (d, 1H, 5″-H), 7.26 (dd, 1H, 6′-H), 7.56 (d, 1H, 2′-H), 7.83 (m, 2H, 6-H, 7-H), 7.94 (s, 1H, 3-H), 8.29 (m, 2H, 5-H, 8-H), 8.51 (d, 2H, 4″-H, 6″-H), 8.58 (d, 1H, 5′-H), 7–11 (br, 2H, 1-NH$_2$), 10.03 (br, 1H, 4′-NH), 12.09 (br, 1H, 4-NH). ^{13}C-NMR (DMSO-d$_6$) δ 108.89 (C-9a), 110,56 (C-4a), 112.61 (C-5″), 119.67 (C-5′), 122.41 (C-3, C-2′), 124.33 (C-6′), 125.76 (C-5, C-8), 131.23 (C-1′), 132.37 (C-6), 132.67 (C-7), 133.54 (C-4′, C-10a), 134.01 (C-8a), 136.06

(C-3′), 141.78 (C-4), 142.98 (C-2), 144.12 (C-1), 157.80 (C-4″, C-6″), 159.24 (C-2″), 181.51 (C-9), 181.84 (C-10). ESI-MS (m/z): 283 [M-2Na]$^{2−}$, 566 (100%) [M-2Na+H]$^−$, 588 [M-Na]$^−$.

1-Amino-4-{4-[4-chloro-6-(2-sulfonatophenylamino)-[1,3,5]triazine-2-ylamino]-2-sulfonatophenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid trisodium salt (18). A solution of 2-aminobenzenesulfonic acid (24) (38 mg, 0.35 mmol) in water (10 ml) was added to a stirred solution of 12 (50 mg, 0.07 mmol) in water (20 ml) and acetone (10 ml). Then the temperature was increased to 40–60 °C and stirring was continued for approximately 4 h. The pH was kept at 5–7 by dropwise addition of 2 M Na$_2$CO$_3$. The formation of product was monitored by RP-TLC with a mobile phase of methanol/water (2:3). After completion of the reaction the solvents were evaporated to dryness and the anhydrous residue was purified by FCC on RP-18 silica gel (MeOH/H$_2$O 2:3, R$_f$ = 0.49) to give the blue solid 18 (50 mg, 81%). ^{1}H-NMR (DMSO-d$_6$) δ 7.00 (t, 1H, 4‴-H), 7.14 (d, 1H, 6′-H), 7.48 (t, 1H, 5‴-H), 7.69 (d, 1H, 3‴-H), 7.71 (d, 1H, 5′-H), 7.83 (m, 2H, 6-H, 7-H), 8.03 (d, 1H, 3′-H), 8.08 (s, 1H, 3-H), 8.27 (m, 2H, 5-H, 8-H), 8.39 (d, 1H, 6‴-H), 7-9 (br, 2H, 1-NH$_2$), 9.94 (s, 1H, 6″-NH), 10.08 (s, 1H, 4′-NH), 11.5–12.5 (s, 1H, 4-NH). ^{13}C-NMR (DMSO-d$_6$) δ 109.42 (C-9a), 112.64 (C-4a), 121.14 (C-3′), 121.83 (C-6‴), 121.93 (C-4‴), 122.03 (C-5′), 122.74 (C-6′), 124.53 (C-3), 125.66 (C-5), 125.78 (C-8), 126.54 (C-3″), 129.57 (C-5‴), 132.35 (C-4′), 132.48 (C-6, C-7), 133.15 (C-1′), 133.76 (C-10a), 133.96 (C-8a), 134.53 (C-1‴), 135.88 (C-2‴), 139.43 (C-4), 140.54 (C-2′), 141.61 (C-2), 144.27 (C-1), 162.56 (C-2″, C-6″), 168.30 (C-4″), 180.99 (C-10), 181.84 (C-9). ESI-MS (m/z): 772/774 (100%) [M(^{35}Cl/^{37}Cl)-3Na+2H]$^−$, 794/796 [M(^{35}Cl/^{37}Cl)-2Na+H]$^−$, 816/818 [M(^{35}Cl/^{37}Cl)-Na]$^−$.

1-Amino-4-{4-[4,6-bis(2-sulfonatophenylamino)-[1,3,5]triazine-2-ylamino]-3-sulfonato-phenylamino}-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid tetrasodium salt (19). A solution of 2-aminobenzenesulfonic acid (24) (32 mg, 0.3 mmol) in water (10 ml) was added to a stirred solution of CB 3GA (17) (50 mg, 0.06 mmol) in water (15 ml). The mixture was heated at 90 °C for 5 h, keeping the pH at 5–7 by dropwise addition of 2 M Na$_2$CO$_3$. The formation of product was monitored by RP-TLC using a mobile phase of methanol/water (2:3). After completion of the reaction the solvents were evaporated to dryness and the residue was purified by FCC on RP-18 silica gel using a methanol/water (1:2) eluent (R$_f$ = 0.34) to give 19 (52 mg, 88%) as a blue solid. ^{1}H-NMR (DMSO-d$_6$) δ 6.96 (t, 2H, 4‴-H), 7.23 (dd, 1H, 6′-H), 7.38 (t, 2H, 5‴-H), 7.59 (d, 1H, 2′-H), 7.71 (dd, 2H, 3‴-H), 7.84 (m, 2H, 6-H, 7-H), 8.02 (s, 1H, 3-H), 8.30 (m, 2H, 5-H, 8-H), 8.46 (d, 2H, 6‴-H), 8.54 (d, 1H, 5′-H), 7–11 (br, 2H, 1-NH$_2$), 9.88 (s, 1H, 4′-NH), 9.93 (s, 2H, 4″-NH, 6″-NH), 12.12 (s, 1H, 4-NH). ^{13}C-NMR (DMSO-d$_6$) δ 108.98 (C-9a), 110.83 (C-4a), 120.52 (C-6‴), 120.81 (C-4‴), 121.45 (C-5′), 122.04 (C-2′), 122.56 (C-3), 123.78 (C-6′), 125.77 (C-5, C-8), 126.68 (C-3‴), 129.03 (C-5‴), 132.13 (C-1′), 132.39 (C-6, C-7), 132.69 (C-4′), 133.54 (C-10a), 134.01

(C-8a), 135.36 (C-2‴), 135.68 (C-1‴), 136.56 (C-3′), 141.42 (C-4), 142.91 (C-2), 144.19 (C-1), 163.47 (C-2″), 163.62 (C-4″, C-6″), 181.57 (C-9), 181.97 (C-10). ESI-MS (m/z): 374 [M-2SO$_3$Na+2H-2Na]$^{2-}$, 414 [M-SO$_3$Na+H-3Na+H]$^{2-}$, 454 [M-4Na+2H]$^{2-}$, 753 [M-SO$_3$Na+H-C$_6$H$_5$+H-3Na+2H]$^-$, 909 (100%) [M-4Na+3H]$^-$, 931 [M-3Na+2H]$^-$, 953 [M-2Na+H]$^-$, 975 [M-Na]$^-$.

6.2. Pharmacology

6.2.1. Methods

Male Wistar rats (200–500 g, Charles River, Sulzfeld, Germany) or guinea-pigs of either sex (400–800 g) were decapitated, the vasa deferentia (rat) or the ventral taenia coli (guinea-pig) removed and cleaned of adherent tissue. The incubation medium contained (mM): NaCl 118, KCl 4.8, CaCl$_2$ 2.5, KH$_2$PO$_4$ 0.9, NaHCO$_3$ 25, glucose 11, ascorbic acid 0.3, and disodium EDTA 0.03. It was saturated with 95% O$_2$/5% CO$_2$ and kept at 37 °C.

6.2.2. Contraction of rat vas deferens

Prostatic thirds of the vas deferens were suspended vertically in a 5.9 ml organ bath. The lower end was fixed and the upper end attached to an isometric force transducer (K30, Hugo Sachs Elektronik, Hugstetten, Germany). The initial tension applied was 9.8 mN (ink recorder R-50-Series, Rikadenki, Freiburg, Germany, or thermal pen recorder WR 3310, Graphtec, Ettlingen, Germany), but tissues subsequently relaxed to approximately 3 mN during a 60 min equilibration period. The medium was replaced every 15 min. Contractions were elicited by α,β-meATP which was added every 60 min at a concentration of 3.2 µM (approximately an EC$_{50}$ concentration under present conditions) and washed out immediately after contractions had peaked.

6.2.3. Relaxation of guinea-pig taenia coli

Strips of about 10 mm of the taenia coli were suspended vertically in a 5.9 ml organ bath. The lower end was fixed and the upper end attached to a K30 transducer. The resting tension was repeatedly adjusted to 9.8 mN during an initial 60 min equilibration period. The medium was replaced every 15 min. After equilibration, carbachol was added to the medium at 15 min intervals for 2 min each. Initially, the maximal contraction of each strip was determined by a single addition of carbachol (300 nM). During the following two to four applications, the concentration of carbachol giving about 80% of the maximum was determined. This concentration (50–90 nM) was used for the remainder of the experiment, the 15 min rhythm being kept throughout. Relaxations were elicited by ADPβS which was added every 60 min at a concentration of 0.1 µM (approximately an EC$_{50}$ concentration under present conditions) during the plateau of the carbachol response and washed out together with the latter after the ensuing relaxation was maximal. Relaxations were expressed as a percentage of the respective carbachol contraction.

6.2.4. Statistics

Antagonist inhibition curves were obtained by using a fixed agonist concentration. Inhibitory effects were expressed as a percentage of the respective control responses. IC$_{50}$ values were calculated for each single experiment by interpolation from the two nearest data points. The resulting antagonist IC$_{50}$ values are expressed as the arithmetic mean ± S.E.M. from n = 4–12 experiments.

6.2.5. Materials

Adenosine 5′-O-(2-thiodiphosphate) trilithium (ADPβS), carbachol chloride, and α,β-methylene adenosine 5′-triphosphate dilithium (α,β-meATP) were purchased from Sigma (Taufkirchen, Germany). All drugs were dissolved in distilled water. Solutions of drugs were added to the organ bath in aliquots not exceeding 100 µl.

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