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Synthesis and biological activity of aminophthalazines and aminopyridazines as novel inhibitors of PGE_2 production in cells

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ABSTRACT

This Letter reports the synthesis and biological evaluation of a collection of aminophthalazines as a novel class of compounds capable of reducing production of PGE_2 in HCA-7 human adenocarcinoma cells. A total of 28 analogs were synthesized, assayed for PGE_2 reduction, and selected active compounds were evaluated for inhibitory activity against COX-2 in a cell free assay. Compound **2xxiv** (R^1 = H, R^2 = *p*-CH_3O) exhibited the most potent activity in cells (EC_{50} = 0.02 μM) and minimal inhibition of COX-2 activity (3% at 5 μM). Furthermore, the anti-tumor activity of analog **2vii** was analyzed in xenograft mouse models exhibiting good anti-cancer activity.

Prostaglandin E_2 (PGE_2) is well known to play a pivotal role in processes associated with inflammation, pain and pyresis and is over expressed in various tumors where chronic inflammation has been linked to the growth of various cancerous tissues. Indeed, PGE_2 has been identified as the major prostaglandin associated with the progression of various tumor malignancies including that of the colon, lung, and breast.[1–5]

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used as analgesics that function by inhibiting the activity of cyclooxygenase (COX) enzymes which are involved in the biosynthesis of a variety of prostaglandins, including PGE_2. At high doses NSAIDs have been shown to reverse the growth of colorectal tumors and patients using NSAIDs over long periods of time for chronic disorders (i.e rheumatoid arthritis) exhibit a lower risk of developing colon cancer.[1–4] It is postulated that the anti-cancer properties elicited by NSAIDs are primarily due to a reduction in PGE_2 levels supported by the observation that exogenous treatment with PGE_2 is shown to impede NSAID induced tumor regression. Historically, side effects with non-selective COX-1/2 inhibitors (Aspirin, Tylenol, Ibuprofen) driven by COX-1 mediated gastrointestinal intolerance, promoted efforts to develop specific COX-2 inhibitors (Celebrex®, Bextra® and Vioxx®). However, the latter two drugs were subsequently withdrawn from the market due to a drug induced imbalance of prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) leading to cases of myocardial infarction and thrombosis.[1–4] Thus the search for colon cancer modifying therapeutics is now geared towards the development of PGE_2 reducing drugs that do not alter COX activity.[6–11]

In search of novel anti-tumor small molecules with the capacity to reduce cellular levels of PGE_2 without affecting in vitro COX-2 activity, our group has recently reported a series of 2-aminothiazoles with interesting biological profiles (Fig. 1).[11] In particular, **1a** exhibited the most potent cellular PGE_2 reducing activity of the entire series (EC_{50} 90 nM), with only a nominal COX-2 inhibition [IC_{50} >5 μM]. Furthermore, analog **1b** exhibited promising anti-cancer activity in mouse xenograft models.[11] In continuation of our studies, we herein report the biological evaluation of a novel series of aminophthalazines in Table 1 analogs **2** with PGE_2 reducing character in HCA-7 colon cancer cells, Figure 1.

The aminophthalazines Table 1 (**2i**–**xxiv**), were assembled using methodology depicted in Scheme 1.[12,13] Briefly, 1,4-dichlorophthalazine **3** was treated at reflux with an equivalent amount of aniline **4** affording intermediates **5** in good yield. Compounds **5** smoothly underwent Suzuki-coupling with select boronic acids enabling formation of final aminophthalazine analogs **2i**–**xxiv** in moderate yields. Analogs **6i**–**ii** were obtained via standard N-methylation methodology from **2vi** and **2vii** respectively. Pyridazines **9i**–**ii** were prepared from commercially available 1,4-dichloropyridazine **7** and *p*-toluidine **4** in a similar fashion to the

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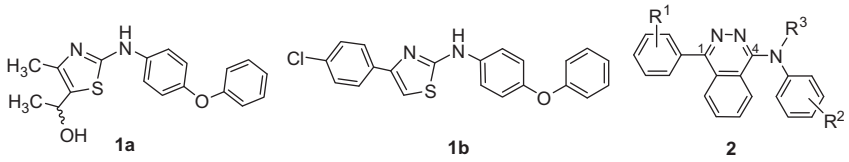
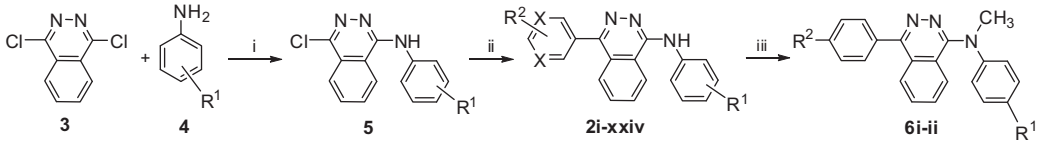
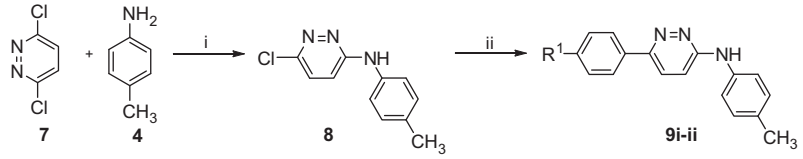


Figure 1. Structures of 2-aminothiazoles **1a**–**b** and general structure of aminophthalazines **2**.



Scheme 1. Synthesis of aminophthalazines **2i**–**xxiv** and **6i**–**ii** (R^1, R^2 and X vary, see Table 1).[13] Reagents and conditions: (i) (a) EtOH, reflux, 0.5 h, (b) NaOH (aq); (ii) Boronic acid (2 equiv), K_2CO_3 (2 equiv), Bis(triphenylphosphine)palladium(II) dichloride (0.05 equiv), dioxane–H_2O (4:1), microwave irradiation, 100 °C, 1 h; (iii) NaH (1.5 equiv), MeI (1.5 equiv), DMF, 0 °C to rt.



Scheme 2. Synthesis of aminopyridazines **9i**–**ii**. Reagents and conditions: (i) (a) EtOH, reflux, 0.5 h, (b) NaOH (aq); (ii) Boronic acid (2 equiv), K_2CO_3 (2 equiv), Bis(triphenylphosphine)palladium(II) dichloride (0.05 equiv), dioxane–H_2O (4:1), microwave irradiation, 100 °C, 1 h.

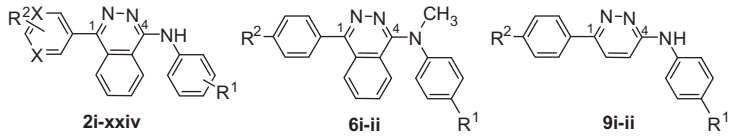
aminophthalazines in Scheme 2. Final compounds exhibited purities >95%, as judged by LC/MS (254 and 214 nm) and evaporative light scattering (ELS).

All compounds were screened for their ability to reduce PGE_2 production in HCA-7 colon cancer cells at 1 μM concentration and activities are summarized as percentage reduction of PGE_2 levels Table 1.[14] Compounds that exhibited reduction of PGE_2 levels higher than 70% were tested for COX-2 inhibition at 5 μM in an in vitro cell free assay, with Celecoxib incorporated as a positive control in both PGE_2 and COX-2 assays.[15] IC_{50} values for COX-2 inhibition were not determined, as no inhibitory activity against COX-2 >50% was observed. Compounds exhibiting >70% reduction of PGE_2 levels, and <50% COX-2 inhibition were pushed forward for EC_{50} determinations of PGE_2 reducing level ability.

Aminophthalazines **2i**–**v**, characterized by various polar substituents at both the C-1 phenyl (R^2) and C-4 aniline (R^1) rings, generally exhibited poor reduction of cellular PGE_2 levels (0–39%), possibly due to a reduced ability to permeate the cellular membrane. Replacement with R^1 = *p*-CH_3 (**2vi**–**xvii**) resulted in good to high levels of inhibition of cellular PGE_2 levels (59–99%), the only exception being **2x** (R^2 = H, X = N, 15%), characterized by a polar pyrimidine ring instead of the phenyl ring. In this group of analogs, the activity was influenced by the nature and position of the substituent R^2 on the C-1 phenyl ring. In detail, when this ring was unsubstituted (**2vi**), a strong reduction of PGE_2 levels was observed (99%; EC_{50} 0.031 μM). Surprisingly, a *para*-methoxy substituent (R^2) led to the same level of inhibitory activity as **2vi** (**2vii**, 98%; EC_{50} 0.032 μM). Movement of the methoxy group R^2 to the *meta* and *ortho* positions resulted in a reduction of activity (**2viii** and **2ix**, 62 and 79%, respectively; **2ix** EC_{50} 0.59 μM). Similar to what was observed for analog **2vii**, a dimethylamine substituent at the *para* position of the C-1 phenyl ring (R^2) led to potent reduction of PGE_2 levels (**2xi**, 94%; EC_{50} 0.42 μM), albeit 10-fold less than **2vii**. Enlargement of the *para*-methoxy (R^2) to ethoxy (**2xii**) or isopropoxy (**2xiii**) resulted in partial loss of activity (75 and 98%; EC_{50} 0.57 and 0.70 μM, respectively). A consistent reduction in activity was also observed when a bulkier 3,4-methylenedioxy substituent was added to the phenyl ring R^2 (**2xiv**, 59%).

Introduction of a bulky hydrophobic substituent at the *meta* position of the C-1 phenyl ring (R^2), including chloro (**2xv**, 75%; EC_{50} 0.86 μM) or phenyl (**2xvii**, 85%; EC_{50} 0.54 μM) led to moderate activity, whilst movement of a chlorine atom from the *meta* to the *ortho* position (**2xvi**) improved activity (97%; EC_{50} 0.38 μM). This result may be explained by restricted rotation of the phenyl ring and associated lowering of entropic barriers to binding, resulting from bulky substituent in the *ortho* position. Elongation of the *para* methyl group on the C-4 of the phenyl ring (R^1) to ethyl (**2xviii**, 85%; EC_{50} 0.93 μM) resulted in slightly reduced levels of activity when compared to **2vii** (R^2 *p*-CH_3O; EC_{50} 0.032 μM). Movement of the *para* methyl group on the C-4 aniline ring to the *meta* position led to a decrease in the observed activity (R^1, **2xix** and **2xx**, 74 and 87%; EC_{50} 0.76 and 0.64 μM, respectively), compared with the activity previously observed for **2vi**–**vii** (99 and 98%; EC_{50} 0.031 and 0.032 μM). Replacement of the *meta* methyl R^1 with a bioisosteric chlorine (**2xxi**, 79%; EC_{50} 0.5 μM) resulted in a similar level of activity to **2xix**. Introduction of a *para* phenoxy substituent on the C-1 phenyl ring (R^2, **2xxii**–**xxiii**) maintained strong reduction of PGE_2 cellular levels, surprisingly only when the C-4 anilino ring (R^1) was substituted with a *para* phenoxy group (92%; EC_{50} = 0.09 μM). Interestingly, removal of the methoxy group from the aniline ring (R^1 = H, R^2 = *p*-CH_3O) resulted in slight increase of cellular activity (**2xxiv**, 97%; EC_{50} = 0.02 μM), delivering the most functionally potent compound observed in cells.

Table 1
Biological activity of aminophthalazines analogs **2i–xxiv**, **6i–ii** and pyridazines **9i–ii**[abcdef]



Compd	R^1	R^2	X	PGE_2 (%)[d]	COX-2 (%)[e]	PGE_2 EC_{50}/μM[f]
2i	*p*-CH_3O	*p*-$C(O)NH(CH_2)_2OH$	C	39.2 ± 13	—	—
2ii	*p*-OH	H	C	26.7 ± 5	—	—
2iii	*p*-SO_2NH_2	H	C	NR	—	—
2iv	*p*-COOEt	H	C	18.4 ± 3	—	—
2v	*p*-$OCH_2C(O)NH_2$	*p*-CH_3O	C	NR	—	—
2vi	*p*-CH_3	H	C	98.9 ± 1	23.0 ± 1	0.031 ± 0.07
2vii	*p*-CH_3	*p*-CH_3O	C	98.3 ± 3	NR	0.032 ± 0.02
2viii	*p*-CH_3	*m*-CH_3O	C	62.0 ± 8	—	—
2ix	*p*-CH_3	*o*-CH_3O	C	77.2 ± 0	NR	0.59 ± 0.04
2x	*p*-CH_3	H	N	15.1 ± 4	—	—
2xi	*p*-CH_3	*p*-$N(CH_3)_2$	C	94.4 ± 1	14.2 ± 1	0.42 ± 0.002
2xii	*p*-CH_3	*p*-OEt	C	75.3 ± 9	NR	0.57 ± 0.06
2xiii	*p*-CH_3	*p*-$OCH(CH_3)_2$	C	76.7 ± 3	NR	0.70 ± 0.25
2xiv	*p*-CH_3	3,4-OCH_2O-	C	59.0 ± 8	—	—
2xv	*p*-CH_3	*m*-Cl	C	75.2 ± 8	NR	0.86 ± 0.10
2xvi	*p*-CH_3	*o*-Cl	C	97.2 ± ± 1	NR	0.38 ± 0.01
2xvii	*p*-CH_3	*m*-Ph	C	84.2 ± 1	NR	0.54 ± 0.05
2xviii	*p*-Et	*p*-CH_3O	C	85.0 ± 11	NR	0.93 ± 0.01
2xix	*m*-CH_3	H	C	74.1 ± 6	NR	0.76 ± 0.30
2xx	*m*-CH_3	*p*-CH_3O	C	87.5 ± 5	NR	0.64 ± 0.34
2xxi	*m*-Cl	H	C	79.4 ± 9	10.1 ± 7	0.50 ± 0.4
2xxii	*p*-PhO	H	C	71.2 ± 15	NR	—
2xxiii	*p*-PhO	*p*-CH_3O	C	92.6 ± 5	NR	0.09 ± 0.05
2xxiv	H	*p*-CH_3O	C	97.3 ± 1	3.0 ± 10	0.02 ± 0.01
6i	CH_3	CH_3O	—	87.0 ± 2	NR	0.05 ± 0.02
6ii	CH_3O	H	—	92.4 ± 1	21.4 ± 27	0.220 ± 0.006
9i	CH_3	H	—	62.5 ± 1	—	—
9ii	CH_3	CH_3O	—	49.0 ± 4	—	—

[a] Previously described inhibitor of cellular PGE_2 levels, **1b**, was used as a positive control (PGE_2 10.9 ± 4.5 and EC_{50} = 0.33 ± 0.09 μM).[11]
[b] NR, no observed reduction.
[c] — Not determined.
[d] % of inhibition of PGE_2 levels in HCA-7 cells at 1 μM concentration ± SD (n = 3).
[e] % of inhibition of COX-2 levels in vitro at 5 μM concentration ± SD (n = 3).
[f] EC_{50} for PGE_2 level reduction in HCA-7 cells ± SD (n = 3).

N-Methylation of **2vi–vii** to afford **6i** and **6ii** respectively, interestingly only slightly impaired the capacity to reduce cellular PGE_2 levels (87 and 92%; EC_{50} 0.05 and 0.220 μM) when compared to structurally related analogs **2vi–vii**. Finally, replacement of the phthalazine scaffold with a pyridazine ring resulted in reduced level of activity (62 and 49%, **9i** and **9ii**, respectively) and no further efforts were pursued with this heterocycle.

Remarkably for this class of compounds, the most functionally active compounds in cells exhibited only negligible inhibition of COX-2 activity in vitro at 5 μM, with % inhibition comprised between 0 and 30%. As such, no dose response curves for COX-2 were determined.

In conclusion, we have prepared and evaluated twenty-six aminophthalazine and two pyridazine analogs for their capacity to reduce cellular levels of PGE_2 in HCA-7 cells. The in vitro inhibitory activity against COX-2 was also determined, leading to the identification of potent inhibitors of PGE_2 levels with negligible activity against COX-2. Compounds **2vi–vii**, **xvi** and **xxiv** exhibited the highest reduction of PGE_2 levels, with percentage of PGE_2 reduction between 97.2 and 98.9% and EC_{50} values comprised between 0.038 and 0.02 μM respectively. Furthermore, **2vii** was tested for its effect on tumor growth in mouse xenograft models expressing HCA-7 cells and was confirmed to have good anti-cancer activity with tumor versus control value (T/C) of 34% for HCA-7 human colonic adenocarcinoma cell lines, at a dosing schedule of 100 mg/kg ip over 10 days. Work is now on going to elucidate the mode of action of these molecules with tagged probes. Future synthetic efforts will focus on further development of new scaffolds with improved physicochemical properties to further develop established SAR and promote discovery of new candidates for in vivo evaluation in mouse xenograft models.

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13. *General procedure for the preparation of analog* ***2xxiv***: 1,4-Dichlorophthalazine (**3**, 1.50 g, 7.57 mmol, 1 equiv) and *para*-anisidine (**4**, 940 mg, 7.57 mmol, 1 equiv) were mixed together in ethanol (10 mL) and the reaction stirred at reflux for 0.5 h. After cooling down to room temperature, aqueous NaOH (aq) was added at once and the product **5** precipitated as a white microcrystalline solid which was isolated by suction filtration and used in the next reaction without further purification (1.72 g, 6.05 mmol, 80%). **5** (200 mg, 0.70 mmol, 1 equiv) was dissolved in dioxane-H_2O (4:1, 5 mL) and phenylboronic acid (171 mg, 1.40 mmol, 2 equiv), Bis(triphenylphosphine)palladium(II) dichloride (25 mg, 0.03 mmol, 0.05 equiv) and K_2CO_3 (193 mg, 1.39 mmol, 2 equiv) were added. The reaction was subjected to microwave irradiation for 1 h at 100 °C. After filtering through celite, H_2O (50 mL) was added and the aqueous layer extracted with DCM (3 × 50 mL). The organic layers were collected, washed with brine (50 mL), dried ($MgSO_4$) and the solvent removed under reduced pressure. The final product **2xxiv** was purified by silica-gel column chromatography (hexane/EtOAc, 0–50%) using an ISCOTM system and obtained as a yellow microcrystalline powder (50 mg, 0.33 mmol, 48%). Mp: 210–213 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.11 (br s, 1H), 8.61 (d, J = 8.3 Hz, 1H), 7.97 (ddd, J = 8.3, 6.8, 1.5 Hz, 1H), 7.89 (ddd, J = 8.0, 6.8, 1.1 Hz, 1H), 7.86–7.77 (m, 3H), 7.66–7.60 (m, 2H), 7.58–7.47 (m, 3H), 6.98–6.92 (m, 2H), 3.75 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.4, 153.3, 152.4, 137.4, 134.0, 132.4, 131.9, 130.1, 128.9, 128.8, 126.1, 126.1, 123.6, 123.0, 118.7, 114.1, 55.7. LC–MS $[MH]^+$ 328.00. Purity (LC–MS): >99% (ELSD-LT); >99% (254 nm); 90% (214 nm).
14. *PGE_2 production assay*: Cells were seeded in 6-well plates and incubated overnight in DMEM/10% FBS. They were serum starved for the next 18 h. Cells were then treated with 10 ng/ml IL-1β and increasing concentration of compounds (dissolved in DMSO) in 1 mL serum-free medium. After 72 h of incubation, the supernatants were collected for PGE_2 level detection using the PGE_2 EIA kit (R&D Systems).
15. *COX-2 cell-free assay*: COX-2 activity was measured by a COX Fluorescent inhibitor screen assay kit following the manufacturer's instructions (Cayman Chemical, http://www.caymanchem.com).