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Effect of Catechol Derivatives on Cell Growth and Lipoxygenase Activity

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Dedicated to Professor David Crout to mark the occastion of his retirement.

Abstract—Derivatives of salicylic acid have been synthesized as potential lipoxygenase inhibitors. Agents containing a phenolic dihydroxy moiety showed potent ($IC_{50}10^{-6}$–10^{-7} M) inhibition of the growth of murine colonic tumour cells in vitro, and were effective inhibitors of 5-, 12- and 15-lipoxygenase in intact cells. The catechols were also potent inhibitors of rabbit reticulocyte 15-lipoxygenase (IC_{50} ~1 μM).

Lipoxygenase metabolites of arachidonic and linoleic acids play an important role in tumourigenesis,[1] cellular proliferation,[2] and metastasis of tumour cells.[3] The lipoxygenases (LOX) constitute a family of non-haeme iron containing dioxygenases, which catalyse regio and stereospecific reactions between oxygen and the polyunsaturated fatty acid to form hydroperoxides, which can undergo reduction to form hydroxyeicosatetraenoic acids (HETE). Three regio isomers of 5-, 12- and 15-HETE are produced from arachidonic acid, via the corresponding 5-, 12- and 15-LOX. 12(*S*)-HETE has been shown to augment the invasiveness of rat prostatic tumour cells,[4] and appears to mediate tumour-induced endothelial cell retraction, important in the exodus of tumour cells from the circulation into the target organ parenchyma.[5] Endogenously synthesized 12(*S*)-HETE may mediate epidermal growth factor/insulin-stimulated DNA synthesis,[6] while both 12(*S*)- and 15(*S*)-HETE suppress apoptosis in tumour cells,[7] and pharmacological inhibition of both 5- and 12-LOX pathways induces apoptosis in pancreatic cancer cell lines.[8] Inhibition of tumour cell growth by LOX inhibitors may result from an imbalance of metabolism of arachidonic acid between the 5-, 12- and 15-LOX pathways.[9] Our own studies have concentrated on the LOX inhibitor 2,3,5-trimethyl-6-(3-pyridylmethyl) 1,4-benzoquinone (CV-6504), which is capable of inducing profound suppression of the growth of the chemoresistant cachexia-inducing MAC16 tumour[10] by inhibition of the 12- and 15-LOX pathways.[11] However, this agent is subject to rapid metabolism and plasma clearance[12] making it difficult to maintain effective therapeutic levels. This suggests the development of alternative LOX inhibitors. Whereas there is a range of 5-LOX inhibitors there are few inhibitors of 12- and 15-LOX. Modification of the structure of caffeic acid, a 5-LOX inhibitor, produced more selective 12-LOX inhibitors.[13] Catechol derivatives of salicylic acid were found to be potent 15-LOX inhibitors.[14] In this study we have examined further catechol derivatives in an effort to produce more specific 15-LOX inhibitors.

Tait et al.[14] reported the rabbit reticulocyte 15-lipoxygenase inhibitory activity for a series of catechols of the general structure shown in Figure 1. The catechol unit was linked to a salicylamide derivative via a variable length methylene chain.

The best inhibitory activity (IC_{50} = 0.3 μM) was observed for a two-carbon chain and all heteroatoms unmasked. Methylation of the catechol oxygens reduced the inhibitory activity by two orders of magnitude. Removal or modification of the salicylic acid hydroxyl group slightly decreased the 15-lipoxygenase inhibitory

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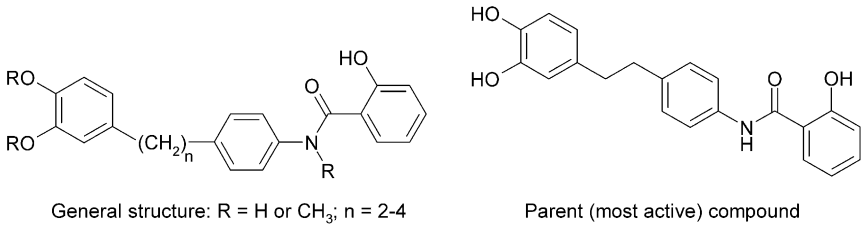
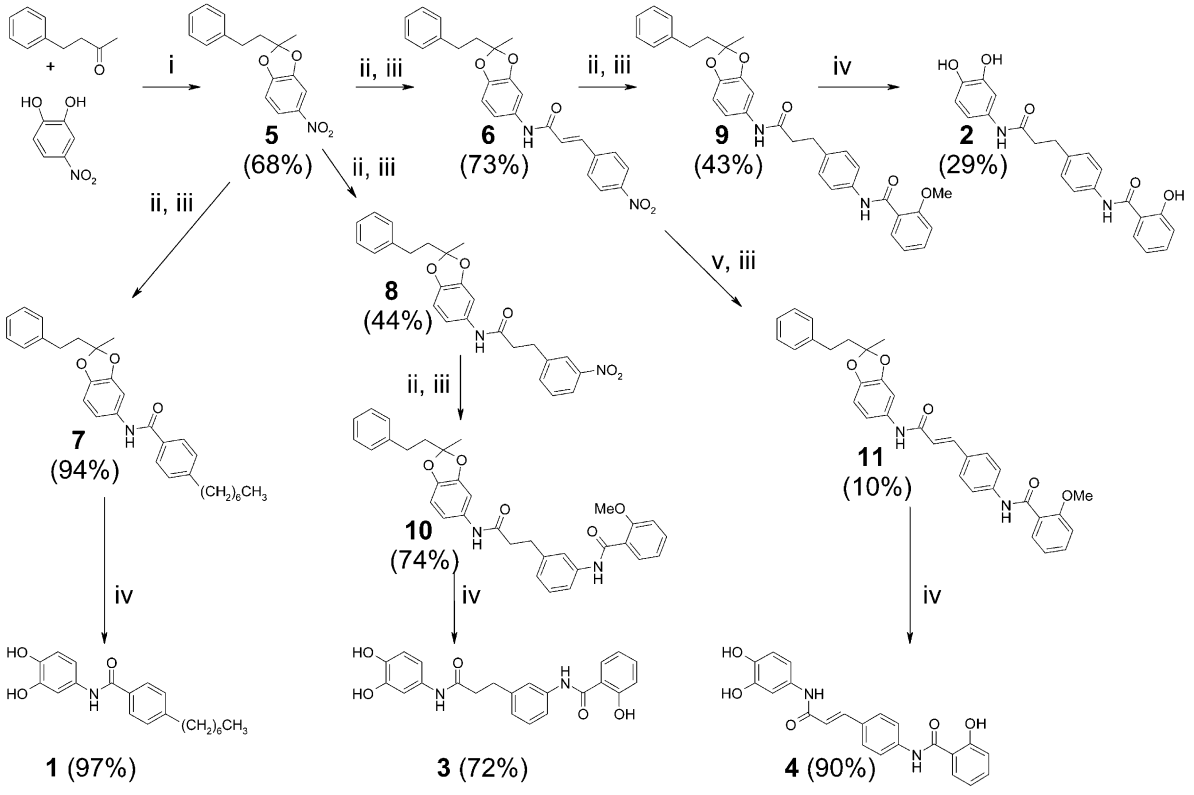


Figure 1.

activity whereas methylation of the amide nitrogen significantly reduced the inhibitory activity. The length of the chain connecting the catechol and the aromatic ring was also investigated and found to have no significant effect on inhibitory activity when varied between two to four carbons in length, there being a marginal advantage with the two-carbon chain.

In this paper, we report the synthesis and lipoxygenase inhibitory activity of a set of analogous compounds wherein functionality has been added to the methylene chain in the form of a carboxamide, a urea, a thiourea or a secondary amine. The first three variations have the advantage of reducing the conformational flexibility of the molecules relative to those containing an unsubstituted methylene chain. It was hoped that this would entropically enhance their binding to the lipoxygenase enzyme.

The synthetic sequence for the preparation of analogues **1**–**4** is given in Scheme 1. The starting point was the protection of 4-nitrocatechol as the racemic phenethyl methyl ketal **5**. This not only masked the hydroxyl functions but also imparted the necessary solubility to what would otherwise have been poorly-soluble intermediate compounds. The nitro group was hydrogenated to reveal the amino function which was acylated in situ under argon to give the carboxamides **6**–**8** in good yield. The inert atmosphere was necessary since in the presence of oxygen very poor yields of these carboxamides were obtained. In the case of **6** and **8**, a further nitro group was available for reduction and acylation. These were reduced as before and coupled with 2-methoxybenzoyl chloride to give the fully-protected compounds **9** and **10**. In the former case the hydrogenation step concomitantly produced a saturated two-carbon linkage. In order to explore the effect of unstauration here, the nitro group of compound **6** was reduced selectively using sodium dithionite and coupled with 2-methoxybenzoyl chloride, whilst leaving the exocyclic double bond intact to give compound **11**. In the final step compounds **7**, **9**, **10** and **11** were deprotected by means



Scheme 1. Reagents and conditions: (i) *p*-TsOH/toluene/refux; (ii) 10% Pd-C/THF/H_2; (iii) RCOCl/Et_3N/DMF or THF/argon; (iv) BBr_3/CH_2Cl_2/reflux; (v) sodium dithionite/EtOH (aq)/reflux or $SnCl_2$/DMF/60 °C.

of boron tribromide in dichloromethane at reflux to give the catechols **1**, **2**, **3** and **4** respectively.

A set of compounds was also prepared wherein the chain connecting the catechol and the aromatic ring was foreshortened to comprise simply an amide (**12**), a urea (**13**), a thiourea (**14**) or a methylene amino group (**15**) (Scheme 2). To that end the nitro group in compound **5** was reduced to reveal an amino function which was acylated to give the protected intermediate compounds; the amide (**16**), the urea (**17**) and the thiourea (**18**). Each of these contained a nitro group which was sequentially reduced and coupled with 2-methoxybenzoyl chloride to give the next stage of protected compounds **19**, **20** and **21** respectively. Finally, the catechols were revealed by boron tribromide-mediated cleavage of the ketal and methoxy functions. The secondary amino compound **15** was prepared by reduction of the nitro group of **16** to amine **22** followed by reductive amination by means of 2-hydroxybenzaldehyde and tetramethylammonium triacetoxyborohydride[15] to give the intermediate amine **23** which was deprotected under strongly acidic conditions to give the target catechol **14**.

Table 1. IC_{50} of compounds against the MAC13 and MAC16 cell lines[a]

Compd	IC_{50} (M)	IC_{50} (M)
	MAC13	MAC16
1	$9.0\pm0.2\times10^{-7}$	$1.0\pm0.3\times10^{-6}$
3	$3.3\pm1.2\times10^{-7}$	$2.6\pm0.0\times10^{-6}$
4	$3.0\pm1.0\times10^{-6}$	$3.0\pm0.3\times10^{-4}$
9	$>5\times10^{-5}$	$>5\times10^{-5}$
CV6504	$6.3\pm0.5\times10^{-6}$	$4.75\pm0.6\times10^{-6}$

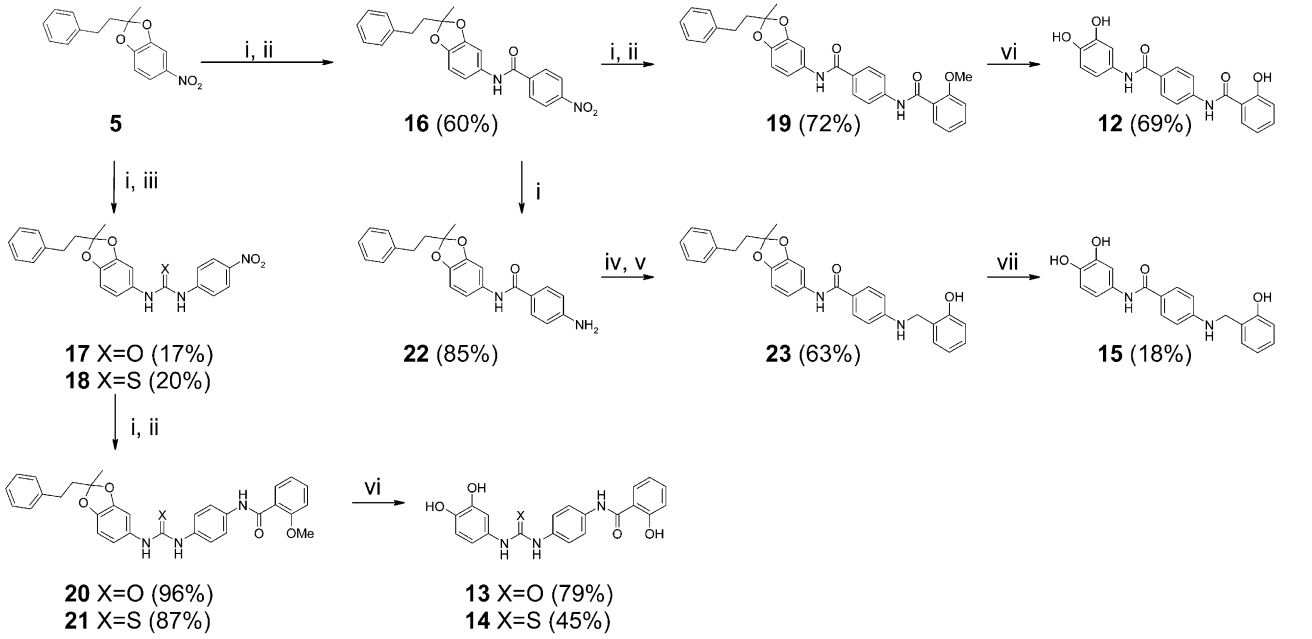
[a]Results are expressed as mean ± SD for between three and five repeats for each individual compound.

Four diverse analogues from the set in hand (compounds **1**, **3**, **4** and **9**) were screened against the MAC16 and MAC13 cell lines.[16] The IC_{50} values in comparison with that for CV-6504 are shown in Table 1. CV-6504 was equally effective against the two cell lines, while the catechol derivatives were more effective against the MAC13 cell line. Highest potency was shown by the compounds **1** and **3**, while **9**, lacking the phenolic dihydroxy moiety, showed no inhibition of either cell line at concentrations less than 5×10^{-5} M. Previous studies[10,11] have established that while the MAC16 cell line is refractory to most cytotoxic agents, it is particularly sensitive to LOX inhibitors. This suggests that the catechol derivatives act as LOX inhibitors, and this was confirmed by in vitro studies[17] on rabbit reticulocyte 15-LOX, which showed the catechols to be effective inhibitors with an IC_{50} ~1 μM (Table 2).

The results and the general trends of the inhibition of rabbit reticulocyte 15-lipoxygenase activity we observed for compounds **2**–**4** and **12**–**15** (Table 2) are in line with those found by Tait et al.[14] for related compounds. All

Table 2. Inhibitory activity of catechol analogues towards 15-lipoxygenase

Compd	% Inhibition of rabbit reticulocyte 15-LOX at 30 μM	IC_{50} for rabbit reticulocyte 15-LOX (μM)
2	99	1.45
3	102	1.67
4	102	1.47
12	100	1.27
13	104	1.51
14	103	1.95
15	103	1.01
CV-6504	3	—



Scheme 2. Reagents and conditions: (i) 10% Pd-C/THF/H_2; (ii) ArCOCl/Et_3N/DMF/argon; (iii) 4-NO_2PhNCO or 4-NO_2PhNCS; (iv) 2-OH-PhCHO/THF/4Å molecular sieves/reflux; (v) $Me_4NBH(OAc)_3$/THF/reflux; (vi) BBr_3/CH_2Cl_2/reflux; (vii) concd HCl (aq)/THF/reflux.

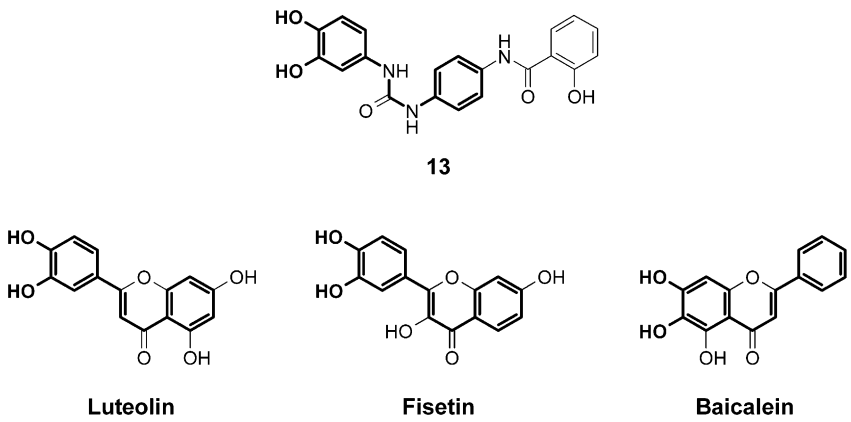


Figure 2. Compound **13** compared with luteolin, fisetin and baicalein (common features emphasised).

Table 3. Effect of test compounds on the metabolism of arachidonic acid through the 5-, 12- and 15-LOX pathways

Compd	Eicosanoid production (pmole/2 h)		
	5-HETE	12-HETE	15-HETE
None (control)	33.5	33.6	67.5
CV6504	13.3	15.0	22.2
3	4.7	7.8	5.3
9	91.5	78.0	110.0

the compounds were potent inhibitors of the enzyme over a relatively narrow range, IC_{50} 1–2 μM. The activity appears to be insensitive to the length of the chain connecting the catechol to the rest of the molecule and even to the functionality within the chain (amide, α,β-unsaturated amide, urea and thiourea). In addition, the precise location of the salicylamide connection to the central aromatic ring appears not to matter (compare **2**, a 1,4-connection and **3**, a 1,3-connection). This tolerance in the non-catechol part of the molecules invites comparison between the catechols in this report and other 15-LOX inhibiting catechols from different classes. For example, a recent study by Sadik et al.[18] provides the 15-LOX inhibitory structure–activity data for a set of flavonoid compounds wherein the best inhibitory activity is found in cases where an aromatic 1,2-dihydroxy system is present. The most potent was luteolin (IC_{50} 0.6 μM) followed by baicalein (1 μM) and fisetin (1.5 μM). See Figure 2 for a comparison of the features common to luteolin, fisetin, baicalein and compound **13**. In contrast, flavone, a non-catechol, was some 300-fold less active.

In order to measure the effect of our compounds on the flux of arachidonic acid through the LOX pathways, cells were pulsed with [^{3}H] arachidonic acid[19] for 2 h after 24-h incubation with 50 μM CV-6504, **3** and **9**. Formation of 5-, 12- and 15-HETE during this time interval is shown in Table 3. No effect upon cell survival or growth was observed for **3** or **9** on the timescale of the experiment. Both **3** and CV-6054 effectively inhibited 5-, 12- and 15-HETE formation. In contrast, **9** promoted their formation by a mechanism as yet unknown. All that can be said at this point is that **9** differs from **3** in that the catechol function and the terminal phenol are both blocked in the former but exposed in the latter. Further work is required to elucidate the mechanism of this effect. Compound **3** was a more effective inhibitor of all three LOX pathways than CV-6504, and it did not appear to have any specificity for a particular pathway. Thus the effect of these agents on the LOX pathways mirrors their effect on cell-growth inhibition. As in previous studies the catechol moiety appeared to be essential for inhibition of LOX.

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16. MAC16 and MAC13 cell lines were derived from the solid tumours and maintained in vitro in RPMI-1640 medium supplemented with either 5% (MAC16) or 10% (MAC13) foetal calf serum at 37 °C under an atmosphere of 5% CO_2 in air. For cell growth assays cells were seeded at either 0.5 (MAC13) or 2.0×10^4 cells per well (MAC16) in 1 mL of growth medium and left for 24 h before drug addition. All drugs were dissolved in DMSO and diluted in culture medium such that the final concentration of DMSO did not exceed 1%. Control wells contained solvent alone. Cell numbers were determined 72 h after drug addition using a Coulter Counter model ZM.
17. Conducted by Panlabs Pharmacology Services, Taiwan. The test compounds were screened initially at a concentration of 30 μM. All the compounds at this concentration gave an inhibition of $\geq$99% apart from the control compound CV-6504. The active compounds were then subjected to a five-point IC_{50} determination ($n=2$) on rabbit reticulocyte 15-lipoxygenase.
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19. MAC16 cells (5×10^6) were incubated with the drugs (5×10^{-5} M) for 24 h, after which they were centrifuged (300*g* for 5 min), washed with phosphate-buffered saline (PBS) and resuspended in fresh RPMI 1640 medium (100 mL) containing 20 μCi of [^{3}H] arachidonic acid (specific activity 206.6 Ci $mmol^{-1}$) together with unlabelled arachidonic acid to give a final concentration of 10 μM. After 2 h at 37 °C, the cells were separated from medium by low speed centrifugation, washed twice with ice-cold PBS, resuspended in 0.5 mL ice-cold PBS, and sonicated for 3×15 s on ice. The pH was adjusted to 3.5 with 1N HCl and was resuspended in $CHCl_3/CH_3OH$ (1:2, v/v). The mixture was vortex mixed for 1 min and left for 30 min at room temperature. Chloroform (1 mL) was added and then vortexed for 10 s, followed by the addition of 0.001 N HCl, and by further vortexing for 10 s. The layers were separated after centrifugation at 2000*g* for 10 min, and the aqueous phase was extracted twice with chloroform (2 mL). The combined chloroform extracts were evaporated under a stream of nitrogen, and the residue was dissolved in CH_3CN (0.1 mL). Cell lipids were fractionated by reverse phase high-performance liquid chromatography with a Waters μBondapak C_{18} column (3.9×300 mm) by an isocratic elution at 1.5 mL min^{-1} with 58% acetonitrile/water/acetic acid (20:100:0.05 v/v) and 42% acetonitrile:acetic acid (100:0.05 v/v)[11]. Radioactivity and UV absorbance at 237 nm were monitored. Peaks were identified based on the retention times of authentic standards.