High-Affinity, Non-Nucleotide-Derived Competitive Antagonists of Platelet $P2Y_{12}$ Receptors

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Anthraquinone derivatives related to the moderately potent, nonselective $P2Y_{12}$ receptor antagonist reactive blue 2 (**6**) have been synthesized and optimized with respect to $P2Y_{12}$ receptor affinity. A radioligand binding assay utilizing human blood platelet membranes and the $P2Y_{12}$ receptor-selective antagonist radioligand [^{3}H]2-propylthioadenosine-5′-adenylic acid (1,1-dichloro-1-phosphonomethyl-1-phosphonyl) anhydride ([^{3}H]PSB-0413) was applied for compound testing. 1-Amino-2-sulfoanthraquinone derivatives bearing a (*p*-phenylamino)anilino substitution in the 4-position and an additional acidic function in the *meta*-position of the aniline ring showed high $P2Y_{12}$ receptor affinity. These new anthraquinone derivatives became accessible by a recently developed copper(0)-catalyzed Ullmann coupling reaction of 1-amino-4-bromoanthraquinone derivatives with anilines in phosphate buffer under microwave irradiation. The most potent compounds exhibited K_i values of 24.9 nM (1-amino-4-[4-phenylamino-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate, PSB-0739, **39**), and 21.0 nM (1-amino-4-[4-phenylamino-3-carboxyphenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate, PSB-0702, **41**), respectively. 1-Amino-2-sulfo-4-anilinoanthraquinone derivatives appeared to be noncytotoxic, as shown for selected derivatives at two human cell lines (melanoma and astrocytoma). Compounds **39** and **41** represent new lead structures for the development of antithrombotic drugs.

Introduction

The P2 purinergic receptors are a major class of receptors in the human body.[1] They are subdivided into two main families: G protein-coupled or metabotropic P2 receptors, designated P2Y, and ligand-gated ion channels or ionotropic receptors, termed P2X.[2] So far, seven subtypes in the P2X family ($P2X_{1-7}$) and eight subtypes in the P2Y family ($P2Y_{1,2,4,6,11,12,13,14}$) have been identified. Blood platelets express one P2X receptor subtype, $P2X_1$, activated by ATP, and two P2Y receptor subtypes, $P2Y_1$ and $P2Y_{12}$, both of which are activated by the nucleotide ADP, which induces platelet aggregation.[3] The combined action of both P2Y receptor subtypes on thrombocytes is required for a full aggregation response following stimulation by ADP. $P2Y_1$, coupled via the heterotrimeric GTP-binding protein G_q to phospholipase C_β is responsible for the mobilization of calcium ions from internal stores mediating platelet shape change and the initial wave of rapidly reversible platelet aggregation induced by ADP. $P2Y_{12}$, on the other hand, is coupled to inhibition of adenylate cyclase through G_i protein and mediates a progressive and sustained aggregation not preceded by shape change. The latter receptor also plays an important role in the potentiation of platelet secretion induced by several agonists, and its congenital deficiency has been shown to result in a lifelong bleeding disorder.[4] Modulation of P2 receptors in platelets appears to be of paramount importance in regulating platelet function and, as a consequence, in controlling thrombotic diseases, which are the most common cause of morbidity and mortality in the Western world.[5] The $P2Y_{12}$ receptor is expressed in very high density on blood platelets.[6] Apart from that, it shows a limited expression pattern with no or only low expression in peripheral tissues and cells and some expression in the brain, making it an ideal drug target for selective therapeutic intervention.[7] The $P2Y_{12}$ receptor on blood platelets is the target of the thienotetrahydropyridine antithrombotic drugs ticlopidine (5-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine), clopidogrel (**1**, Figure 1), and the newly developed analogue prasugrel (5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl acetate),[8] representing platelet aggregation inhibitors that are effective in the prevention and treatment of arterial thrombosis. These compounds are not direct $P2Y_{12}$ antagonists. Clopidogrel, for example, has to be bioactivated by oxidation via cytochrome P450 (CYP3A4, CYP3A5, and CYP2C19) enzymes followed by ring-opening to the corresponding highly unstable thiol or sulfenic acid derivatives, which are believed to covalently bind to the receptor protein forming a disulfide bond and thus presumably act as covalent, possibly allosteric antagonists at $P2Y_{12}$ receptors[9] (see Supporting Information, Scheme 1). Major drawbacks of clopidogrel and related thienotetrahydropyridine derivatives are: (i) slow onset of action (up to several days) due to the required metabolism, (ii) long duration of action due to irreversible inhibition, (iii) "drug resistance" in a high percentage of patients (up to 30%), (iv) moderate potency (therefore high doses are required), and (v) difficulties in steering and controlling the effects.

Therefore, it is highly desirable to develop $P2Y_{12}$ antagonists that are lacking the drawbacks associated with the standard $P2Y_{12}$ antagonists such as clopidogrel and other thienotetrahydropyridine derivatives. Several groups have recently been developing competitive, reversible $P2Y_{12}$ antagonists that may be superior to clopidogrel and related drugs. Most approaches started from the adenine nucleotides as lead structures, ADP,

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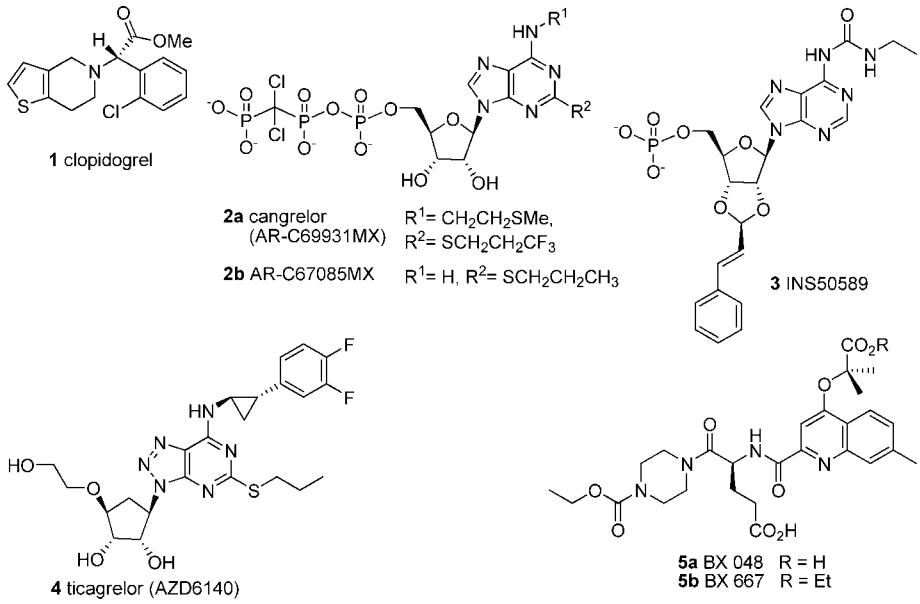
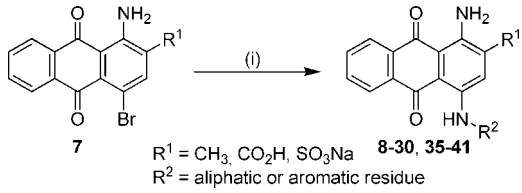


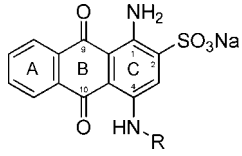
Figure 1. Structures of P2Y$_{12}$ receptor antagonists with antithrombotic activity.

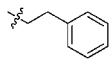
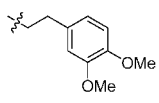
Scheme 1. General Synthesis of 4-Substituted Anthraquinones (**8**−**30**, **35**−**41**)[a,b]



[a] Reagents and conditions: (i) amine, phosphate buffer, Cu^0, microwave, 80−120 °C, 5−20 min. [b] For R^1, R^2, see Tables 1−4.

Table 1. Affinity of Anthraquinone Derivatives for the Human Platelet P2Y$_{12}$ Receptor: Part I

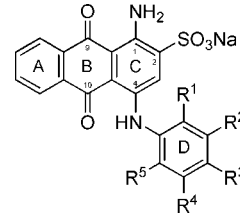


compd	R	K_i ± SEM (μM) vs. [^{3}H]PSB-0413 (% inhibition at 10 μM)
8 (AB25)		**9.83** ± 2.43
9		>> 10 (11 ± 3%)
10		>> 10 (16 ± 2%)
11		> 10 (23 ± 5%)
12		> 10 (25 ± 4%)

the physiological agonist, or ATP, which is an antagonist at the P2Y$_{12}$ receptor. Astra-Zeneca developed a series of ATP analogues, including cangrelor (AR-C69931MX, **2a**) and AR-C67085MX (**2b**) (Figure 1).[10] These compounds are very polar because they are negatively charged at a physiologic pH value of 7.4 and therefore cannot be perorally applied. In addition, they are metabolically unstable due to hydrolysis by nucleotide pyrophosphatases and therefore exhibit only a short half-life in vivo.[11] Furthermore, they are not easy to purify in large amounts as required for use as drugs. Compound **2b** was reported to possess high selectivity (>10^4-fold) for P2Y$_{12}$ receptors versus other P2Y and P2X receptor subtypes,[10a] but its selectivity versus other nucleotide binding sites is unclear.

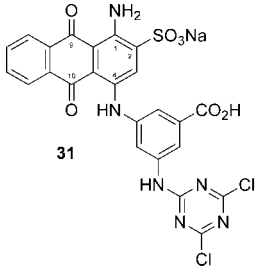
Inspire Pharmaceuticals Inc. developed nucleoside 5′-monophosphates as P2Y$_{12}$ antagonists for intravenous application, e.g.,

Table 2. Affinity of Anthraquinone Derivatives for the Human Platelet P2Y$_{12}$ Receptor: Part II



compd	R^1	R^2	R^3	R^4	R^5	K_i ± SEM (μM) vs [^{3}H]PSB-0413 (% inhibition at 10 μM)
13	NH_2	H	H	H	H	>10 (37 ± 1%)
14	H	H	NH_2	H	H	>10 (30 ± 3%)
15	H	Cl	H	H	H	**6.76** ± 2.07
16	H	H	Cl	H	H	**7.35** ± 1.72
17	H	NO_2	H	H	H	≫10 (17 ± 2%)
18	CO_2H	H	H	H	H	**25.1** ± 7.0
19	H	H	SO_3Na	H	H	**3.13** ± 0.39
20	OH	H	H	H	H	≈10 (45 ± v5%)
21	H	H	OH	H	H	≈10 (50 ± 5%)
22	H	H	NHC(=O)CH_3	H	H	≫10 (4 ± 14%)
23	H	NH_2	SO_3Na	H	H	**2.10** ± 0.47
24	H	SO_3Na	NH_2	H	H	**2.39** ± 0.65
25	H	CO_2H	H	NH_2	H	**7.07** ± 1.70
26	SO_3Na	H	NH_2	H	H	**19.74** ± 8.27
27	CO_2H	H	Cl	H	H	**12.3** ± 1.7
28	CO_2H	H	H	Cl	H	**12.2** ± 0.3
29	CH_3	H	CH_3	H	CH_3	>10 (30 ± 7%)
30	CH_3	NH_2	CH_3	H	CH_3	>10 (35 ± 5%)

Table 3. Affinity of Anthraquinone Derivatives for the Human Platelet $P2Y_{12}$ Receptor: Part III



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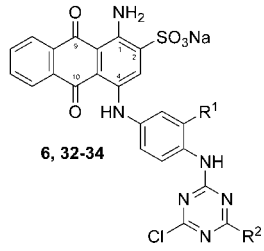
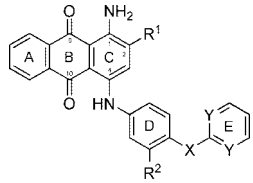


6, 32-34

compd	R^1	R^2	$K_i \pm$ SEM [μM] vs [^{3}H]PSB0413
31			**3.76** ± 1.03
32	SO_3Na	Cl	**2.45** ± 1.00
33	H	Cl	**1.90** ± 0.24
34	SO_3Na	OMe	**0.66** ± 0.12
6 (RB-2)	SO_3Na	*m*-/*p*-sulfophenylamino (1:2)	**0.68** ± 0.26

Table 4. Affinity of Anthraquinone Derivatives for the Human Platelet $P2Y_{12}$ Receptor: Part IV



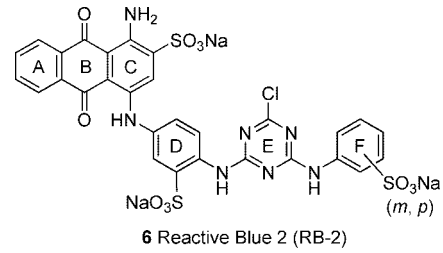
compd	R^1	R^2	X	Y	$K_i \pm$ SEM [μM] vs [^{3}H]PSB0413 (% inhibition at 10 μM)
35	SO_3Na	H	CH_2	C	**0.614** ± 0.095
36	SO_3Na	H	NH	C	**1.85** ± 0.57
37	SO_3Na	H	S	C	**0.884** ± 0.525
38	SO_3Na	SO_3Na	O	C	**0.063** ± 0.009
39	SO_3Na	SO_3Na	NH	C	**0.0249** ± 0.0032
40	CH_3	SO_3Na	NH	C	**0.541** ± 0.085
41	CO_2H	SO_3Na	NH	C	**0.021** ± 0.003
42	SO_3Na	SO_3Na	NH	N	**0.0507** ± 0.0160
43	SO_3Na	H	NH	N	~10 (57 ± 4%)

for blood product transfusion.[12] INS50589 (**3**, Figure 1) has been evaluated in clinical trials.[13] These compounds including **3** are also not perorally applicable and have very short half-lives due to enzymatic dephosphorylation, leading to inactive nucleosides or nucleoside analogues.[11] The development of **3** has meanwhile been discontinued.[14]

Furthermore, 2-substituted AMP, ADP, and ATP derivatives have been evaluated for their effects on platelets, and 2-phenylethynyladenosine di- and triphosphates, but not the corresponding monophosphates, inhibited ADP-induced platelet aggregation.[15]

An important progress was achieved by the development of the nucleoside analogue ticagrelor (AZD6140, **4**, Figure 1) and related compounds, which are perorally active $P2Y_{12}$ antagonists, and **4** is currently developed for clinical application as an antithrombotic agent.[16] However, the drug molecule is stereochemically sophisticated and requires a demanding, multistep synthesis. Furthermore, recent studies have indicated dyspnoea and bradycardia as potential side effects of **4**, possibly in part due to the formation of adenosine receptor-activating metabolites.[17] Very recently, the quinoline derivative **5a** was described as a novel competitive $P2Y_{12}$ receptor antagonist and its ethyl ester prodrug **5b** was shown to be orally bioavailable.[18]

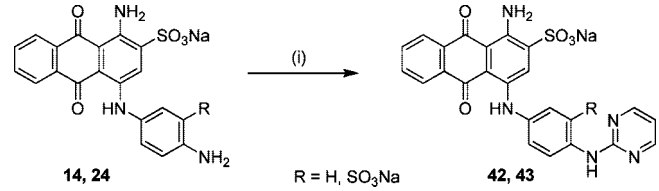
The anthraquinone derivative reactive blue 2 (RB-2, **6**, Figure 2) has been found to interact with a variety of nucleotide-binding



6 Reactive Blue 2 (RB-2)

Figure 2. Structure of reactive blue 2 (RB-2).

Scheme 2. Synthesis of Pyrimidylamino-Substituted Anilinoanthraquinones **42** and **43**[a]



[a] Reagents and conditions: (i) 2-bromopyrimidine, acetone:water (1:2), 0−5 °C 1 h, then reflux, 1−3 d, yield 90−97%.

proteins in the human body, including a number of different P2 receptor subtypes and ectonucleotidases.[19,20] RB-2 was described to antagonize $P2Y_{12}$ receptor activation and may thus be acting as a direct $P2Y_{12}$ receptor antagonist.[21]

Some RB-2 derivatives have been synthesized and investigated in functional studies at $P2Y_1$ and P2X receptors.[22] However, no data have been published on their potency at the $P2Y_{12}$ receptor subtype. In a recent review article, RB-2 was described as a $P2Y_{12}$ antagonist with a K_i value of 1.3 μM.[2b] In our laboratory, we determined a similar K_i value of 0.68 μM.[23] Although one anthraquinone derivative, RB-2, has shown promise as a $P2Y_{12}$ antagonist, a systematic evaluation of this class of compounds is still lacking, which may result in enhanced potency and selectivity.

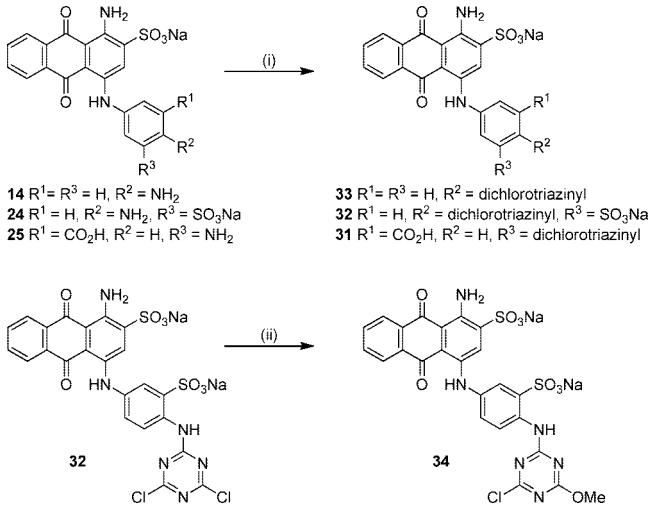
Novel $P2Y_{12}$ receptor antagonists, particularly those with high affinity and stability, chemically and enzymatically, are very interesting not only due to their antithrombotic properties. Because the $P2Y_{12}$ receptor is also expressed in brain, e.g., in microglial cells,[24] specific $P2Y_{12}$ antagonists may in addition be used to prevent or treat CNS diseases, including neuroinflammatory conditions and neurodegenerative disorders, e.g., Alzheimer's and Parkinson's disease.

Results and Discussion

Chemistry. The anthraquinone derivatives were synthesized as depicted in Schemes 1−3. The synthesis of compounds **8**, **10**−**30**, **32**−**34**, **36**, and **42** has previously been described.[25] The new compounds (**9**, **35**, and **37**−**41**) and the precursors of the new compounds (**31** and **43**) were obtained in analogy to the published new method by microwave-assisted Ullmann reaction.[26] Coupling of 4-bromo-substituted anthraquinone derivatives (bromaminic acid: $R^1 = SO_3H$; 1-amino-4-bromo-2-carboxyanthraquinone: $R^1 = CO_2H$; 1-amino-4-bromo-2-methylanthraquinone: $R^1 = CH_3$) with the appropriate amine in phosphate buffer (pH 6−7) in the presence of Cu^0 under microwave irradiation at 80−120 °C for 5−20 min yielded the target compounds in good to excellent yields (Scheme 1).

Two of the compounds, **14** and **24**, which bear a primary amino group in the *para*-position of the N^4-phenyl substituent, were further reacted with 2-bromopyrimidine yielding compounds **42** and **43** (Scheme 2).

Scheme 3. Synthesis of the Dichlorotriazinylaminoanthraquinone Derivatives **31**−**33**[a]



[a] Reagents and conditions: (i) cyanuric acid chloride, acetone:water (1:2), 0−5 °C, 1−6 h, yield 80−95%, (ii) MeOH, 60 °C, 30 min, yield 96%.

Three anilinoanthraquinone derivatives, **14**, **24**, and **25**, which bear a primary amino group in the *meta*- or *para*-position of the N^4-phenyl ring, were treated with cyanuric acid chloride (Scheme 3) to afford the desired compounds **31**−**33**. Compound **32** was treated with methanol at 60 °C for 30 min to yield product **34** (Scheme 3).

Purification and Analysis of the Synthesized Anthraquinone Derivatives. The synthesized anthraquinone derivatives were relatively polar compounds. Their purification was achieved by flash column chromatography using reversed-phase C18 material. A typical purification procedure is described in the Experimental Section and shown in Figure 2 of the Supporting Information. The structures of the synthesized anthraquinone derivatives were confirmed by MS and ^{1}H and ^{13}C NMR spectra. The purity of the compounds was determined by HPLC analysis coupled with UV detection at 254 nm. The purity of all products was at least 95% (see Experimental Section and Figure 3 in the Supporting Information).

Biological Assays. We have recently developed the first P2Y$_{12}$-selective radioligand, a [^{3}H]-labeled form of **2b** named [^{3}H]PSB-0413[6] and developed a P2Y$_{12}$ receptor radioligand binding assay at human platelet membranes.[6] This allows the fast screening of potential P2Y$_{12}$ receptor ligands.

All anthraquinone derivatives were investigated in this assay initially at a concentration of 10 μM. For compounds that showed at least 50% inhibition of radioligand binding, full concentration−inhibition curves were obtained and K_i values ($n = 3$) were determined.

Selected compounds were investigated for their functionality in GTP shift experiments.[27] Radioligand binding studies as described above were performed in the presence and absence of GTP (100 μM). Agonists, such as ADP, show a significant right-shift of the concentration−inhibition curve, while antagonists do not.

Two anthraquinone derivatives were investigated in cell proliferation assays at human 1539 melanoma cells and at human 1321N1 astrocytoma cells using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in order to assess their cytotoxic effects.[28]

Structure−Activity Relationships. A series of 36 anthraquinone derivatives was tested in radioligand binding assay (Tables 1−4).

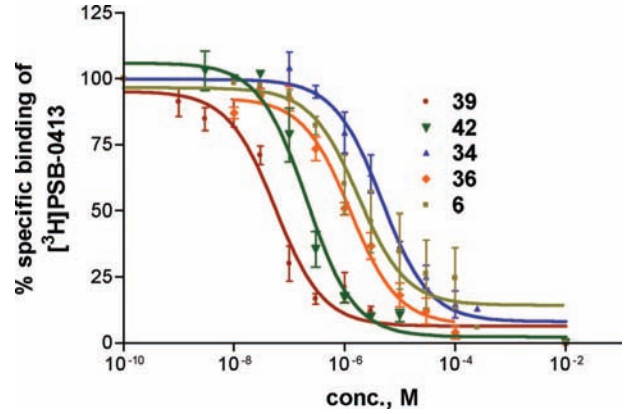


Figure 3. Competition curves of selected anthraquinone derivatives vs 5 nM [^{3}H]PSB-0413 at human platelet P2Y$_{12}$ receptors.

Determined K_i values at human platelet P2Y$_{12}$ receptors are collected in Tables 1−4. Selected concentration−inhibition curves are shown in Figure 3. It was found that the simple 1-amino-4-phenylamino-2-sulfoanthraquinone, known as acid blue 25 (AB25, **8**), showed some affinity for the P2Y$_{12}$ receptor ($K_i = 9.83$ μM). Because it is relatively small (MW = 416.38) as compared to **6** (MW = 840.10), we selected **8** as the new lead structure. Replacing the phenylamino ring by benzylamino, phenethylamino, or 3′,4′-dimethoxyphenethylamino (**9**−**11**) dramatically decreased the affinity. An α-naphthylamino group in the 4-position (**12**) was also poorly tolerated (Table 1).

These results suggested that a phenylamino substituent at the 4-position is essential for P2Y$_{12}$ affinity. Therefore, a small library consisting of 18 derivatives of lead structure **8** bearing various substituents on the phenyl ring in the N^4-position was investigated next (see Table 2).

Introducing a hydrophilic group (OH) at the ortho- or para-position of the phenyl ring was tolerated, as shown for **20** and **21** (Table 2), while introducing an amino group (NH_2) in the meta- or para-position as for **13** and **14** was less favorable. Acetylation of the *para*-amino group (**22**) or introducing a nitro group in the *meta*-position (**17**) abolished the affinity. Interestingly, introducing a more lipophilic, polar function (chlorine) in the meta- or para-position increased the potency somewhat (**15**, **16**). A negatively charged group (sulfonate) in the *para*-position (**19**) increased affinity by 3-fold, while a carboxylate in the ortho-position (**18**) decreased the activity by 2.5-fold compared to **8**.

Disubstitution of the phenyl ring appeared to be even somewhat more promising than mono-substitution. Especially the combination of a sulfonate and an amino group in the meta- and para-position, respectively (**23**, **24**), increased the affinity by 4.5-fold as compared to the lead structure (**8**). The *ortho*-sulfonate isomer **26** showed decreased affinity by more than 2-fold. Compound **25** (*meta*-amino, *meta*-carboxy-substituted) was also quite potent (Table 2). A combination of *para*- or *meta*-chloro-substitution with a carboxylate group in the ortho-position was tolerated (**27**, **28**), whereas introducing lipophilic substituents such as 2,4,6-trimethyl-phenylamino or 2,4,6-trimethyl-3-amino-phenylamino (**29**, **30**) abolished the affinity (Table 2).

In the next step, compounds with two aromatic rings attached to the anthraquinone 4-position connected via a nitrogen (NH) linker were investigated. The dichlorotriazinyl derivatives of arylaminoanthraquinones exhibiting an **ABCDE** ring system (**31**−**33**) were found to be more active than the ABCD ring compounds (**14**, **24**, and **25**). Interestingly, introduction of the dichlorotriazinyl residue into the para-position (**32** and **33**)

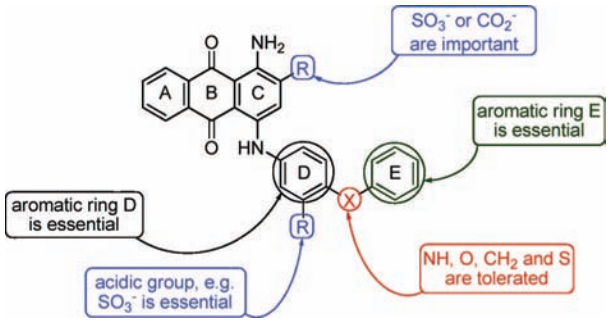


Figure 4. Summary of structure−activity relationships of anthraquinone derivatives as high-affinity $P2Y_{12}$ antagonists.

resulted in higher affinity (2−8-fold) than in the meta-position (**31**). Replacement of one chlorine in compound **32** by a methoxy group (**34**) led to a similarly potent compound as **6**, indicating that it was unnecessary to introduce an extra ring (ring **F**) (Table 3 and Figure 2) to obtain high $P2Y_{12}$ affinity.

Replacing the triazinyl ring by a phenyl ring (ring **E**) in the para-position of the phenyl ring **D** led to potent compounds with affinities reaching the nanomolar range (Table 4). Different bridging atoms between rings D and E (methylene (CH_2), amino (NH), or thioether (S) (**35**−**37**)) resulted in similarly high affinity (Table 4), with the methylene bridge being slightly superior to the S, and 3-fold better than NH. Introducing a sulfonate group in the meta-position of ring D (compare **36** and **39** (PSB0739)[29]) dramatically increased the affinity by ca. 75-fold, leading to a compound with a K_i value in the low nanomolar range (Table 4 and Figure 3).

In the next step, the analogue of the most potent compound so far (**39**, K_i = 24.9 nM), in which the sulfonate at position C-2 in the anthraquinone moiety was replaced by a methyl group (**40**), was synthesized. The affinity decreased dramatically by 24-fold, indicating the importance of a negatively charged group, such as sulfonate, at the C-2 position.

The most potent compound of the present series was **41** (PSB0702),[29] in which the sulfonate function in the 2-position of the anthraquinone core was replaced by a carboxylate group, exhibiting a K_i value of 21 nM in binding studies. This result shows that the sulfonate can be replaced by a carboxylate without any reduction in affinity.

Figure 4 summarizes the structure−activity relationships (SAR) of the investigated anthraquinone derivatives at human platelet $P2Y_{12}$ receptors. The **ABCDE** aromatic ring system proved to be very important for the affinity, and acidic groups such as sulfonate or carboxylate in the 2 position of the anthraquinone scaffold and in the meta-position of ring D play a crucial role because they are required for high affinity.

Selected anthraquinone derivatives, namely compounds **6** and **42**, were investigated in GTP shift assays in order to investigate whether they behaved as agonists or antagonists at $P2Y_{12}$ receptors. For the agonist ADP, a statistically significant, 2.8-fold increase of the IC_{50} value was detected in the presence of GTP (100 μM) in radioligand binding studies at human platelet membranes versus [^{3}H]PSB-0413. In contrast, GTP did not show any significant effect on the competition curves of compounds **6** and **42**. The GTP shift values defined as IC_{50} value in the presence of 100 μM GTP divided by IC_{50} without GTP added were as follows: 0.95 ± 0.22 for **6** and 0.76 ± 0.11 for **42**. Statistical analysis revealed that the shifts were not significantly different from 1. These results indicated that the compounds behave as antagonists rather than agonists at $P2Y_{12}$ receptors. One of the most potent compounds of the present series, the

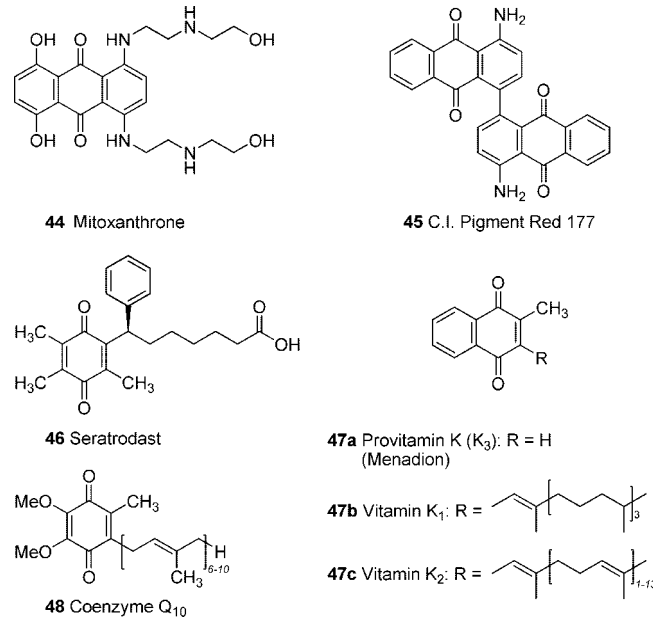


Figure 5. Selected marketed anthraquinone, naphthoquinone, and quinone derivatives Tables 1−4.

prototypical **39**, has recently been further characterized in functional studies at human $P2Y_{12}$ receptors recombinantly expressed in astrocytoma cells by von Kügelgen et al. (preliminary results have been published as a conference abstract).[30] Compound **39** caused a parallel shift of the concentration−response curve of the agonist to the right with a corresponding apparent pK_B value of 9.4 (K_B value ca. 0.4 nM). These results confirmed a competitive mechanism of inhibition and very high potency. To our knowledge, this is one of the most potent non-nucleotide antagonists at the human $P2Y_{12}$ receptor reported so far.

Initial results indicate that the most potent compounds of this series are also quite selective versus other P2 receptor subtypes. For example, **42** was found to be at least 100-fold selective versus human $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_2$, and $P2X_4$ receptors (data not shown). Although the lead structures **6** and **8** are promiscuous, interacting with many different targets,[25] it appears that the right substitution pattern on the anthraquinone scaffold can impart selectivity for a certain target such as the $P2Y_{12}$ receptor.

Cytotoxicity. Anthraquinones are an important class of natural products found for example in medicinal plants used as laxatives, such as senna, frangula, cascara, and rheum. A synthetic anthraquinone derivative bearing basic substituents, mitoxanthrone (**44**), is used in cancer therapy (Figure 5). It is derived from the anthracyclinesm, which are natural products from *Streptomyces* strains, which also show cytotoxic properties. On the other hand, C.I. pigment red 177 (**45**), an anthraquinone dye that is widely used for coloration of plastics and industrial paints, has been reported as not being acutely toxic by the oral route (LD_{50} value >5000 mg kg^{-1}, Figure 5).[31]

Another quinone derivative, seratrodast (**46**), is therapeutically applied as an antiasthmatic drug (Figure 5).[32] Another example for nontoxic quinone derivatives are the naphthoquinones belonging to the family of vitamin K derivatives (**47a**−**c**, Figure 5). Coenzyme Q10 (also known as ubiquinone, **48**), a benzoquinone (Figure 5) that is present in most eukaryotic cells, primarily in the mitochondria as a component of the electron transport chain.[33,34] Some studies indicate that coenzyme Q10 protects the brain from neurodegenerative disease such as Parkinson's due to its antioxidative properties.[35] Interestingly,

in coenzyme Q10 as well as in seratrodast, the α,*β*-positions of the quinone or naphthoquinone, respectively, are blocked by alkylation (methyl or long alkyl chain). Similarly the α,*β*-positions of anthraquinone are blocked by two benzene rings that prevent Michael addition, which is the main cause of cellular damage that occurs through alkylation in related molecules containing a free *β*-position.

To further investigate the toxicity of the anthraquinone scaffold (1-amino-4-anilino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate) present as a partial structure in most of our newly developed P2Y$_{12}$ antagonists, we selected two structurally simple derivatives: compound **16** (N^4-*p*-chlorophenyl-substituted), which had shown some P2Y$_{12}$ affinity, and compound **12** (N^4-α-napththyl-substituted), which was inactive. Both compounds were investigated in the MTT assay at two cell lines, human 1539 melanoma cells and human 1321N1 astrocytoma cells. The colorimetric MTT assay can be taken as a measure of mitochondrial activity and cell toxicity. The cytotoxic anticancer drug 5-fluorouracil was included as a control. While 5-fluorouracil inhibited the proliferation of both cell lines, with IC_{50} values of 49 ± 14 μM (astrocytoma cells) and 5.7 ± 0.8 μM (melanoma cells) after 72 h of incubation (similar values were obtained after longer incubation times of up to 144 h), both anthraquinone derivatives did not show any effects at the tested concentrations of 0.1, 1, and 100 μM on both cell lines (for data see Supporting Information). This indicates that the anthraquinone scaffold present in the described class of compounds is not cytotoxic; the compounds had no effects on cell proliferation and appeared not to inhibit mitochondrial activity.

Interaction of Therapeutically Used Anthraquinone Drugs with P2Y$_{12}$ Receptors. As a next step, we investigated whether therapeutically used natural and synthetic products with anthraquinone structure interact with platelet P2Y$_{12}$ receptors. Frangulin B, an anthraquinone glycoside (emodin-6-*O*-*β*-D-apiofuranoside) had previously been found to inhibit collagen-induced platelet aggregation.[36] Thus, several anthraquinone derivatives present in medicinal plants were tested, including aloe emodin (1,8-dihydroxy-3-(hydroxymethyl)anthraquinone), emodin (6-methyl-1,3,8-trihydroxyanthraquinone), chrysophanol (1,8-dihydroxy-3-methylanthraxquinone), frangulin (mixture of isomers A and B: emodin-6-*O*-α-L-rhamnopyranoside (A) and emodin-6-*O*-*β*-D-apiofuranoside (B)), physcion (1,8-dihydroxy-3-methoxy-6-methylanthraquinone), and rhein methyl ester (1,3-dihydroxy-anthraquinone-3-carboxylic acid methyl ester). In addition, the related anthrone derivatives aloin (1,8-dihydroxy-10-(*β*-D-glucopyranosyl)-3-(hydroxymethyl)-9(10*H*)-anthracenone) and emodin anthrone were investigated. None of these compounds showed a significant inhibition of [^{3}H]PSB-0413 binding to human platelet membranes at a test concentration of 10 μM. The cytotoxic anthracyclines mitoxantrone (**44**) and doxorubicin ((8*S*,10*S*)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2*H*-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione) as well as the related tetracyclines, tetracycline and chlorotetracycline, also showed no effect in P2Y$_{12}$ receptor binding assays at 10 μM concentration (for structures and data see Supporting Information).

Conclusions

In conclusion, we have developed a new class of highly potent, competitive non-nucleotide derived P2Y$_{12}$ antagonists. The most potent compounds exhibited K_i values for the human platelet P2Y$_{12}$ receptor in the lower nanomolar range and selectivity versus other P2 receptor subtypes. The new compounds will be useful as pharmacological tools for studying the role of peripheral as well as brain P2Y$_{12}$ receptors. Several members of this class of compounds have been identified, e.g., **39** and **41**, which constitute novel lead structures for the development of antithrombotic drugs.

Experimental Section

Chemistry

Material and Methods. All commercially available chemicals and drugs were obtained from Sigma-Aldrich or Acros, Germany, and used as purchased. Thin-layer chromatography was performed using TLC aluminum sheets with silica gel 60 F_{254}, or TLC aluminum sheets RP silica gel 18 F_{254} (Merck, Darmstadt, Germany). Colored compounds were visible at daylight; other compounds were visualized under UV light (254 nm). Flash chromatography was performed on a Büchi system using silica gel RP-18 (Merck, Darmstadt, Germany). ^{1}H and ^{13}C NMR data were collected on a Bruker Avance 500 MHz NMR spectrometer at 500 MHz (^{1}H) or 126 MHz (^{13}C), respectively. DMSO-d_6 was used as a solvent. Chemical shifts are reported in parts per million (ppm) relative to the deuterated solvent, i.e., DMSO, δ ^{1}H, 2.49 ppm; ^{13}C, 39.7 ppm. Coupling constants *J* are given in hertz and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad).

The purities of isolated products were determined by HPLC-UV obtained on an LCMS instrument (Applied Biosystems API 2000 LCMS/MS, HPLC Agilent 1100) using the following procedure: the compounds were dissolved at a concentration of 0.5 mg/mL in H_2O:MeOH = 1:1, containing 2 mM NH_4CH_3COO. Then, 10 μL of the sample was injected into an HPLC column (Phenomenix Luna 3 μ C18, 50 mm × 2.00 mm). Elution was performed with a gradient of water:methanol (containing 2 mM NH_4CH_3COO) from 90:10 to 0:100 for 30 min at a flow rate of 250 μL/min, starting the gradient after 10 min. UV absorption was detected from 200 to 950 nm using a diode array detector. Purity of the compounds was determined at 254 nm and proved to be ≥95%. A second HPLC method was developed using a Beckman Coulter HPLC for the determination of the synthesized anthraquinone derivatives' purity as follows: the compounds were dissolved at a concentration of 0.5 mg/mL in H_2O. Then, 10 μL of the sample was injected into an HPLC column (Ultrasphere 5 μm Spherical 80 Å Pore C-18, 250 mm × 4.6 mm). Elution was performed with a gradient of water:acetonitrile from 90:10 to 0:100 for 30−40 min at a flow rate of 500 μL/min, starting the gradient after 10 min. UV absorption was detected at 254 nm using a UV detector. The purity of the compounds proved to be ≥95%. For a typical HPLC spectrum see Figure 3 in the Supporting Information. For microwave reactions a CEM Focused Microwave Synthesis type Discover apparatus was used. A freeze-dryer (CHRIST ALPHA 1-4 LSC) was used for lyophilization. The syntheses of compounds **8**, **10**−**30**, **32**−**34**, and **42** were previously described.[20,22,26,37]

General Procedures for the Preparation of 4-Substituted Anthraquinones (8−30, 35−41): General Procedure A. To a 5 mL microwave reaction vial equipped with a magnetic stirring bar were added halogen-substituted compounds **7** (bromaminic acid, sodium salt, carboxy isomer, or the methyl isomer) (0.20 mmol) and the appropriate aniline and/or amine derivative (0.40 mmol), followed by a buffer solution of Na_2HPO_4 (pH 9.6) (4 mL) and NaH_2PO_4 (pH 4.2) (1 mL). A catalytic amount (ca. 0.002−0.003 g) of finely powdered elemental copper was added. The mixture was capped and irradiated in the microwave oven (80−100 W) for 5−20 min at 80−120 °C. Then the reaction mixture was cooled to rt and the product was purified using the following procedure. The contents of the vial were filtered to remove the elemental copper. Then ca. 200 mL of water was added to the filtrate and the aqueous solution was extracted with dichloromethane (200 mL). The extraction procedure was repeated until the dichloromethane layer became colorless (2−3 times). Then the aqueous layer was reduced by rotary evaporation to a volume of 10−20 mL, which was

subsequently submitted to flash column chromatography using RP-18 silica gel and water as an eluent. The polarity of the eluent was then gradually decreased by the addition of methanol in the following steps: 5, 10, 20, 40, 60, 80, and 100%. Fractions containing blue product were collected. For some compounds, the last step of purification (RP-18 flash chromatography) had to be repeated two to three times to obtain pure product (≥95% purity as determined by LC-MS). The pooled product-containing fractions were evaporated under vacuum to remove the methanol, and the remaining water was subsequently removed by lyophilization to yield (up to 90%) as blue powders.

General Procedure B. Coupling reaction of cyanuric acid chloride with the amino function in phenyl ring D: synthesis of ABCDE ring anthraquinone derivatives **31**−**33**. An ice-cooled solution of cyanuric acid chloride (0.5−1.0 mmol) in water (25 mL) and acetone (25 mL) was added to a stirred solution of amine-containing compound (**14**, **24**, and **25**) (0.5 mmol) in water (25 mL) at 0−5 °C. The resulting mixture was stirred for 1 h at 0−5 °C, then allowed to warm to rt and kept stirring at rt for 4−6 h. The formation of product was monitored by RP-TLC using a mobile phase of acetone:water (2:3). After completion of the reaction, the solvents were evaporated and the residue was purified by flash column chromatography on RP-18 silica gel using acetone:water as an eluent to obtain the desired products (**31**−**33**) (Scheme 3). It should be noted that the reaction did not require the addition of sodium carbonate to keep the solution basic as described in the literature.[22,37] Interestingly, a higher yield was obtained without adding the basic salt.

General Procedure C. Coupling reaction of 2-bromopyrimidine with the amino function in phenyl ring D: synthesis of **ABCDE** ring anthraquinone derivatives **42** and **43**. An ice-cooled solution of 2-bromopyrimidine (0.5−1.0 mmol) in water (25 mL) and acetone (25 mL) was added to a stirred solution of the respective compound (**14** or **24**) (0.5 mmol) in water (25 mL) at 0−5 °C. Then the temperature was gradually increased to refluxing at 120 °C for 1−3 d. 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 and the residue was purified by flash column chromatography on RP-18 silica gel using a methanol:water eluent to obtain the desired products **42** and **43**. Following the previously described synthesis[22b] was not successful in our hands because it failed to yield the desired product. It is worth mentioning that the yield of **42** was very strongly improved (97%) in comparison to that reported in the literature (47%), while **43** has not been previously described.

Sodium 1-Amino-4-(benzylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (9). According to general procedure A, bromaminic acid (0.2 mmol) and benzylamine (3 equiv, 0.6 mmol) were heated in the microwave oven at 120 °C for 10 min. Analytical data: yield 30%, mp >300 °C, blue powder. ^{1}H NMR: δ 4.67 (s, 2H, 4-NHCH_2), 7.29 (br, 1H, 4′-H), 7.38 (m, 4H, 2′-H, 3′-H, 5′-H, 6′-H), 7.77 (s, 1H, 3-H), 7.80 (m, 2H, 6-H, 7-H), 8.24 (m, 2H, 5-H, 8-H), 10.07 (br, 2H, 1-NH_2), 10.96 (s, 1H, 4-NH). ^{13}C NMR: δ 46.05 (4-NHCH_2), 109.28 (C-9a), 109.42 (C-4a), 121.21 (C-3), 125.90 (C-5), 126.04 (C-8), 127.33 (C-2′, C-4′, C-6′), 128.83 (C-3′, C-5′), 132.69 (C-6, C-7), 134.02 (C-10a), 134.15 (C-8a), 138.92 (C-1′), 143.35 (C-4), 143.66 (C-2), 145.13 (C-1), 181.39 (C-9), 181.80 (C-10). LC-MS (*m*/*z*): 426 [M − Na + NH_4^+]$^+$, 409 [M − Na]$^+$, 407 [M − Na]$^-$. Purity by HPLC-UV (254 nm) ESI-MS: 97%.

Sodium 1-Amino-4-[3-(4,6-dichloro-[1,3,5]triazine-2-ylamino)-5-carboxyphenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (31). According to general procedure B. Analytical data: yield 95%, blue powder, mp >300 °C. ^{1}H NMR: δ 7.56 (td, 1H, H-6′), 7.69 (td, 1H, H-3), 7.85 (m, 2H, H-6, H-7), 7.97 (s, 1H, H-3), 7.95 (d, 1H, H-2′), 8.26 (m, 2H, H-5, H-8), 10.80 (br, 1H, NH-4′), 11.39 (br, 2H, NH_2-1), 11.88 (br, 1H, NH-4). ^{13}C NMR: δ 109.57 (C-9a), 112.68 (C-4a), 117.58 (C-2′), 118.54 (C-5′), 119.11 (C-3), 122.96 (C-6′), 126.21 (C-5, C-8), 132.88 (C-4′), 133.03 (C-6), 133.55 (C-1), 133.62 (C-10a), 134.29 (C-8a), 139.33 (C-1′), 139.77 (C-3′), 140.55 (C-4), 142.77 (C-2), 144.67 (C1), 154.11 (C-1″), 166.66 (C-3″, C-5″), 182.20 (C-9), 183.22 (C-10), 183.30 (COOH). LC-MS (*m*/*z*): 618 [M − Na + NH_4^+]$^+$, 599 [M − Na]$^-$. Purity by HPLC-UV (254 nm) ESI-MS: 95%.

Sodium 1-Amino-4-(4-benzylphenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (35). According to general procedure A, bromaminic acid (0.2 mmol) and 4-benzylaniline (3 equiv, 0.6 mmol) were heated in the microwave oven at 120 °C for 5 min. Analytical data: yield 51%, mp >300 °C, blue powder. ^{1}H NMR: δ 3.97 (s, 2H, CH_2), 7.20, 7.29 (2 m, 6H and 3H, 2′-H, 3′-H, 5′-H, 6′-H, 2″-H, 3″-H, 4″-H, 5″-H, 6″-H), 7.83 (m, 2H, 6-H, 7-H), 7.98 (s, 1H, 3-H), 8.26 (m, 2H, 5-H, 8-H), 12.05 (s, 1H, 4-NH). ^{13}C NMR: δ 40.6 (CH_2), 109.2 (C-9a), 111.2 (C-2′, C-6′), 122.7 (C-4a), 123.4, 126.05, 126.1, 128.6, 128.9, 130.0 (C-3, C-5, C-8, C-4′, C-3′, C-5′, C-4″), 132.9, 133.2, 133.7, 134.3, 137.2, 137.8 (C-8a, C-10a, C-6, C-7, C-2″, C-6″, C-3″, C-5″), 141.3 (C-1′), 143.0 (C-4), 144.4 (C-2, C-1), 181.9 (C-9), 182.4 (C-10). LC-MS (*m*/*z*): 502 [M − Na + NH_4^+]$^+$, 485 [M − Na]$^+$, 483 [M − Na]$^-$. Purity by HPLC-UV (254 nm) ESI-MS: 99.8%.

Sodium 1-Amino-4-(4-phenylthiophenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (37). According to general procedure A, bromaminic acid (0.2 mmol) and 4-phenylthioaniline (3 equiv, 0.6 mmol) were heated in the microwave oven at 120 °C for 10 min. Analytical data: yield 48%, mp >300 °C, blue powder. ^{1}H NMR: δ 7.31 (m, 5H, 2″-H, 3″-H, 4″-H, 5″-H, 6″-H), 7.36 (d, 2H, 2′-H, 6′-H), 7.41 (d, 2H, 3′-H, 5′-H), 7.85 (m, 2H, 6-H, 7-H), 8.05 (s, 1H, 3-H), 8.26 (m, 2H, 5-H, 8-H), 10.1 (br, 2H, 1-NH_2), 11.92 (s, 1H, 4-NH). ^{13}C NMR: δ 109.5 (C-9a), 112.5 (C-4a), 123.1 (C-2′, C-6′), 123.3, 126.1, 126.2 127.1, 128.6, 129.6, 129.9 (C-3, C-5, C-8, C-4′, C-5′, C-3′, C-2″, C-4″, C-6″), 133.0, 133.3, 133.5, 133.6, 134.3, 136.0 (C-8a, C-10a, C-6, C-7, C-3″, C-5″, C-1″), 139.6 (C-1′), 139.7 (C-4), 142.7, (C-2), 144.7 (C-1), 182.1 (C-9), 183.0 (C-10). LC-MS (*m*/*z*): 520 [M − Na + NH_4^+]$^+$, 502 [M − Na]$^+$, 501 [M − Na]$^-$. Purity by HPLC-UV (254 nm) ESI-MS: 99.5%.

Disodium 1-Amino-4-[4-phenoxy-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (38). According to general procedure A, bromaminic acid (0.2 mmol) and 4-phenoxy-3-sulfoaniline (2 equiv, 0.4 mmol) were heated in the microwave oven at 120 °C for 5 min. Analytical data: yield 35%, mp >300 °C, blue powder. ^{1}H NMR: δ 6.84 (d, 1H, 5′-H), 6.98 (d, 2H, 2″-H, 6″-H), 7.06 (dd, 1H, 4″-H), 7.21 (dd, 1H, 6′-H), 7.34 (dd, 2H, 3″-H, 5″-H), 7.63 (d, 1H, 2′-H), 7.84 (m, 2H, 5-H, 8-H), 7.91 (s, 1H, 3-H), 8.27 (m, 2H, 6-H, 7-H), 10.15 (br, 2H, 1-NH_2), 12.07 (br, 1H, 4-NH). ^{13}C NMR: δ 109.22, 111.20, 119.01, 121.31, 122.61, 122.72, 124.26, 125.18, 126.14, 129.63, 132.87, 133.22, 133.80, 134.28, 140.86, 141.60, 143.14, 144.44, 150.71, 158.12, 181.91, 182.47. LC-MS (*m*/*z*): 565 [M − 2Na]$^-$, 282 [M − 2Na]$^{2-}$, 584 [M − 2Na + NH_4^+]$^+$. Purity by HPLC-UV (254 nm) ESI-MS: 99%.

Disodium 1-Amino-4-[4-phenylamino-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (39). According to general procedure A, bromaminic acid (0.2 mmol) and 4-anilino-3-sulfoaniline (2 equiv, 0.4 mmol) were heated in the microwave oven at 120 °C for 5 min. Analytical data: yield 46%, mp >300 °C, blue powder. ^{1}H NMR: δ 6.91 (dd, 1H, 4″-H), 7.12 (m, 3H, 2″-H, 6″-H, 5′-H), 7.30 (m, 3H, 3″-H, 5″-H, 6′-H), 7.50 (b, 1H, 2′-H), 7.83 (s, 1H, 3-H), 7.83 (m, 2H, 5-H, 8-H), 8.28 (m, 2H, 6-H, 7-H), 12.12 (br, 1H, 4-NH). ^{13}C NMR: δ 109.11 (C-9a), 110.42 (C-4a), 115.79 (C-4″), 118.28 (C-2″, C-6″), 120.98 (C-6′), 122.68 (C-3), 124.10 (C-5′), 126.07 (C-2′), 126.13 (C-1″), 129.51 (C-3″, C-5″), 132.78 (C-6), 133.01 (C-7), 133.89 (C-10a), 134.28 (C-8a), 135.31 (C-4′), 137.88 (C-3′), 142.38 (C-1′), 142.76 (C-4), 143.25 (C-2), 144.25 (C-1), 181.75 (C-9), 181.88 (C-10). LC-MS (*m*/*z*): 564 [M − 2Na]$^-$, 281[M − 2Na]$^{2-}$, 583 [M − 2Na + NH_4^+]$^+$. Purity by HPLC-UV (254 nm) ESI-MS: 100%.

1-Amino-2-methyl-4-[4-phenylamino-3-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene Sodium Salt (40). According to general procedure A, 1-amino-4-bromo-2-methylanthraquinone (0.2 mmol) and 4-anilino-3-sulfoaniline (2 equiv, 0.4 mmol) were heated in the microwave oven at 120 °C for 5 min. Analytical data: yield 30%, mp >300 °C, blue powder. ^{1}H NMR: δ 2.25 (s, 3H,

CH_3), 6.91 (dd, 1H, 4″-H), 7.11 (d, 2H, 2″-H, 6″-H), 7.18 (dd, 1H, 6′-H), 7.28 (m, 2H, 3″-H, 5″-H), 7.33 (d, 1H, 5′-H), 7.51 (d, 1H, 2′-H), 7.81 (m, 2H, 5-H, 8-H), 8.27 (m, 2H, 6-H, 7-H), 8.55 (s, 1H, 3-H), 12.27 (br, 1H, 4-NH). ^{13}C NMR: δ 18.77, 107.9, 108.6, 115.9, 118.2, 120.9, 123.9, 124.3, 125.9, 126.1, 126.2, 129.4, 129.5, 132.7, 134.1, 135.2, 137.6, 137.7, 142.4, 143.7, 147.0, 181.2, 181.8. LC-MS (*m*/*z*): 498 [M − Na]$^-$, 500 [M − Na]$^+$. Purity by HPLC-UV (254 nm) ESI-MS: 96%.

Sodium 1-Amino-4-[4-phenylamino-3-carboxyphenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (41). According to general procedure A, 1-amino-4-bromo-2-carboxyanthraquinone (0.2 mmol) and 4-anilino-3-sulfoaniline (2 equiv, 0.4 mmol) were heated in the microwave oven at 120 °C for 5 min. Analytical data: yield 55%, mp >300 °C, blue powder. ^{1}H NMR: δ 6.93 (dd, 1H, 4″-H), 7.13 (d, 2H, 2″-H, 6″-H), 7.21 (d, 1H, 6′-H), 7.31 (dd, 2H, 3″-H, 5″-H), 7.35 (d, 1H, 5′-H), 7.56 (d, 1H, 2′-H), 7.88 (m, 2H, 6-H, 7-H), 8.19 (s, 1H, 3-H), 8.29 (m, 2H, 5-H, 8-H), 11.71 (br, 1H, 4-NH). ^{13}C NMR: δ 110.45 (C-9a), 113.86 (C-4a), 115.91 (C-4″), 118.25 (C-2″, C-6″), 121.00 (C-6′), 122.97 (C-3), 123.69 (C-5′), 125.92 (C-2′), 126.18 (C-1″), 126.29 (C-6), 129.17 (C-7), 129.56 (C-3″, C-5″), 133.19 (C-10a), 133.63 (C-8a), 134.13 (C-4′), 135.25 (C-3′), 137.72 (C-1′), 140.82 (C-4), 142.37 (C-2), 147.24 (C-1), 167.68 (COOH), 182.29 (C-9), 183.13 (C-10). LC-MS (*m*/*z*): 263 [M − Na]$^{2-}$, 527 [M − Na]$^-$, 529 [M − Na]$^+$, 547 [M − Na + NH_4^+]$^+$. Purity by HPLC-UV (254 nm) ESI-MS: 98%.

Sodium 1-Amino-4-[4-([1,3]diazine-2-ylamino)phenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (43). According to the general procedure C, a solution of 160 mg (1 mmol) of 2-bromopyrimidine and 255 mg (0.5 mmol) of 1-amino-4-(4-aminophenylamino)-9,10-dioxo-9,10-dihydroanthracene 2-sulfonic acid sodium salt (**14**) was stirred at 0−5 °C and then the temperature was increased to refluxing at 120 °C for one day. RP-FCC was performed with methanol:water (2:3) as an eluent. Analytical data: yield (295 mg) 90%, blue powder, mp >300 °C. ^{1}H NMR: δ 6.84 (dd, 1H, 4″-H), 7.21 (d, 2H, 6′-H, 2′-H), 7.84 (m, 2H, 6-H, 7-H), 7.96 (s, 1H, 3-H), 8.28 (m, 4H, 5-H, 8-H, 3′-H, 5′-H), 8.48 (d, 2H, 3″-H, 5″-H), 9.72 (br, 1H, 4′-NH), 10.12 (br, 2H, 1-NH2), 12.13 (br, 1H, 4-NH). ^{13}C NMR: δ 109.1 (C-9a), 110,5 (C-4a), 112.5 (C-4″) 120.0 (C-2′, C-6′), 122.8 (C-3), 124.4 (C-3′, C-5′), 126.0 (C-5), 126.2 (C-8), 132.4 (C-1′), 132.8 (C-6), 133.1 (C-7), 133.8 (C-10a), 134.3 (C-8a), 137.9 (C-4′), 142.2 (C-4), 143.1 (C-2), 144.3 (C-1), 158.2 (C-3″, C-5″), 160.1 (C-1″), 181.7 (C-9), 181.9 (C-10). LC-MS (*m*/*z*): 505 [M − 2Na + NH_4^+]$^+$. Purity by HPLC-UV (254 nm) ESI-MS: 95%.

Pharmacology: Human Platelet Membrane Preparation. Membranes were prepared from human outdated platelets from the blood bank. Platelets were obtained from the University of Bonn blood bank 4−5 days after donation. Platelet rich plasma was washed by centrifugation (10 min at 1000*g*) in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 150 mM NaCl. The supernatant was centrifuged twice (48400*g* for 60 min), and the resulting platelet pellets were resuspended in 5 mM Tris-HCl (pH 7.4) containing 5 mM EDTA. The platelets were homogenized, and the final suspension was stored frozen as multiple aliquots at −80 °C until needed. Protein concentrations were determined by the method of Lowry using a Sigma Chemie protein assay kit.

P2Y$_{12}$ Radioligand Binding Assay. The precursor of [^{3}H]PSB-0413 was synthesized as described[6] and custom-labeled by GE Healthcare UK Ltd., Whitchurch, Cardiff, UK,[6] with a specific activity of 74 Ci/mmol, or 94 Ci/mmol, respectively. Radioligand binding assays were performed using 5 nM [^{3}H]PSB-0413 and 100 μg of protein in Tris-HCl buffer 50 mM, pH 7.4, as previously described in a final volume of 400 μL.[6] Competition by 10 μM of test compound was initially determined. The mixture was incubated for 1 h at rt, followed by filtration through GF/B filters. Nonspecific binding was determined with 1 mM ADP. For potent compounds, concentration−inhibition curves were determined using at least 6−7 different concentrations spanning 3 orders of magnitude. At least three independent experiments were performed each in triplicate.

Cell Culture. Cell lines (human 1539 melanoma cells and human 1321N1 astrocytoma cells) were grown as previously described.[38] Cells were cultured in 175 cm^2 cell culture flasks and maintained in an incubator at 37 °C, 5% CO_2, and 95% humidity. Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) was used as a growth medium.

MTT Assays. Cells were detached from the 175 cm^2 culture flask and counted using a Neubauer hemocytometer. Then they were resuspended in the growth medium to give a total of 2×10^5 cells in 10 mL of the medium. Cell suspension (100 μL) was added into each well of a 96-well plate to obtain a final concentration of 2000 cells per well and incubated for 24 h at 37 °C, 5% CO_2, and 95% humidity. The outer wells of the 96-well plate were filled with 200 μL of medium without cells to prevent evaporation. After 24 h, the medium was removed and 180 μL of fresh growth medium per well was added. Stock solutions (10 mM) of test compounds (5-fluorouracil, **12**, and **16**) were prepared in DMSO and diluted with medium to give 10× of final concentrations. Then test compound solution (20 μL) was added to each well. The final DMSO concentration was 1%. Compounds **12** and **16** were tested in concentrations of 0.1, 1, and 100 μM. For 5-fluorouracil, full dose−inhibition curves were determined. The cells were incubated in the presence of the appropriate drug for 72−144 h. Then 40 μL from a freshly made stock solution of MTT in phosphate-buffered saline (5 mg/mL) was added to each well and the cells were incubated for 1 h. After the incubation time, the medium containing MTT was removed and 100 μL of DMSO was added to each well in order to dissolve crystals that were formed. The spectrophotometric absorbance was subsequently measured at 570 nm using a UV-Multiwellreader Multiscan spectrophotometer (Thermo Electron, Dreieich) with a reference filter of 690 nm. The data were analyzed using Microsoft Excel and Graphpad Prism 4. Results were evaluated by comparing the absorbance of the wells containing compound-treated cells with the absorbance of wells containing 1% DMSO without any drug (= 100% viability). All experiments were performed in triplicates in at least 3−8 separate experiments.

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Supporting Information Available: Mechanism of clopidogrel activation, results from cell proliferation studies, results from P2Y$_{12}$ receptor radioligand binding studies of therapeutically used anthraquinone derivatives and related drugs, additional experimental details on the synthesis of anthraquinone derivatives, ^{1}H NMR and ^{13}C NMR spectroscopic data, HPLC-UV analyses and purification procedure by reversed phase flash chromatography. This material is available free of charge via the Internet at http://pubs.acs.org.

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