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Novel di-*tertiary*-butyl phenylhydrazones as dual cyclooxygenase-2/5-lipoxygenase inhibitors: Synthesis, COX/LOX inhibition, molecular modeling, and insights into their cytotoxicities

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ABSTRACT

Although dual inhibition of Cyclooxygenase-2 (COX-2) and 5-Lipoxygenase (5-LOX) enzymes is highly effective than targeting COX or LOX alone, there are only a few reports of examining such compounds in case of colorectal cancers (CRC). In the present work we report that the novel di-*tert*-butyl phenol-based dual inhibitors **DTPSAL, DTPBHZ, DTPINH, and DTPNHZ** exhibit significant cytotoxicity against human CRC cell lines. Molecular docking studies revealed a good fit of these compounds in the COX-2 and 5-LOX protein cavities. The inhibitors show significant inhibition of COX-2 and 5-LOX activities and are effective against a panel of human colon cancer cell lines including HCA-7, HT-29, SW480 and intestinal Apc10.1 cells as well as the hyaluronan synthase-2 (Has2) enzyme over-expressing colon cancer cells, through inhibition of the Hyaluronan/CD44v6 cell survival pathway. Western blot analysis and qRT-PCR analyses indicated that the di-*tert*-butyl phenol-based dual inhibitors reduce the expression of COX-2, 5-LOX, and CD44v6 in human colon cancer HCA-7 cells, while the combination of CD44v6shRNA and **DTPSAL** has an additional inhibitory effect on CD44v6 mRNA expression. The synergistic inhibitory effect of Celecoxib and Licofelone on CD44v6 mRNA expression suggests that the present dual inhibitors down-regulate cyclooxygenase and lipoxygenase enzymes through CD44v6. The compounds also exhibited enhanced antiproliferative potency compared to standard dual COX/LOX inhibitor, viz. Licofelone. Importantly, the HA/CD44v6 antagonist CD44v6shRNA in combination with synthetic compounds had a sensitizing effect on the cancer cells which enhanced their antiproliferative potency, a finding which is crucial for the *anti*-proliferative potency of the novel synthetic di-*tert*-butyl phenol based dual COX–LOX inhibitors in colon cancer cells.

Amongst all cancers known to affect mankind, colorectal cancer (CRC) is the third most cause of cancer deaths with an estimated 103,107 new cases and 51,690 deaths in 2012 in USA.[1] CRC is comparatively less prevalent in Asia, Africa and parts of South America. Although there have been some improvements in screening techniques and early detection, the overall rate of mortalities due to CRC still remains high. Presently, primary and metastatic CRC are treated effectively only with surgical procedure.[2] Clinical results show that combination therapy of 5-FU with irinotecan and oxaliplatin can only prolong the survival time of CRC patients.[3] Hence, it is essential to identify novel therapeutic lead molecules for the treatment of CRC.

The strong association existing between sporadic or chronic inflammation of bowels and CRC offers a basis for evolving new therapeutic agents for this type of cancer. Increased levels of cyclooxygenase (COX) and lipoxygenase (LOX) family of enzymes that are involved in the conversion of arachidonic acid to prostaglandins or leukotrienes have been shown to have implications in patients with familial adenomatous polyposis[4,5] and in

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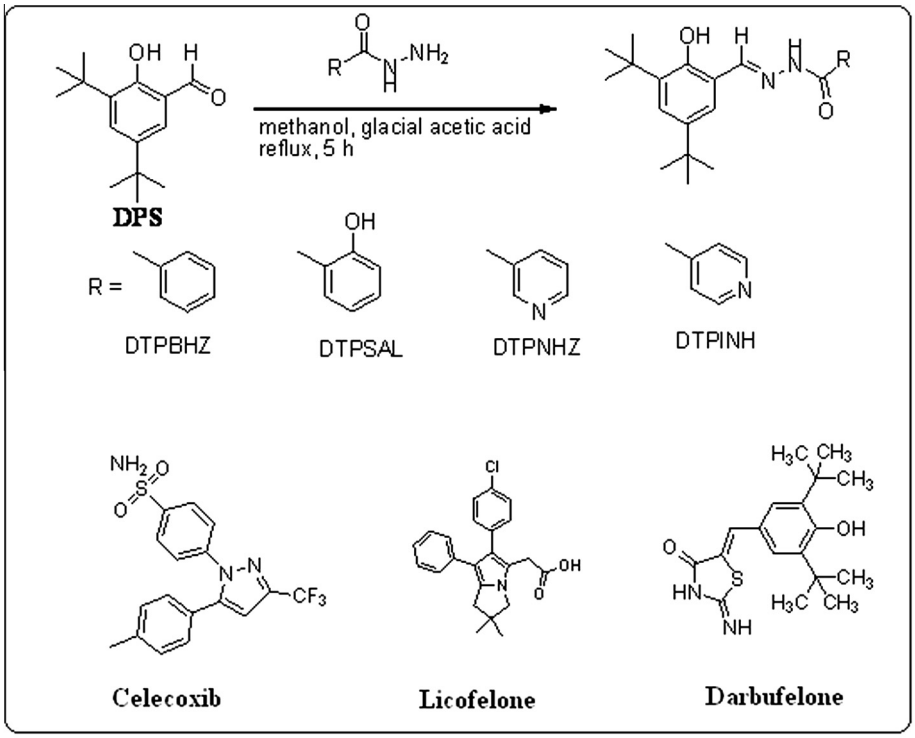
experimental rodent model.[6] The cancer growth-promoting effects of downstream products of 5-LOX pathway such as 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene E4 (LTB4) have also been recorded.[7] It thus appears logical that inhibition of both COX and LOX pathways may lead to more effective control of colon cancer growth and associated signal transduction processes. Use of non-steroidal *anti*-inflammatory agents (NSAIDs) as chemopreventive strategy for colorectal cancers has generated great deal of interest as they have been shown to offer protective effects during different stages of colorectal tumorigenesis including apoptosis, angiogenesis and cell-cycle progression. Most of these effects have been attributed to the inhibition of COX enzymes responsible for prostaglandin biosynthesis as these have been found to be elevated in majority of colorectal cancers. However, safety considerations restrict their use for cancer prevention. Similarly, although selective COX-2 inhibitors exhibit good benefits in controlling various types of cancers including those of colon, the compounds also show increased risk of cardiovascular events thereby limiting their use in the clinics. Under such circumstances inhibition of lipoxygenase pathway (LOX) involving leukotrienes, might be useful for targeting the CRC. More recently, involvement of the 5-LOX enzyme and its products have been implicated in development and progression of colon cancer.[8,9] Both COX-2 as well as 5-LOX are up-regulated during colon carcinogenesis[10] and are related to tumor size, depth and vessel invasion.[8] Since both these enzymes use arachidonic acid as the substrate for eicosanoids synthesis, controlling the amount of free arachidonic acid as well as the activity of the metabolizing enzymes can potentially alter the production of prostaglandin and leukotriene.[11] It may also perhaps explain why the *anti*-inflammatory and chemopreventive properties of Celecoxib may be compromised in cells and tissues in which arachidonic acid is shunted into the 5-LOX pathway contributing to its cardiovascular complications.[12] Such cardiovascular risks can be minimized by employing dual COX/5-LOX inhibitors rather than COX-2 inhibitors alone.

Several clinically effective NSAIDs have been structurally modified to yield potent dual COX–LOX inhibitors. Knaus and co-workers have reported novel Aspirin analogs recently for their in vitro as well as in vivo *anti*-inflammatory efficacy.[13] This group has also studied several analogs of other known NSAIDs including Indomethacin,[14] Rofecoxib[15] and Celecoxib[16,17] which have exhibited higher efficacy as COX–LOX dual inhibitors. Among the dual COX/LOX inhibitors studied so far, Licofelone has advanced into clinical trial.[18] Its safety and efficacy, in comparison with clinically used NSAIDs like Naproxen and Rofecoxib have recently been reviewed.[19,20] Rao and co-workers have shown that Licofelone is able to inhibit both small intestinal and colon tumorigenesis in $Apc^{Min/+}$ mice,[21] where it is reported to trigger apoptosis in a dose- and time-dependent manner in human colon cancer cell HCA-7.[22] Subsequently in an attempt to identify molecules that are specifically expressed by epithelial tumor cells which correlate with tumor growth and drug resistance it was found that a major extra-cellular component (ECM) hyaluronan (HA)[23,24] and its interaction with its receptor variant isoform CD44v6[23,25–27] play crucial role in regulating COX-2/PGE2 mediated cell survival, motility, and drug resistance.[26,28–32] We and others have demonstrated that the reversal of HA/CD44v6 signaling modulates the cancer phenotype and adenoma growth in $Apc^{Min/+}$ mice by inhibiting CD44v6/ErbB2/COX-2-PGE2 pathway. Additionally, our study indicates that CD44v6/HA interaction has a regulatory effect on COX-2-induced prostaglandins E2 (PGE2) which in turn controls HA synthesis and hence the HA/CD44v6 signaling. Further it was also observed that, elevated HA production resulted in the increase of COX-2 expression (3 to 4-fold) as evident by RT-PCR, suggesting the potential of HA/CD44v6 as target for anticancer/chemoprevention therapy.[30,33,34]

The di-*tert*-butyl phenols represent a potent class of antioxidants and dual COX/LOX inhibitors[35,36] which are represented by experimental drugs like Darbufelone, Licofelone and KME4, respectively. Darbufelone has recently been found to inhibit growth of non-small cell lung cancer cell lines, inducing cell cycle arrest at G0/G1 phase and apoptosis by activating caspase-3 and caspase-8,[37] respectively. However, this class of potent COX–LOX dual inhibitors has remained inadequately explored for inhibition of colon cancer. In the present work, we describe synthesis, characterization of four 3,5-di-*tert*-butyl phenols appended with hydrazinic chain, viz. **DTPBHZ**, **DTPSAL**, **DTPNHZ** and **DTPINH** as shown in Scheme 1 where the side chain contributes towards enhanced lipophilicity and metabolic stability. The compounds were evaluated for their antiproliferative potential against COX-positive human colon cancer HT-29, HCA7 cells,[30] as well as COX-negative SW480 human colon cancer cells and Has2 over-expressed murine intestinal epithelial Apc10.1 Has2 cells.[30] An in vitro assay was also performed to elucidate their potential against inhibition of COX-1, COX-2 and 5-LOX enzymes. The compounds were docked into COX-2 and 5-LOX protein cavities in order to estimate their binding energies in these protein cavities which revealed that the synthesized compounds had higher binding energies than the standard drugs like Celecoxib and Licofelone, respectively. It was also observed that CD44v6shRNA sensitizes these cells to **DTPSAL** as well as to the chemotherapeutic drugs like Celecoxib and Licofelone. Our results suggest that the di-*tert*-butyl moiety with hydrazinic side chain represents an active class of COX/5-LOX dual inhibitors with chemo-preventive potential against colon cancer.

The di-*tert*-butyl-phenylhydrazone ligands **DTPBHZ**, **DTPSAL**, **DTPNHZ** and **DTPINH** (Scheme 1) were prepared by mixing equimolar amounts of 3,5-di-*tert*-butyl-2-hydroxy benzaldehyde (**DPS**) with respective hydrazides in methanolic solvent and re-crystallizing the resulting compounds using methanol–water mixtures. The details of spectroscopic assignments are given in Supplementary data. The mass spectral (MS) data for the ligand **DTPBHZ** showed molecular ion peak at 352 corresponding to $M\bar{1}$ peak. Similarly the MS data for other ligands **DTPSAL**, **DTPNHZ** and **DTPINH** revealed peaks at 368, 353 and 353, respectively, confirming formation of the respective Schiff bases. The IR spectrum of the parent **DPS** compound exhibited a sharp intense band at 1663 cm^{-1} due to aldehyde carbonyl group which disappeared upon Schiff base formation replaced by the appearance of the C═N imines stretching vibration in the region 1560–1552 cm^{-1} confirming successful reaction.[38] All ligands showed a band due to hydroxyl stretching vibration of the aldehyde in the region 3348–3230 cm^{-1}. The hydrazinic N—H stretches were located in the region 3002–3196 cm^{-1}, while the hydrazinic carbonyl was observed at 1641–1652 cm^{-1}, respectively.

The ^{1}H NMR signal at δ 9.19–11.87 ppm was attributed to the hydroxyl group on the di-*tert*-butyl phenyl ring. The sharp signal of the aldehyde proton appearing at δ 10.00–10.50 ppm was found to be absent upon hydrazone formation. The signal for the protons of one of the *tert*-butyl groups was observed as a singlet with downfield shift between 1.41 and 1.46 ppm due to presence of adjacent hydroxyl group while signal for the other *tert*-butyl group appears as a singlet at 1.27–1.34 ppm. This suggests that hydrazide substitution induced asymmetry in the hydrazonate ligands.[39] A new signal appearing at δ 7.14–8.24 ppm confirmed the Schiff base formation. All ligands exhibit a broad signal at δ 8.49–10.66 ppm due to presence of the hydrazinic–NH proton. The aromatic protons appeared in the range of δ 6.0–8.2 ppm for all compounds. The ^{13}C NMR spectra for the present ligands showed a singlet in the range δ 136.72–154.53 ppm ascribed to the imines carbon (–C═N)



Scheme 1. Synthesis of 3,5-di-*tert*-butyl-2-hydroxy phenylhydrazone ligands.

confirming formation of the Schiff base functionality. The six methyl carbons of di-*tert*-butyl groups were observed as singlet with maximum intensity of the peak at δ 34.68–39.65 ppm.[40,41] Strong de-shielding in the aromatic carbon linked with the hydroxyl group of the di-*tert*-butyl phenol moiety was observed at δ 113.16–128.11 ppm compared to other aromatic carbons.

The ligand **DTPNHZ** (CCDC 926966) crystallized as a monomeric species having triclinic space group $P\bar{1}$ with crystal parameters $a = 10.843(3)$ Å, $b = 11.659(3)$ Å, $c = 17.737(5)$ Å, respectively. Interestingly the crystal structure of **DTPNHZ** possesses two crystallographically independent molecules in the same unit cell. The C═N– azomethine bond length [N (3)–C (7)] is 1.282(2) Å in **DTPNHZ** has a double bond character,[42] whereas N—N bond length for [N (2)–N (3)] is 1.3845(19). The bond length of the hydrazinic carbonyl [C (6)–O (1)] is found to be 1.230(2) Å. The crystal structure shows intra-molecular hydrogen bonding [N (3)–H (2c)–O (2)] 2.5931 Å (19). One of the molecules in the unit cell forms a hydrogen bond with the methanol solvent molecule through carbonyl and hydroxyl oxygen atoms. The details of the bond lengths and bond angles are provided in the Supplementary data.

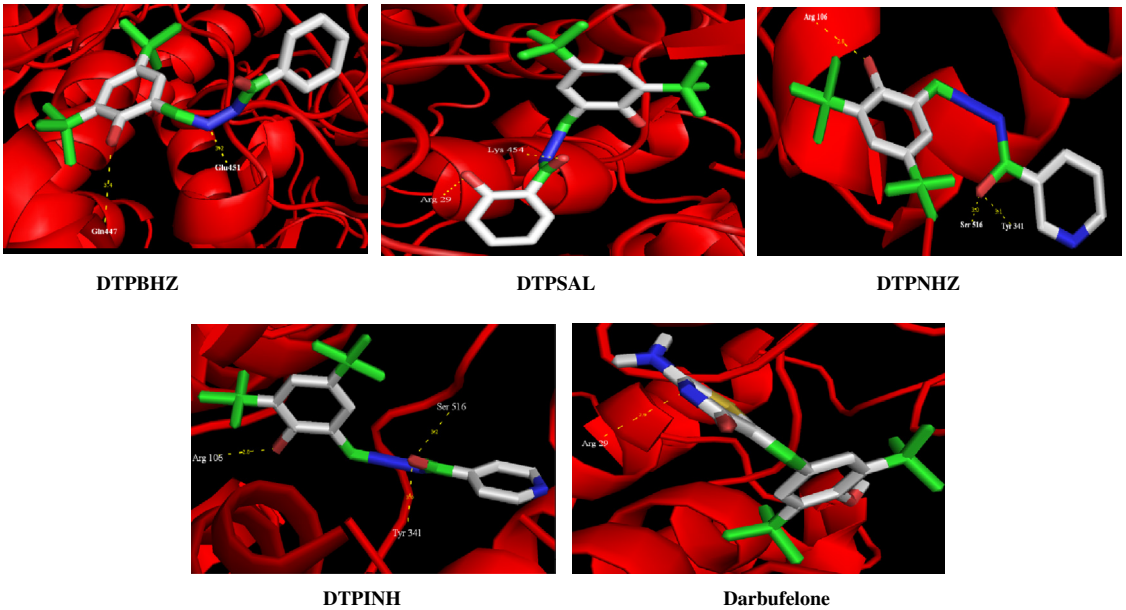


Figure 1a. Docking figures of 3,5-di-*tert*-butyl-2-hydroxyphenylhydrazones in COX-2 protein cavity.

In order to evaluate the efficacy of all synthesized COX–LOX dual inhibitors and obtain information about the binding energies within the COX cavity and their interactions with protein residues we performed homology modeling in COX-2 protein cavity for all of the ligands using AutoDock Vina software (Fig. 1a). It was observed that the ligand **DTPBHZ** showed the best fit in the protein cavity with binding energy of −10.1 Kcal/mol while **DTPSAL** was the second best-fit compound with binding energy of −9.7 Kcal/mol, respectively. Additionally **DTPBHZ** was able to form 2 hydrogen bonds with Lys454 and Arg29 amino acid residues while the standard compound Darbufelone containing analogous di-*tert*-butyl moiety, was able to form only 1 hydrogen bond with LYS437 residue with binding energy of −8.3 Kcal/mol. Comparing all molecular descriptors arising from the docking studies it may be concluded that **DTPBHZ** efficiently fits in the COX-2 cavity followed by **DTPSAL** and Celecoxib.

The enzyme 5-lipoxygenase having PDB ID: 3O8Y was also considered for the docking studies with present COX–LOX dual inhibitors. It was observed that the ligand **DTPSAL** exhibited binding energy of −8.2 Kcal/mol, which is lower than that of the well-known dual COX–LOX inhibitor, Darbufelone (−7.73 Kcal/mol) (Fig. 1b). The compound forms 4 hydrogen bonds with residue ARG 370 whereas Darbufelone forms 4 hydrogen bonds with LEU 244, ILE 283, ASP 285 and GLU 287 residues, respectively. The second best compound in the series was **DTPNHZ**, which exhibited binding energy of −7.8 Kcal/mol undergoing two hydrogen bonding interactions with ARG 246 and ARG 370 residues. The docking results of all compounds in COX-2 and 5-LOX cavities are summarized in Table 1. From the docking studies it is clear that the present ligands have high binding affinities for COX and LOX cavities, which is reflected in their in vitro activity.

The potential of all synthesized compounds to inhibit conversion of arachidonic acid to prostaglandin H_2 (PGH_2) by ram seminal vesicle COX-1 was determined (data not shown). Amongst ligands studied, **DTPSAL** showed maximum efficacy in inhibiting COX-2 with IC_{50} value of 5.13 μM followed by **DTPBHZ** (COX-2 IC_{50} = 6.49 μM) and **DTPINH** (COX-2 IC_{50} = 7.11 μM), respectively, on the immobilized enzymes in ELISA plates (Table 2a). All compounds exhibited higher values for COX-1 inhibition, indicating that they have preferential affinity for COX-2 enzyme (Table 2a). The compounds were also evaluated in vitro for their 5-LOX enzyme, wherein the most potent ligand **DTPSAL** exhibited IC_{50} value of 8.0 μM on the immobilized enzymes in ELISA plates (Table 2a). Overall; the new ligands exhibited good COX–LOX dual inhibitory profiles. We would like to clarify that the docking studies and the IC_{50} determinations have been carried out for two different purposes. In the docking studies virtual interactions between synthetic compounds and the active sites of the enzymes were studied in silico to predict activities against COX-2 and 5-LOX enzymes (Table 1). The effects of these compounds on COX-2 and

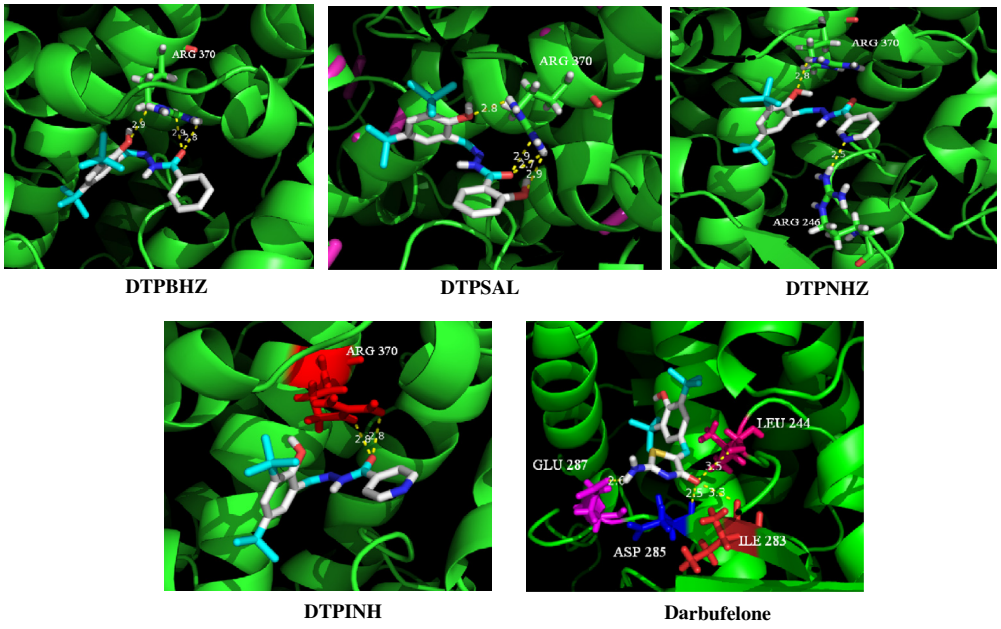


Figure 1b. Docking figures of 3,5-di-*tert*-butyl-2-hydroxyphenylhydrazones in human 5-LOX protein cavity.

Table 1
Docking results and consensus scores of 3, 5-di-*tert*-butyl-2-hydroxy phenylhydrazone ligands in COX-2 and LOX-5 protein cavity

Molecule	COX-2						5-LOX				
	B.E.	D.E.	Log*P*	H bond	H bond residues	Distance (Å)	B.E.	D.E.	H bond	H bond residues	Distance (Å)
DTPBHZ	−8.57	−9.19	6.27	1	LYS473	1.874	−6.50	−6.19	1	TYR181	2.098
DTPSAL	−8.60	−9.58	6.44	1	ASN87	2.007	−4.48	−3.91	1	GLN557	1.722
DTPNHZ	−8.62	−9.56	5.4	3	GLU520	1.977	−8.72	−7.92	2	GLN557	2.196
					LYS473	2.181				ASN425	1.811
					GLU520	2.114					
DTPINH	−8.45	−9.10	5.13	1	ASN87	1.791	−7.81	−6.80	2	GLN363	2.004
										ASN425	2.132
Darbufelone	−7.08	−6.75	4.98	1	LYS437	2.215	−9.94	−8.24	2	ASN425	2.117
										HIS600	2.073

Table 2a
The effects of synthetic di-*tert*-butyl phenol based dual inhibitors on COX-2 and 5-LOX enzyme activities (IC_{50} in μM by ELISA assay)

Compounds	In vitro COX–LOX inhibition assay (IC_{50} values in μM)		
	COX-1	COX-2	5-LOX
DTPNHZ	>300	8.8	5.55
DTPSAL	44.6	5.13	8.00
DTPINH	>300	7.11	6.25
DTPBHZ	25.48	6.49	5.88
Celecoxib	22.90	0.057	NA
Licofelone	0.8	>30	0.18

5-LOX enzymes (IC_{50}) were tested on the immobilized enzymes in ELISA plates (Table 2a). It is possible that steric hindrance may play a key role as to why the IC_{50} values were in the similar ranges in the case of inhibitions of the two enzymes. Hence, it may not necessarily correlate exactly with the observed experimental results.

The compounds were evaluated for their antiproliferative activity against COX-2 positive (HT29, HCA7, and Apc10.1–Has2) cells[30] as well as COX-2 negative SW480 colon cancer cells. Two standard compounds, viz. Celecoxib (COX-2 selective inhibitor) and Licofelone (COX–LOX dual inhibitor) were also evaluated against these cancer cell lines. In general, IC_{50} values for the present compounds against COX-2 positive (HT29, HCA7, and Apc10.1–Has2) cells[30] were in the range of 1.6–6.4 μM, whereas the standard Celecoxib compound showed IC_{50} value around 4.9 μM (Table 2b). Most active compound in the present series was found to be **DTPSAL**, which contains salicylyl hydrazinic side chain appended to the di-*tert*-butyl moiety. In case of COX-2 negative SW480 cells, **DTPSAL** had higher IC_{50} value (15.5 μM) indicating other inhibitory mechanism than straight forward COX2 inhibition might be operative. In case of Apc10.1 cells, **DTPSAL** showed greater potency with IC_{50} value of 5.6 μM followed by **DTBBHZ** (IC_{50} = 11.9). It was noted that all of the present ligands were highly active against COX-2 positive (HT29, HCA7, and Apc10.1–Has2) cells,[30] whereas they had relatively lesser inhibitory activities on COX-2 negative (SW480) cells (Table 2b).

The functional effect of 5-LOX and COX-2 on CRC cell growth (proliferation) and survival was analyzed by exposing cells to either colon cancer chemotherapeutic drug Celecoxib (known

Table 2b
Sensitization of present dual COX–LOX inhibitors by hyaluronan/CD44v6 interaction antagonist CD44v6shRNA towards *anti*-proliferative activities in colon cancer cells (IC_{50} values in μM of viable cell growth)

Compounds	Cell types					
	HT29			HCA7		
	HT29	HT29 + CD44v6shRNA (100 pmol)	Fold decrease	HCA7	HCA7 + CD44v6shRNA (100 pmol)	Fold decrease
DTPNHZ	4.3 ± 0.5	0.35 ± 0.07	12	3.5 ± 0.4	0.26 ± 0.09	14
DTPSAL	1.8 ± 0.9	0.09 ± 0.02	20	1.6 ± 0.3	0.078 ± 0.044	21
DTPINH	5.8 ± 0.9	0.71 ± 0.17	8	6.4 ± 0.77	0.76 ± 0.15	8.4
DTPBHZ	5.7 ± 1.1	0.85 ± 0.17	6.7	4.3 ± 0.48	0.56 ± 0.08	7.7
Celecoxib	5.7 ± 0.9	0.7 ± 0.17	8	4.9 ± 0.69	0.75 ± 0.067	7
Licofelone	67.0 ± 7.9	21.1 ± 4.8	3	60.9 ± 9	18.5 ± 0.123	3.5

Compounds	Cell types			
	Apc10.1 + Has2			SW480
	Apc10.1 + Has2	Apc10.1 + Has2 + CD44v6shRNA (100 pmol)	Fold decrease	
DTPNHZ	13.8 ± 1.6	1.9 ± 0.78	7.2	37
DTPSAL	5.6 ± 0.9	0.15 ± 0.05	37.3	15.5
DTPINH	18.2 ± 1.3	3.0 ± 0.33	6.1	33.7
DTPBHZ	11.9 ± 1.0	1.8 ± 0.19	6.1	35.7
Celecoxib	13.4 ± 1.5	1.3 ± 1.4	10.3	6.9
Licofelone	70.6 ± 9.0	23 ± 3.8	3	68

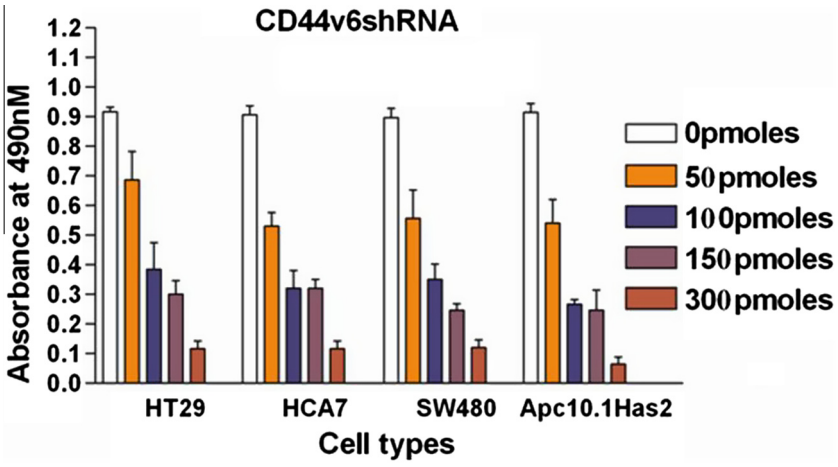


Figure 2. Effect of CD44v6shRNA (pSicoRCD44v6shRNA) on inhibition of proliferation of colorectal carcinoma cells. HT29, HCA-7, and Apc10.1-Has2 cells were treated with various doses of pSiocRCD44v6-shRNA, or pSicoRscrambledshRNA (0 pmol) and grown for a period of 72 h. Cell proliferation was measured by an MTS assay and expressed as the mean absorbance at 490 nm/20 × 10^3 cells/15 min.

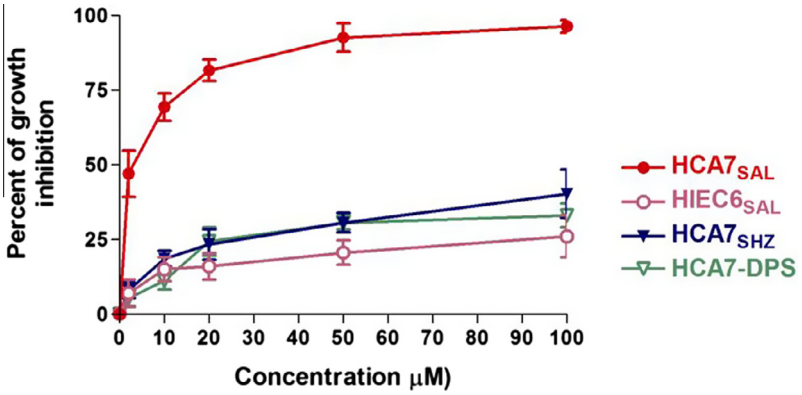


Figure 3. The growth inhibition curves of **DTPSAL** and parent ligands (**SHZ**, and **DPS**) at different concentrations in HCA7 (**$HCA7_{SAL}$**) and HIEC6 (**$HIEC6_{SAL}$**) cells. HCA-7 and HIEC6 cells were treated with various doses of **DTPSAL (HCA7$_{SAL}$, HIEC6$_{SAL}$)**, whereas HCA7 cells were treated with **SHZ**, **DPS** at different test concentrations for a period of 24 h. Cytotoxic activity was measured by an MTT assay and expressed as the percent inhibition of growth (cells without drug treatment was set to 0% inhibition of growth).

specific COX-2 inhibitor),[43] or dual COX/5-LOX inhibitor Licofelone,[21] respectively. The COX-2 inhibitor Celecoxib is known to exhibit IC_{50} values in the range 4.9–13.4 μM, whereas Licofelone, exhibits IC_{50}-values in range of 60–70 μM against COX-2/5-LOX positive HT29, HCA7, and Apc10.1–Has2 transformed cells (Tables 2a and b).[30,44,45] Since CD44v6/HA signaling is known to regulate COX-2 expression and activity, we transfected both COX-2 positive HT29, HCA7, and Apc10.1–Has2 cells and COX-2 negative SW480 cells with pSiocRCD44v6shRNA[30] (CD44v6shRNA) (Fig. 2), or pSiocRscrambledshRNA (data not shown since data for pSiocRscrambledshRNA is more or less similar to that of pSiocRCD44v6shRNA transfection at 0 pmol). Results in Figure 2 indicate that these antagonist drugs inhibited all colon cancer cell proliferation in a dose-dependent manner. This indicates that 5-LOX and COX-2 in addition to HA/CD44v6 could be involved in the mechanisms of COX-2 positive (HT29, HCA7 and Apc10.1–Has2)[30] cell carcinogenesis (Fig. 2). In case of COX-2 negative CRC SW480 cell, CD44v6shRNA (pSicoRCD44v6shRNA) substantially reduced the cell proliferation (Fig. 2) while present synthetic ligands have very little or no effect on cell proliferation indicating that, CD44v6shRNA affects the cell survival by a different route other than COX-2/5-LOX pathway.

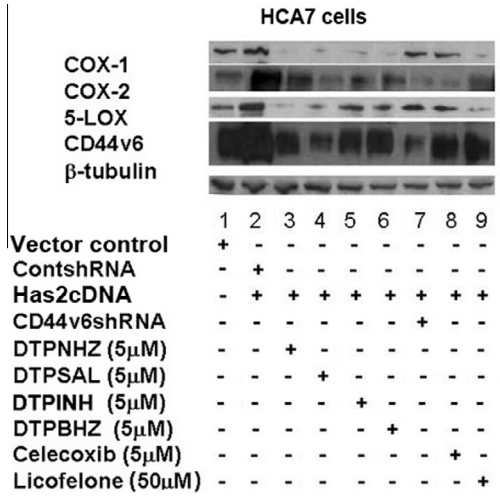


Figure 4. Effect of COX/LOX inhibitors on HA-induced CD44v6 expression associated with COX-1, COX-2 and 5-LOX proteins in HCA7 cells. Western blot analysis of COX-1, COX-2, CD44v6 and 5-LOX protein levels in HCA7 and HCA7-Has2 cells. In order to show the expression of COX-2 and 5-LOX in all the cells, longer exposure times were used. Equal loading was confirmed by reprobing the same blot for β-tubulin. HCA7 cells were transiently transfected with vector control shRNA (ContshRNA), or vector control (pCIneo), or Has2cDNA, or CD44v6shRNA, or first transfected with Has2cDNA followed by additional transfection with CD44v6shRNA. Transfected cells were grown for 72 h before they are further treated with **DTPNHZ** (5 μM), or **DTPSAL** (5 μM), or **DTPINH** (5 μM), or **DTPBHZ** (5 μM), or Celecoxib (5 μM), or licofelone (50 μM), or a combination of Celecoxib (5 μM) and licofelone (50 μM) for 24 h as indicated in the figure. The indicated concentrations of the compounds are close to their IC_{50} concentrations. The results of CD44v6shRNA that inhibits the basal CD44v6 expression were not shown.

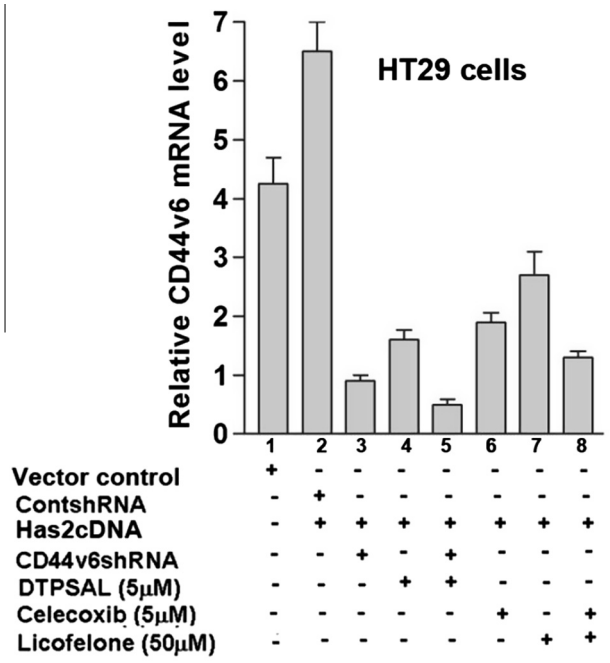


Figure 5. Effect of COX/LOX inhibitors on expression of CD44v6 detected by qRT-PCR analysis in HT29 and HT29-Has2 cells. HT29 cells were transiently transfected with vector control shRNA (ContshRNA), or vector control (pCIneo), or Has2cDNA, or controlshRNA (ContshRNA), or CD44v6shRNA, or first transfected with Has2cDNA followed by additional transfection with CD44v6shRNA. Transfected cells were grown for 72 h before they are further treated with **DTPSAL** (5 μM), or treated with **Celecoxib** (5 μM), or treated with licofelone (50 μM), and a combination of Celecoxib (5 μM) and licofelone (50 μM) for 24 h as indicated in the figure. The indicated concentrations of the compounds are close to their IC_{50} concentrations. The results of the effects of DTPNHZ (5 μM), or DTPINH (5 μM), or DTPNHZ (5 μM) on inhibition of CD44v6 expression were carried out. These three compounds inhibit CD44v6 expression to a lesser extent compared to that of DTPSAL (the data not shown). The results of CD44v6shRNA that inhibits the basal CD44v6 expression were not shown. RNAs were isolated using RNA purification kit from Norgen Biotek Corporation. Using Sybergreen fluorogenic probes and the primers for CD44v6 (Genbank # L05415): Forward-CCAGGCAACTCCTAGTAGTA-CAAC; Reverse: GGGAGTCTTCTCTGGGTGTTTG. qRT-PCR assay was carried out in three individual experiments. Each experiment has triplicate samples.

In order to confirm whether the HA/CD44v6 antagonist CD44v6shRNA is able to reverse constitutive multi-drug resistance in tumor cells, we examined the effect of HA/CD44v6 antagonist CD44v6shRNA with present synthetic ligands as well as Celecoxib and Licofelone through cell proliferation assays on COX-2 positive (HT29, HCA7 and Apc10.1–Has2) cells.[30] It was observed that these cells were sensitized to different extent by present ligands (Table 2b) which probably indicates differential recognition of the compounds by COX-2 and 5-LOX proteins. Among the present ligands **DTPSAL** was found to be the most potent in reversing the drug resistance while **DTPBHZ** has the least potency to reverse the drug resistance which, however, is comparable to Celecoxib (Table 2b). Licofelone has the least capability in reversing the drug resistance compared to all the compounds. This indicates that HA-CD44v6 interaction promotes *anti*-apoptosis and cell survival in COX/5-LOX positive colon tumor cells in the presence of present chemotherapeutic compounds. Moreover, down-regulation of CD44v6 by transfecting tumor cells with CD44v6shRNA but not scrambled sequence shRNA effectively attenuates HA/CD44v6-mediated colon tumor cell *anti*-apoptosis/survival and enhances multi-drug sensitivity in these cells (Fig. 2 and Table 2b). Together, these findings indicate that the HA-CD44v6-mediated COX-2 and 5-LOX signaling pathways provide new drug targets to sensitize tumor cells to undergo enhanced cell growth inhibition and to overcome drug resistance in colon tumor cells.

In order to further confirm the cytotoxic activity of the most potent compound **DTPSAL**, we have carried out another set of growth inhibition measurement using MTT assay. The compound **DTPSAL** was tested by MTT assay in HCA7 cells that endogenously express COX-2, 5-LOX and CD44v6 as well as in normal human intestinal epithelial cells (HIEC6) that do not express these. We have also tested the parent building blocks of compounds, such as salicylyl hydrazide (**SHZ**) and 3,5 di-*tert* butyl—2-hydroxy benzaldehyde (**DPS**) in HCA7 cells by MTT assay. The compound **DTPSAL** exhibited cytotoxic activity (Fig. 3) in this assay since the HCA7 cells express COX-2, 5-LOX, and CD44v6. Their cell growth-inhibiting activity is thus attributed to CD44v6–COX–LOX axis. Importantly, this assay showed that the **DTPSAL** have little or no effect on normal human intestinal epithelial cells HIEC6, which do not exhibit COX-2, LOX and CD44v6 expression. The IC_{50} values of the parent building block of compounds (**SHZ**, **DPS**) showed moderate level of antiproliferative activity.

COX-2 and 5-LOX are over-expressed during the process of colonic adenoma formation and have been implicated as promoters for tumor development.[6] Investigation of the role of the LOX pathway in colon cancer has been limited. We have previously shown that increased synthesis of hyaluronan regulates COX-2 expression and COX-2 induced PGE2 activity.[34] Similarly we have also reported that using structural analogs of 2,6 di-*tert*-butyl-*p*-benzoquinone (**BQ**) appended with hydrazide side chain the synthesis of hyaluronan also regulates 5-LOX expression in two human CRC cell lines, viz. HT29 and HCA-7 and in Has2 over-expressing Apc10.1–Has2 cells, respectively.[30] Since the present set of compounds inhibited COX-1, COX-2 activities in vitro (Table 2a), and inhibited hyaluronan/CD44v6-mediated colon tumor cell *anti*-apoptosis/survival as well as enhanced multi-drug sensitivity in these cells (Fig. 2 and Table 2b) it is plausible to envision that these compounds will affect the expression of COX-1, COX-2, 5-LOX and CD44v6 in protein levels.

Our previous studies have shown that the over-expression of Has2 can convert the pre-neoplastic Apc10.1 cells to metastatic phenotype[30] and hence increased COX-2 and 5-LOX levels in HCA7-Has2 cells correlate with the increased hyaluronan and may contribute to increased oncogenic phenotype.[30] Consequently we analyzed the effects of present COX–LOX dual inhibitors on CD44v6, COX-2 and 5-LOX expression in protein level of HCA7 cells. Results included in Figure 4 clearly shows that up-regulation of Has2 in HCA7 cells increases CD44v6, COX-2 and 5-LOX significantly (**Lane 2**) and that CD44v6shRNA decreases this response to control levels (**Lane 7 compared with Lane 2**). Similarly

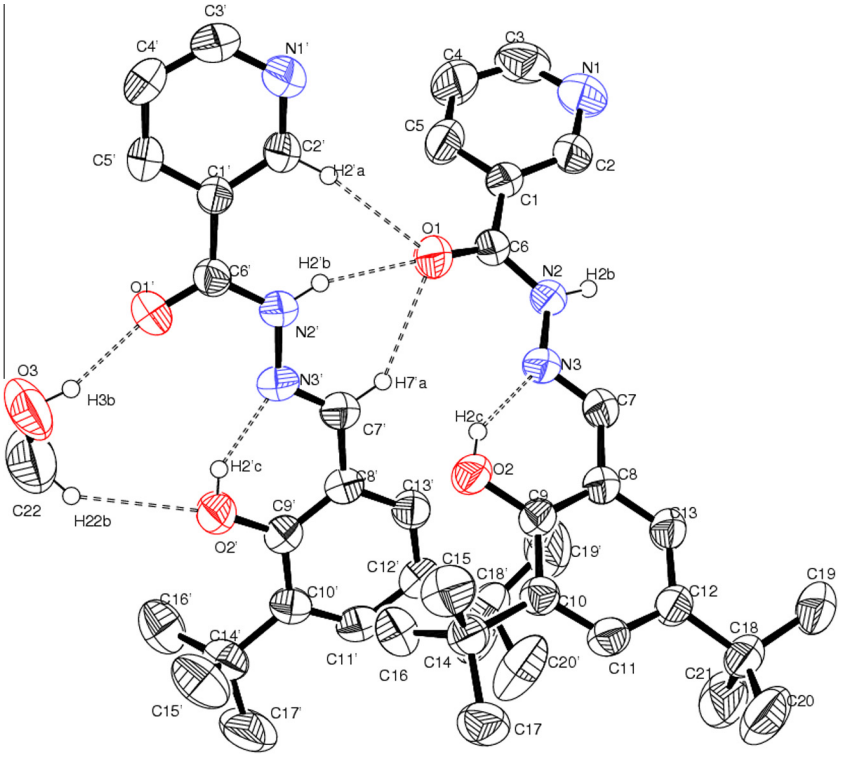


Figure 6. ORTEP diagram for **DTPNHZ** crystal. X-ray crystal structure for **DTPNHZ** (**CCDC deposition code: 926966**); ellipsoids are represented at 50% probability.

up-regulation of Has2 in HT29 cells increases the expression of CD44v6 in the relative mRNA expression (**q-RT-PCR analyses** Fig. 5). It is important to point out that the present compounds down-regulate the hyaluronan induced CD44v6 expression substantially to basal level (Figs. 4 and 5), indicating that their COX–LOX inhibitor action is through HA and CD44v6 supporting our hypothesis that the HA/CD44v6 signaling pathways are critical for regulating COX-2 and 5-LOX activities. On the other hand HA/CD44v6 signaling pathways and Celecoxib have little effect on COX-1 expression (Fig. 4). Results depicted in Figure 4 also show substantial inhibition of HA-induced CD44v6 associated COX-1, COX-2 and 5-LOX proteins by present ligands (**Lanes 3–6**), as well as Licofelone (**Lane 9**), Celecoxib (**Lane 8**), and CD44v6shRNA (**Lane 7**), respectively. We also tested the synergistic inhibitory effect of COX-2 and 5-LOX and CD44v6shRNA and our potent dual COX–LOX inhibitor DTPSAL on CD44v6 mRNA expression (Fig. 5). The present results indicate that combination of CD44v6shRNA and DTPSAL has an additional effect on CD44v6 mRNA expression (Fig. 5; **Lane 5**), while Celecoxib and Licofelone synergistically inhibit CD44v6 mRNA expression (Fig. 5; **Lane 8**, **compared to lanes 6 and7**). These results suggest that present dual inhibitors down-regulate cyclooxygenase and lipoxygenase enzyme through CD44v6. Taken together, results in Figs. 2–5 justify that present inhibitors function through CD44v6 regulated COX-2 and 5-LOX.

In summary, present class of COX–LOX dual inhibitors exhibit potent antiproliferative activity against COX-2 positive (HT29, HCA7 and Apc10.1–Has2) colon cancer cells. The decrease in cell survival in COX-2 negative SW480 cells may be due to cell survival pathway other than COX-2/5-LOX pathway. The drug sensitization experiments indicate that CD44v6shRNA sensitizes these compounds towards higher antiproliferative potency by 7 to 30-fold. The results of the present study, therefore, indicate the potential of CD44v6 and COX–LOX as targets in colon cancer therapy and prevention using our newly synthesized COX–LOX dual inhibitors (Fig. 6).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.11.015.

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