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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Isothiocyanate analogs targeting CD44 receptor as an effective strategy against colon cancer

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Abstract Inflammatory pathway plays an important role in tumor cell progression of colorectal cancers. Although colon cancer is considered as one of the leading causes of death worldwide, very few drugs are available for its effective treatment. Many studies have examined the effects of specific COX-2 and 5-LOX inhibitors on human colorectal cancer, but the role of isothiocyanates (ITSCs) as COX–LOX dual inhibitors engaged in hyaluronan–CD44 interaction has not been studied. In the present work, we report series of ITSC analogs incorporating bioisosteric thiosemicarbazone moiety. These inhibitors are effective against panel of human colon cancer cell lines including COX-2 positive HCA-7, HT-29 cells lines, and hyaluronan synthase-2 (Has2) enzyme over-expressing transformed intestinal epithelial Apc10.1Has2 cells. Specifically, our findings indicate that HA-CD44v6-mediated COX-2/5-LOX signaling mediate survivin production, which in turn, supports anti-apoptosis and chemo-resistance leading to colon cancer cell survival. The over-expression of CD44v6shRNA as well as ITSC treatment significantly decreases the survival of colon cancer cells. The present results thus offer an opportunity to evolve potent inhibitors of HA synthesis and CD44v6 pathway and thus underscoring the importance of the ITSC analogs as chemopreventive agents for targeting HA/CD44v6 pathway.

Keywords Isothiocyanate analogs · CD44/hyaluronan · Colon cancer · Molecular modeling · COX–LOX dual inhibition · Anti-cancer

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Introduction

Although colon rectal cancer (CRC) is the third leading cause of cancer death in men and women in the United States (Siegel *et al*., 2011), the cellular and molecular events associated with its pathogenesis are not known to a certainty. Epidemiological studies indicate that consumption of green leafy vegetables, fruits, broccoli, and cruciferous vegetable is correlated with a lowered risk of colon cancer (Lin *et al*., 1998; Steinmetz and Potter, 1991). Sulforaphane (SFN) is the predominant isothiocyanate (ITSC) found in these cruciferous vegetables as its glucosinolate precursor, which has been shown to inhibit colonic aberrant crypt foci (Chung *et al*., 2000; Kassie *et al*., 2003). The compound has also been found to be effective inhibitor of carcinogen-induced mammary gland tumorigenesis (Zhang *et al*., 1994), lung cancer (Hecht *et al*., 2002), and stomach tumors in rodent model (Fahey *et al*., 2002). Although mechanism of tumor inhibition by SFN is not clear, it has been reported to affect several cellular processes, including apoptosis (Chen *et al*., 2012), cell cycle arrest (Davis *et al*., 2009), disruption of oncogenic signaling (Li *et al*., 2011), and epigenetic regulation of tumor suppressor genes (Bhamre *et al*., 2009; Clarke *et al*., 2008; Dashwood and Ho, 2008; Telang *et al*., 2009).

Despite extensive research, the pathological and molecular markers for prognosis of colon cancer are still lacking. There is now adequate evidence to indicate that cyclooxygenase-2(COX-2) metabolite prostaglandin-E2 (PGE2) and 5-lipoxygenases (5-LOX) metabolite leukotrienes (LTs) play an important role in colon carcinogenesis (Reddy and Rao, 2002; Reddy *et al*., 2000; Kawamori *et al*., 1998; Swamy *et al*., 2003; Bertagnolli *et al*., 2006; Soumaoro *et al*., 2006; Ye *et al*., 2005; Ihara *et al*., 2007). The COX-2 transcription is regulated through transcription factors, especially nuclear factor-κB (NF-κB) (Tsatsanis *et al*., 2006). NF-κB is a ubiquitous and pleiotropic transcription factor closely linked with carcinogenesis by a variety of pathways including cell cycle progression, apoptosis, invasion, metastasis, and inflammation (Bubici *et al*., 2006). SFN has been shown to possess anti-inflammatory activity, resulting in down-regulation of COX-2 expression (Shan *et al*., 2009), probably through inhibition of NFκB-DNA binding and transactivation of NFκB-dependent genes (Heiss *et al*., 2001). Attempts are underway in identifying molecules that are specifically expressed by epithelial tumor cells which correlate with tumor growth and drug resistance. Among such molecules hyaluronic acid (HA), is a major extracellular matrix (ECM) component (Misra *et al*., 2009) whose interaction with the receptor variant isoform CD44v6 (Aruffo *et al*., 1990) has been suggested to play a crucial role in regulating COX-2/PGE2 mediated cell survival, motility, and drug resistance (Lesley *et al*., 2000a, b; Lesley and Hyman, 1992; Naor *et al*., 2002; Yamada *et al*., 2004). We had earlier demonstrated that the reversal of HA/CD44v6 signaling can modulate the cancer phenotype and adenoma growth in Apc Min/+ mice by inhibiting CD44v6/ErbB2/COX-2-PGE2 pathway (Ghatak *et al*., 2008, 2010), suggesting the potential of HA/CD44v6 as target for anti-cancer/chemopreventive drugs. In a recent study, we also demonstrated that CD44v6 is a direct target of COX-LOX dual inhibitors (Misra *et al*., 2013). The regulation of COX-2 expression and NFκB by SFN has been primarily investigated in inflammation wherein it has been shown that CD44v6 has a binding site for NFκB (Damm *et al*., 2010). However, the inter-relationships among COX-2, NFκB, and CD44v6 in CRC cells and the mechanism by which SFN modulates these enzymes or protein complexes has not been adequately explored. Recent studies seem to suggest that SFN offers protection against tumor development during the "post initiation" phase. While studying the inhibitory mechanisms by SFN, it has become apparent that this natural compound is an inhibitor of HA/CD44v6-induced cell growth and proliferation as well as COX-LOX enzyme activity. Although bioavailability of SFN in the colon is unknown, a recent pilot study in human mammary tissue has indicated that an oral dose of broccoli sprout preparation containing 200 mmol SFN 1 h prior to tissue removal showed mean accumulation of 1.45 ± 1.12 pmol/mg tissue in the right breast and 2.00 ± 1.95 pmol/mg in the left breast (Cornblatt *et al*., 2007).

A number of cell-culture studies have been reported in the literature in order to elucidate the mechanism responsible for toxicity, enzyme modulation capability, and metabolic pathways of available isothiocyanates such as SFN, benzylisothiocyanate (BITSC), allylisothiocyanate (AITC), and phenylethyl isothiocyanate (PEITC). These studies revealed that the effects of these ITSCs in cell cultures can vary depending upon the cell line used and experimental conditions employed. The evaluation of these ITSCs in the chemoprevention of breast and other cancers has revealed that these compounds once diffused passively into the cell undergo metabolic conversion pre-dominantly to the glutathione (GSH) conjugate. Of the standard ITSCs available in the market, SFN was found to be the weakest substrate for glutathione *S*-transferase as reflected from the half-life of their glutathione conjugates at pH 7.4 which are being in the order: PEITC-SG (58 min) $\approx$ SFN-SG (58 min) $<$ BITC-SG (67 min) (Lamy *et al*., 2011). Consistent with these findings, the growth inhibition of different cancer cells was found to be in the order of BITC $\approx$ PEITC $>$ AITC $>$ SFN which also correlates well with their LogP values being in the order: ITSCs (SFN (0.72) $<$ AITSC (2.3) $<$ BITSC (2.97) $<$ PEITSC (3.08) (Lamy *et al*., 2011). Although this information is important, the absence of commercially

available ITSC analogs with various chain lengths and substituents influencing bioactivity of ITSCs impedes reliable conclusions on real availability of isothiocyanate for the cells under study both in vitro and in vivo. With this in mind, we synthesized ITSC analogs with various substituents and structural motifs which can influence the bioactivity of these analogs. Such modifications are also anticipated to increase lipophilicity and LogP values of these compounds and influence their reactivity with thiol moieties and consequently increase chemical stability (Van Eylen *et al.*, 2007).

In spite of such studies analogs of ITSC which possess better thermal stability than SFN have remained inadequately explored for the inhibition of colon cancer. In the present study we have, therefore, synthesized novel ITSC analogs by incorporating the analogous thiosemicarbazone motif into some of the naturally occurring bioactive phytochemical motifs such as chromone, coumarin, indole, quinolone, and pyridine, respectively. The efficacies of these conjugates in inhibiting CRC cell proliferation and growth as well as sensitizing such cells for apoptosis through CD44v6 and HA pathways have also been examined. Our results suggest that these compounds may represent a novel class of chemopreventive agents for colon cancer by inhibiting the HA/CD44v6/COX-2-5-LOX pathway.

Results and Discussion

Chemistry

Characterization details for synthesized compounds

FPYITSC [(E)-1-((pyridin-2-yl)methylene)thiosemicarbazide] IR(ν, cm^{-1}): 1725 (C=O), 1617 (C=N imine), 3395 and 3353 (–NH2 free), 3257 (–NH–); 1H-NMR (CDCl3, δ, ppm): 2.08 (2H, s, NH2), 7.7 (1H, s, –NH), 8.13 (1H, s, –CH), 8.35 (1H, ArH), 8.37 (1H, ArH), 8.58(1H, ArH), 8.76(1H, ArH), ESI–MS: *m/z* found 179, Calc 180 (M–) in accordance with C7H8N4S; Anal. Calc. (Found %): C7H8N4S; C, 46.68 (46.65), H, 4.44 (4.47), N, 31.07 (31.09), S, 17.72 (17.79).

APYITSC [(E)-1-(1-(pyridin-2-yl)ethylidene)thiosemicarbazide] IR(ν, cm^{-1}): 1729 (C=O), 1612 (C=N imine), 3348 and 3306 (–NH2 free), 3231 (–NH–); 1H-NMR (CDCl3, δ, ppm): 2.07 (2H, s, NH2), 2.4 (3H, s, –CH3), 7.8 (1H, s, –NH), 8.35 (1H, ArH), 8.41 (1H, ArH), 8.77 (1H, ArH), 10.76 (1H, ArH), ESI–MS: *m/z* found 193, Calc 194 (M–) in accordance with C8H10N4S; Anal. Calc. (Found %): C8H10N4S; C, 49.44 (49.46), H, 5.22 (5.19), N, 28.85 (28.84), S, 16.45 (16.51).

QNLITSC [(E)-1-((quinolin-2-yl)methylene)thiosemicarbazide] IR(ν, cm^{-1}): 1719 (C=O), 1619 (C=N imine), 3471 and 3401 (–NH2 free), 3249 (–NH–); 1H-NMR (CDCl3, δ, ppm): 2.08 (2H, s, NH2), 7.75 (1H, s, –NH), 7.9 (1H, s, –CH), 8.11 (1H, ArH), 8.20 (1H, ArH), 8.31 (1H, ArH), 8.57 (1H, ArH), 8.68 (1H, ArH), 8.77(1H, ArH) ESI-MS: *m/z* found 229, Calc 230 (M–) in accordance with C11H10N4S; Anal. Calc. (Found %): C11H10N4S; C, 57.31 (57.37), H, 4.36 (4.38), N, 24.36 (24.33), S, 13.98 (13.92).

CHRITSC [(1E)-1-((4-oxo-4H-chromen-3-yl)methylene)thiosemicarbazide] IR(ν, cm^{-1}): 1706 (C=O), 1641 (C=N imine), 3477 and 3431 (–NH2 free), 3243 (–NH–); 1H-NMR (CDCl3, δ, ppm): 2.06 (2H, s, NH2), 7.53 (1H, s, –NH), 7.68 (1H, s, –CH), 7.79 (1H, ArH), 8.08 (1H, ArH), 8.17 (1H, ArH), 9.15 (1H, ArH), 11.55 (1H, ArH), ESI–MS: *m/z* found 246, Calc 247 (M–) in accordance with C11H9N3O2S; Anal. Calc. (Found %): C11H9N3O2S; C, 53.41 (53.43), H, 3.59 (3.67), N, 16.94 (16.99), O, 12.92 (12.94) S, 12.93 (12.97).

COUITSC [(1E)-1-(1-(2-oxo-2H-chromen-3-yl)ethylidene)thiosemicarbazide] IR(ν, cm^{-1}): 1718 (C=O), 1603 (C=N imine), 3471 and 3381 (–NH2 free), 3236 (–NH–); 1H-NMR (CDCl3, δ, ppm): 2.06 (2H, s, NH2), 2.25 (3H, s, CH3) 7.40 (1H, s, –NH), 7.60 (1H, s, –CH), 7.75 (1H, ArH), 8.0 (1H, ArH), 8.46 (1H, ArH), 10.45 (1H, ArH), ESI–MS: *m/z* found 260, Calc 261 (M–) in accordance with C12H11N3O2S; Anal. Calc. (Found %): C12H11N3O2S; C, 55.19 (55.16), H, 4.20 (4.24), N, 16.14 (16.08), O, 12.29 (12.25) S, 12.23 (12.27).

INDITSC [(E)-1-(1-(1H-indol-3-yl)ethylidene)thiosemicarbazide] IR(ν, cm^{-1}): 1725 (C=O), 1656 (C=N imine), 3577 and 3554 (–NH2 free), 3254 (–NH–); 1H-NMR (CDCl3, δ, ppm): 2.08 (2H, s, NH2), 2.34 (3H, s, CH3) 7.10 (1H, s, –NH), 7.39 (1H, s, –CH), 7.21 (1H, ArH), 7.91 (1H, ArH), 8.17 (1H, ArH), 10.08 (1H, ArH), 11.53 (1H, –NH heterocyclic) ESI–MS: *m/z* found 231, Calc 232 (M–) in accordance with C11H12N4S; Anal. Calc. (Found %): C11H12N4S; C, 56.85 (56.87), H, 5.26 (5.21), N, 24.09 (24.12), S, 13.77 (13.80).

Molecular Docking Studies

In order to evaluate the efficacy of the synthesized ITSC analogs to inhibit COX-2 activity, they were docked into the cavity of crystallized COX-2 protein from RSPDB (Royal Society Protein Data Bank) http://www.rscb.org/ PDB ID (1PXX). All calculations were performed using AutoDock-Vina software (Trott and Olson, 2010). Grid maps of 50 × 50 × 50 points centered on the active site of the ligand were calculated for each atom types found on the adducts. The AutoDock-Vina program which is an

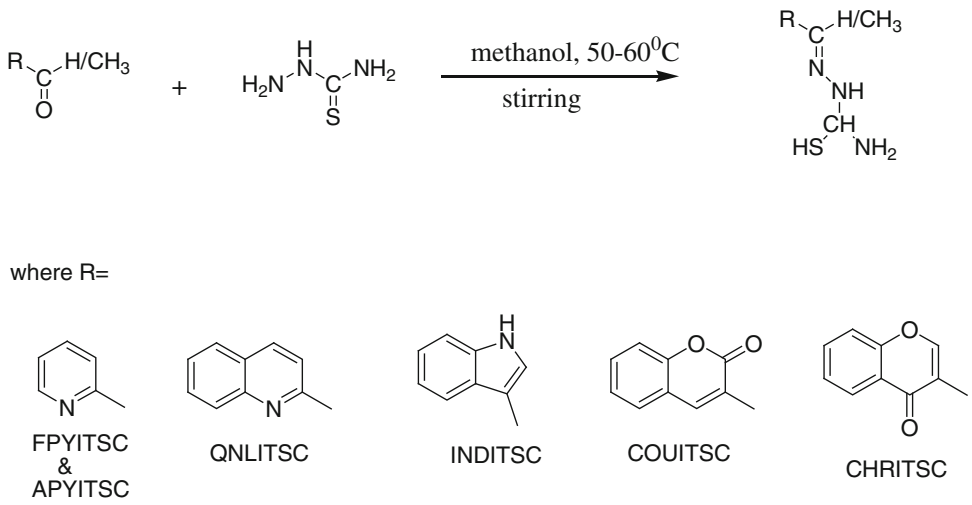

Fig. 1 Synthetic scheme for ITSC analogs

Table 1 Docking results and consensus scores of ITSC analogs in COX-2 protein cavity

Molecule	BE	Number of H bond	H bond formed with	H-bond length
FPYITSC	−5.3	1	Leu352	2.4
APYITSC	−5.6	1	Val523	2.5
QNLITSC	−7.8	2	GLN192	2.2
			SER353	2.3
INDITSC	−6.8	2	ARG44	2.3
			GLY45	2.6
CHRITSC	−7.3	1	ASN382	2.1
COUITSC	−7.4	1	MET522	2.1
Darbufelone	−7.08	1	LYS437	2.2
PITC	−5.4	1	VAL523	3.5
SFN	−4.5	1	TYR385	3.2

BE binding energy (kcal/mol), *H bond* hydrogen bond

automated docking program was used to dock all ligand molecules in the active site of COX-2 enzyme. For each compound, the most stable docking model was selected based upon confirmation of best score predicted by Auto-Dock scoring function. The compounds were energy minimized with MMFF94 force field. From the histogram relevant parameters such as binding energy, total number of hydrogen bonds formed, and hydrogen bonding pattern were determined using defined sets of descriptors and adherence to Lipinski's criterion (Fig. 1a, b). It was observed that the ligand QNLITSC and COUITSC showed best fit in the COX-2 protein cavity with binding energies of −7.80 and −7.4 kcal/mole (Table 1), respectively. The standard COX-LOX dual inhibitor Darbufelone shows (Table 1) slightly less binding energy (−7.08 kcal/mole), whereas far less binding energies were observed for other isothiocyanates like PEITSC (−5.4 kcal/mole) and SFN (−4.5 kcal/mole), respectively. Among the present analogs, QNLITSC having the highest binding energy exhibited two H-bonding interactions involving GIN192 and SEK353 residues, while the next best analog, viz. COUITSC, showed only one H-bonding interaction with MET522 (Table 1).

The X-ray crystal structure of human 5-lipoxygenase protein (5-LOX) has become available only in 2011 due to Gilbert and co-workers (Gilbert *et al*., 2011).It was obtained from Protein Data Bank (PDB) having PDB ID as 3O8Y and used for the present docking studies of present analogs. The active site residues were obtained from the study of PDB sum (Laskowski, 2009). The ligands were energy minimized and partial charges were added using PRODRG algorithms (Schuttelkopf and van Aalten, 2004). The Auto-Dock Tools graphical user interface (Sanner, 1999) was used to add polar hydrogens and partial charges to 5-LOX Atomic solvation parameters using Kollman United charges and fragment volumes which were assigned using the ADDSOL subroutine. The grid map was calculated using the auxiliary program Autogrid-3. Grid maps of 60 × 60 × 60 points centered on the active site of the ligand with 0.375 Å spacing were calculated for each atom type found on the adducts. Lamarckian Genetic Algorithm (LGA) was selected for ligand conformational search. The Genetic Algorithm (GA) population size was set to 150, the maximum number of GA energy evaluations as 2,500,000, GA mutation rate as 0.02, GA crossover rate as 0.8, and GA docking runs was set as 100. The resulting docking conformations were clustered into families of similar conformation, with root mean square deviation (RMSD) clustering tolerance as 1.0 Å. The lowest docking energy conformations were included in the largest cluster as a rule. Flexible torsion in the ligands was assigned with AUTOTORS, an auxiliary module for Auto-Dock Tools. Each ligand was docked individually within 5-LOX protein cavity to obtain the best binding conformation. The docking results of all compounds in COX-2 and 5-LOX cavities are summarized in Tables 1 and 2. Among the present series CHRITSC, COUITSC, and QNLITSC compounds

Table 2 Docking results and consensus scores of ITSC analogs in 5-LOX protein cavity

Molecule	BE	Number of H bond	H bond formed with	H-bond length
FPYITSC	−5.5	3	ARG370	2.2
			SER447	2.4
			VA243	2.5
APYITSC	−6.1	2	VAL243	2.7
			SER447	2.4
QNLITSC	−7.3	3	VAL243	2.3
			ARG370	2.7
			ARG370	2.5
INDITSC	−6.9	3	ARG370	2.7
			ARG370	2.8
			VAL243	2.4
CHRITSC	−7.6	4	VAL243	2.3
			ARG370	2.3
			SER447	2.8
			ARG457	2.8
COUITSC	−7.2	2	GLN549	2.5
			ARG370	2.6
Darbufelone	−9.94	2	ASN425	2.117
			HIS600	2.073
PITSC	−5.6	1	ARG370	2.6
SFN	−4.1	2	LEU448	3.4
			ARG457	2.7

BE binding energy (kcal/mol), *H bond* hydrogen bond

showed higher binding energies than standard COX-LOX dual inhibitor, Darbufelone. Overall, these findings indicate that the new analogs have higher stability in the COX and LOX protein cavities than the natural isothiocyanate SFN and it is commonly employed analog PEITSC.

Anti-cancer activities

Effect of silencing CD44v6 on colon tumor cell proliferative response

It has been established that many cancer chemopreventive agents are known to lower cancer risk by suppressing HA/CD44-signaling pathway (Bourguignon *et al*., 2009; Cordo Russo *et al*., 2008), which subsequently leads to attenuation of pro-inflammatory mediators and their activities. Natural anti-oxidants like ITSCs found in cruciferous vegetables have been under investigation due to their potential anti-cancer effects. However, not many studies have been devoted to examining structure–activity relationships between ITSC structure and HA/CD44 activation as well as subsequent anti-inflammatory action. In the present work we have employed five common phytochemical motifs in conjunction with the thiosemicarbazone side chain containing isothiocyanate group as shown in Fig. 1 and in the Fig. 2.

In order to ascertain whether HA-CD44v6-mediated signaling interaction with COX-2 and 5-LOX contributes to colon tumor cell survival which is the focus of present study, we determined the effect of CD44v6shRNA on colon tumor cell proliferation. The effect of silencing CD44v6 on colon tumor cell survival was studied in HCA7, HT29, and murine intestinal epithelial Apc10.1Has2 cells stably expressing Has2 cDNA (Fig. 3). It was observed that treatment of these colon tumor cells with CD44v6shRNA for 24 h followed by 48 h incubation significantly decreased cell proliferation, whereas treatment with the controlshRNA (scrambled shRNA) rendered cell proliferation unchanged (data not shown). The results in Fig. 3 also indicate that the order of inhibition of CD44v6shRNA in terms of IC_{50} values was: HCA7 (65 pmol) < Apc10.1Has2 (95 pmol) < HT29 (105 pmol), respectively. These results are in agreement with our previous findings that the HA-CD44v6-activated signaling is an important activator of oncogenic growth and chemo-resistance in colon cancer cells.

Expression of CD44v6, COX-2 and 5-LOX proteins are suppressed in ITSC treated cells

It has been shown earlier that commonly available ITSCs such as SFN or PITSC can target COX-2 (Shan *et al*., 2009). Our recently published data have clearly shown that HA/CD44v6 signaling regulates COX-2 expression/activity both in vitro and in vivo (Misra *et al*., 2011). COX-2 also regulates HA/CD44 signaling (Misra *et al*., 2008a, b). These studies collectively suggest that expression of COX-2 in cancer cells is essential for cancer cell growth and that the function of ITSCs including SFN may involve engaging CD44 to target COX-2.

In the present study, we have found that a basal level of CD44v6, COX-2, and 5-LOX expression is present in human colon tumor HCA7 cells (Fig. 4a, b). However, cells pre-treated with CD44v6shRNA down-regulates COX-2 and 5-LOX expressions significantly when compared with the controlshRNA-transfected cells. Moreover, combining the ITSCs with CD44v6shRNA resulted in a pronounced decrease in basal CD44v6, COX-2, and 5-LOX expression. Thus, the reduction of COX-2 and 5-LOX expression (Fig. 4a, b) appears to be CD44v6-specific in colon cancer cells, and ITSCs engage CD44v6 to target COX-2 and 5-LOX. The order of inhibitory effect on COX-2, 5-LOX, and CD44v6 expression in colon tumor cells was found to be in the deceasing order of CD44v6shRNA > COUITSC > Celecoxib > SFN > Licofelone, respectively. Interestingly, CD44v6 also seem to regulate 5-LOX in addition to COX-2 (Fig. 4a, b).

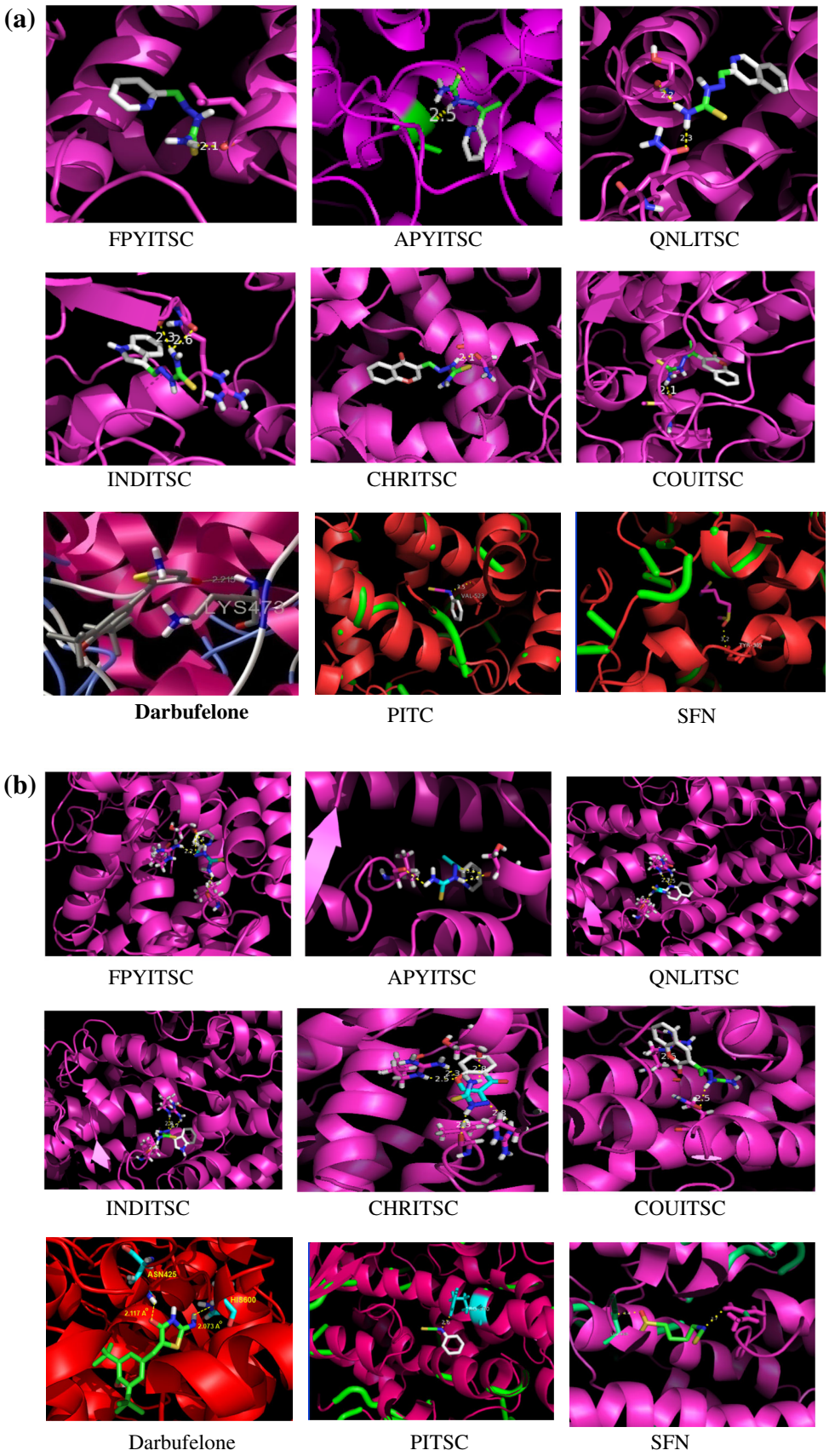

Fig. 2 **a** Docking figures of ITSC analogs in COX-2 protein cavity. **b** Docking figures of ITSC analogs in 5-LOX protein cavity

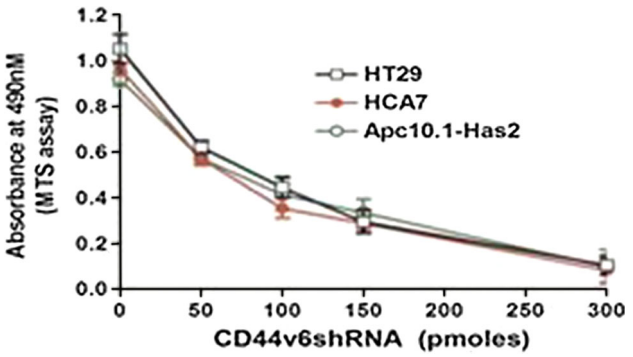

Fig. 3 Effect of silencing CD44v6 on cell proliferation of colon tumor cells. HCA7, HT29, and Apc10.1-Has2 cells were seeded on a 96-well plate cultured in 100 µl medium. Each well was transfected with CD44v6shRNA at the indicated concentrations. After 24 h, 20 µl MTS solution was added. Cell viability was measured as absorbance at 490 nm. Likewise, these cells were transfected with scrambled shRNA (ControlshRNA), and the cell proliferation was measured as described before. ControlshRNA shows similar levels of cell proliferation with the various doses of controlshRNA treatment in HT29, HCA-7, and Apc10.1-Has2 cells (data not shown). The experiments were performed three times with identical outcome and the means ± SD are shown

Effect of synthesized analogs on proliferative activity of colon tumor cells

Since cellular ITSCs have been shown to target COX-2 and 5-LOX levels by the cellular HA-CD44v6 pathway, we analyzed cytotoxicity of these ITSC analogs in HT-29, HCA7 human colon cancer cells using the MTS assay. In order to examine the structural influence of the present ITSCs on the proliferation potential of HA-synthase 2 (Has2), we used the Apc10.1 murine intestinal epithelial cell line (De Giovanni *et al*., 2004), which is stably transfected with Has2 expression plasmid construct, as a screening model for HA. The HT-29, HCA7, and Apc10.1Has2 cells were treated with the indicated concentrations of ITSCs for 24 h and the effect of ITSC analogs on the MTS activity was compared (Fig. 5). A comparative account of the IC_{50} values of all compounds provided in Tables 3 and 4 revealed the following order in their IC_{50} values: COUITSC (2.8 µM) > CHRITSC (3.8 µM) > INDITSC (5.0 µM) > QNLITSC (6.8 µM) > APYITSC (12.0 µM) > FPYITSC (12.5 µM) (Fig. 5; Tables 3, 4) indicating COUTSC to be the most potent analog.

Additionally, we also determined the effect of SFN and standard compound PITSC as well as COX-2 specific inhibitor Celecoxib, and COX/LOX dual inhibitor Licofelone on the proliferation of HT29, HCA7, and Apc10.1-Has2 cells (Fig. 6). The order of inhibition (IC_{50}) of these compounds was found to be: COUITSC (2.8 µM) > Celecoxib (3.8 µM) > PITSC (28 µM) > SFN (35 µM) > Licofelone (68 µM) (Figs. 5, 6; Tables 3, 4). These studies (Figs. 3–6) collectively indicate that apoptosis resistance of the colon tumor cells is most probably due to the enhanced expression of HA-CD44v6 interacting with COX-2 and 5-LOX target genes.

Effect of novel analogs on normal cells

In control experiments, we confirmed that the most potent analog COUITSC exhibited no pronounced cytotoxicity (as determined by MTT assay) to normal primary skin fibroblasts (NSFb) as well as to normal human intestinal epithelial cells (HIEC6) (Fig. 7). Similar results were also found in case of other ITSC analogs as well as CD44v6shRNA and Licofelone compounds (data not shown).

Effect of new ITSC analogs on survivin and caspase-3 proteins

In order to determine the mechanistic details of these changes in the colon cancer cells proliferation, we performed tumor cell apoptosis assays using Apc10.1 (empty

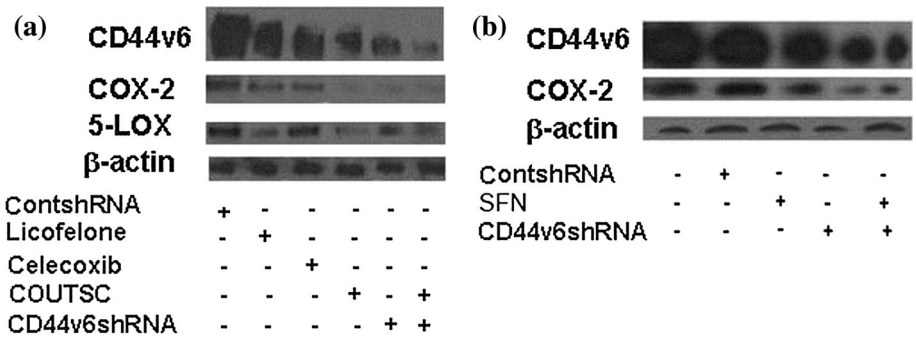

Fig. 4 Inhibition of CD44v6 by ITSCs attenuates COX-2 and 5-LOX expression in colon tumor cells. HCA7 cells were either transfected with ControlshRNA or CD44v6shRNA for 24 h. Transfected cells were incubated for 48 h for growth and then treated with 16 h addition of IC_{50} concentrations (obtained from Table 2a, b) of Licofelone, Celecoxib, SFN, COUITSC, or their combination as indicated in the figure. Western blot analysis for CD44v6, COX-2, 5-LOX, and β-actin was then carried out. Data are representative of three independent experiments. Results indicate that COX-2 up-regulation in colon tumor cells is dependent on the activation of CD44v6 and signaling, while ITSCs attenuates this signaling

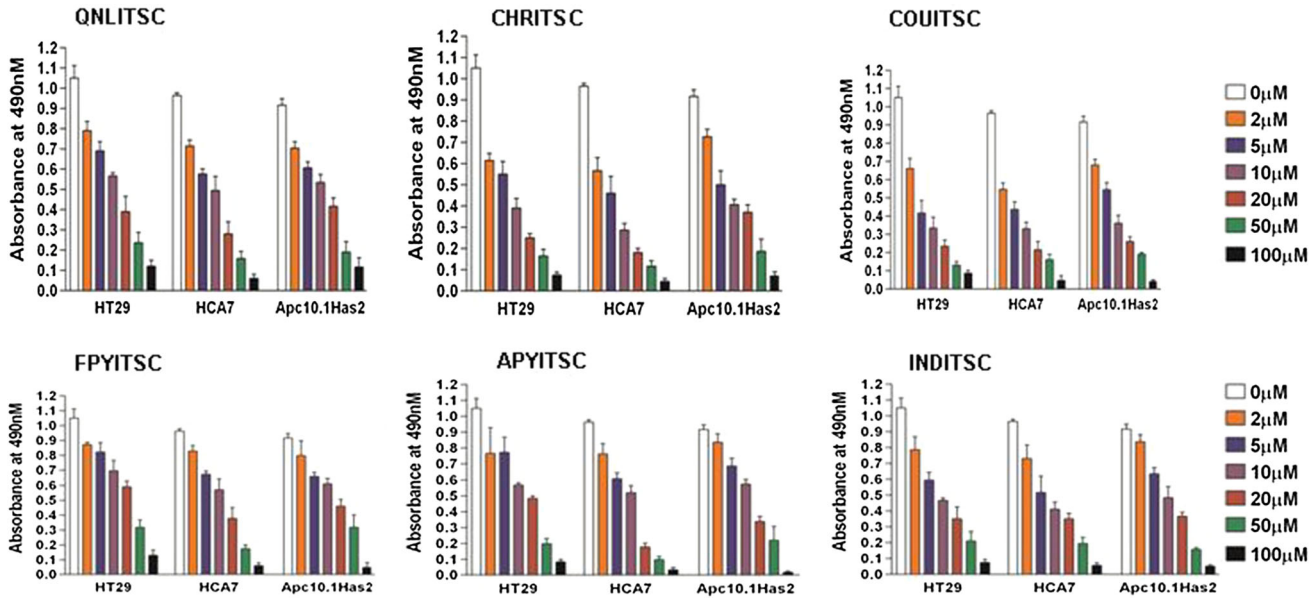

Fig. 5 Anti-proliferative effect of synthesized ITSC analogs on colon cancer cells. HT29, HCA-7, and Apc10.1-Has2 cells (stable transfectant expressing Has2) were transfected with pSicoR scrambled shRNA (controlshRNA), pSicoR-CD44v6shRNA (CD44v6shRNA), were left untreated, treated with various doses of FPY TSC, APY TSC, IND TSC, CHR TSC, COU TSC, or QNL TSC. Cell proliferation was measured by an MTS assay and expressed as the mean absorbance at 490 nm/20 × 10^3 cells/90 min. The experiments were performed three times with identical outcome and the means ± SD are shown. Statistical analysis was done using analysis of variance (ANOVA) as applicable when compared between the various concentration groups of the analogs with untreated group. $P \leq .05$ was considered statistically significant. In case of QNLITSC, CHRITSC, COUITSC, and INDITSC analogs when used for HT29 and HCA7 cells the results with 2–100 μM when compared to that of untreated group are statistically significant, but when FPYITSC and APYITSC analogs were used, the results with 5–100 μM when compared to that of untreated group are statistically significant. In case of Apc10.1Has2 when QNLITSC, CHRITSC, and COUITSC analogs were used the results with 2–100 μM are statistically significant when compared to that of untreated group, but when FPYITSC, APYITSC, and INDITSC analogs were used the results with 5–100 μM are statistically significant when compared to that of untreated group

vector pCIneo transfected) and Apc10.1-Has2 cells which is stably transfected with Has-expression plasmid construct (to examine the effect of HA in the presence or absence of Has2) and controlshRNA, CD44v6shRNA-transfected HCA7, and HT29 cells (to examine the effect of silencing CD44v6 in the presence or absence of CD44v6shRNA), respectively. In the absence of Has2 expression, the Apc10.1 cells treated with COUITSC displayed an increase in apoptotic tumor cells with IC_{50} value of 2.3 μM (Table 3). Results in Fig. 8 indicate that the number of apoptotic cells (Annexin-V positive) was increased in the following order: COUITSC > Celecoxib > SFN > Licofelone. On the other hand, over-expression of Has2 or increased HA levels enhanced cell survival and reduced apoptosis in untreated controls (Tables 3, 4; Fig. 8). Furthermore, pre-treatment of HCA7 cells with CD44v6shRNA, or anti-Has2 alone significantly increased tumor cell apoptosis compared with controlshRNA, or vector control pre-treatment (Fig. 8) indicating that HA and CD44v6 are necessary for cell survival. Moreover, down-regulation of CD44v6 by CD44v6shRNA, or down-regulation of HA by anti-Has2 effectively enhanced drug sensitivity beyond the respective control-treated cells (Fig. 8, colored bars).

These observations strongly suggest that HA is indeed responsible for the increase in tumor cell survival leading to the enhancement of chemo-resistance (Tables 3, 4 and Fig. 8).

Effect of ITSC on survivin, caspase-3, expression

To further assess whether the ITSCs effects on colon HCA7, HT29, and Apc10.1Has2 cells might affect colon tumor cell-specific behavior (e.g., chemo-resistance), we decided to analyze the expression of the chemo-resistance protein survivin and cleaved caspase-3. HA-CD44 interaction has been shown to induce the expression of survivin in tumor cells (Bourguignon *et al.*, 2009). In addition, frequent co-expression of COX-2 and survivin was assessed by immuno-histochemistry in human lung cancer specimens of the patients revealed a marked reduction of tumor survivin levels in patients treated with COX-2 inhibitors prior to surgery (Krysan *et al.*, 2004). The question of whether expression of survivin (induced by HA-CD44-COX-2 interaction signaling) is the target of ITSCs analogs has not been investigated systematically. To answer this question, immunoblot analyses

Table 3 Anti-proliferative potential of ITSC analogs

Cell lines	FPYITSC IC_{50} (μM)		APYITSC IC_{50} (μM)		QNLITSC IC_{50} (μM)		CHRITSC IC_{50} (μM)		COUITSC IC_{50} (μM)		SFN IC_{50} (μM)	
Has2	–	+	–	+	–	+	–	+	–	+	–	+
HT29	28.3 ± 2.9	55.7 ± 5.7	14.9 ± 1.7	42.6 ± 5.1	15.2 ± 1.9	5.4 ± 5.7	5.8 ± 1.2	21.4 ± 3.6	3.9 ± 0.9	12.3 ± 2.3	39.2 ± 5.9	52.8 ± 6.7
HCA-7	12.5 ± 1.1	35.75 ± 2.9	12.0 ± 1.4	39.7 ± 4.9	12 ± 1.5	38.6 ± 3.9	3.8 ± 1.0	13.4 ± 1.5	2.8 ± 0.4	16.4 ± 1.1	35 ± 4.4	46.5 ± 8.5
Apc10.1	8.9 ± 01.1	19.3 ± 1.7	5.39 ± 0.9	10.5 ± 1.3	12 ± 1.5	38.6 ± 3.9	2.9 ± 0.7	6.5 ± 2.5	2.3 ± 0.6	5.7 ± 1.3	29.4 ± 1.6	45.7 ± 5.8

using antibodies (e.g., anti-survivin antibody and β-tubulin) (as loading control antibody) were employed to detect the production of survivin, caspase-3, and β-tubulin in HCA7 cells. Our data indicated that the expression of survivin (Fig. 9) is significantly increased in HCA7 cells that are treated with CD44v6shRNA, COUITSC, Celecoxib, and their combination (Fig. 9) down-regulated survivin level. Likewise results from Fig. 9 indicated that cleaved caspase-3 levels were not detectable in non-apoptotic cells but significantly increased with the treatment of Cd44v6shRNA, COUITSC, or Celecoxib, or their combinations. These results indicate that HA-CD44v6-activated COX-2 signaling actively participate in the up-regulation surviving, and the data are consistent with previously reported data where COX-2 stabilizes survivin in cancer cells (Krysan *et al*., 2004).

Effect of ITSC analogs on PGE2 synthesis

It is becoming clear that cancers are dependent upon a supportive inflammatory microenvironment, and that the cytokine prostaglandin E2 (PGE2) contributes to it and cancer progression (Park *et al*., 2006). The mechanism through which PGE2 contributes to tumor formation is partly through activation of HA-CD44 pathway. Similarly, the development of one significant cell type within the inflammatory environment is enhanced by production of prostaglandin E2 by tumor cells (Apte *et al*., 2006a, b; Krelin *et al*., 2007). These observations led us to develop the hypothesis that ITSCs may reduce the expression of PGE2. Subsequently we investigated whether anti-neoplastic effects of the present analogs as well as Celecoxib on CRC cells are mediated through the inhibition of PGE2 production. ELISA analyses were used to detect the levels of PGE2 concentration from the COX-2 positive (HCA7, HT29) and Apc10.1-Has2 cell lines (Patsos *et al*., 2010). The ELISA analyses revealed high levels of PGE2 in colon cancer cell line HCA7, followed by HT-29 (Fig. 10), which were dramatically decreased following the treatment with two potent ITC analogs, viz. CHRITSC and COUITSC, respectively. These analogs also substantially reduced the PGE2 production in COX-2 positive HCA7, HT29, and Apc 10.1 cells (Fig. 10) suggesting that suppression of cell survival as well as chemo-resistance (Figs. 3–9) by COU-ITSC may be due to the HA/CD44v6 interaction in these cells through COX-2/5-LOX-dependent mechanism (Figs. 3–11; Tables 3, 4).

Our present work thus indicates that among the new ITSC analogs seems to be a potent COX-LOX dual inhibitor which is active against colon cancer cells with IC_{50} values lower than the reference COX-2 specific drug, viz. Celecoxib. It inhibits PGE2 production in these cells indicating that the coumarin motif in it may have some

Table 4 Anti-proliferative potential of ITSC analogs

Cell lines	INDITSC IC_{50} (μM)		Phenyl ITSC (PEITSC) IC_{50} (μM)		Celecoxib IC_{50} (μM)		Licofelone IC_{50} (μM)		CD44v6shRNA IC_{50} (pmoles)	
Has2	−	+	−	+	−	+	−	+	−	+
HT29	6.6 ± 7	24.0 ± 2.4	32.0 ± 4.9	52.6 ± 5.1	4.8 ± 1.6	13.5 ± 1.7	72.2 ± 6.9	123.7 ± 10.9	65.5 ± 6	169.6 ± 15
HCA-7	5.0 ± 1.3	28.4 ± 1.9	28.0 ± 3.7	39.7 ± 4.9	3.8 ± 1.3	10.7 ± 1.5	58.9 ± 6.4	115.9 ± 9.5	58.2 ± 5	135.0 ± 11.6
Apc10.1	4.2 ± 1.1	9.8 ± 19	19.5 ± 2.6	37.5 ± 3.3	2.8 ± 0.8	11.5 ± 0.8	41.5 ± 4.8	72.5 ± 7.9	45.8 ± 5.9	75.5 ± 8.9

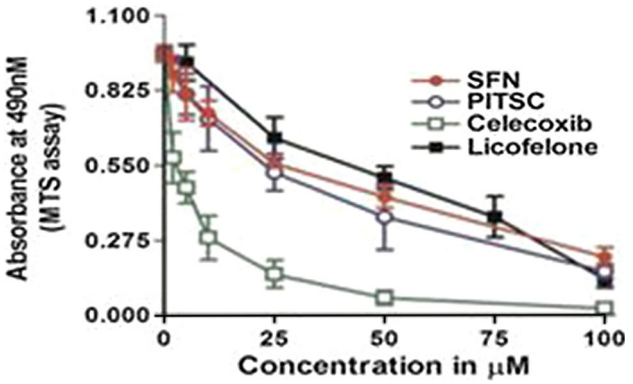

Fig. 6 Anti-proliferative effect of standard ITSCs (SFN and PITSC), Celecoxib, and Licofelone on colon cancer cells. HCA-7 cells were left untreated, or treated with 16 h addition of various doses (as indicated in the figure) of Licofelone, Celecoxib, SFN, or PITSC for a period of 48 h. Cell proliferation was measured by an MTS assay and expressed as the mean absorbance at 490 nm/20 × 103 cells/90 min. The experiments were performed three times with identical outcome and the means ± SD are shown

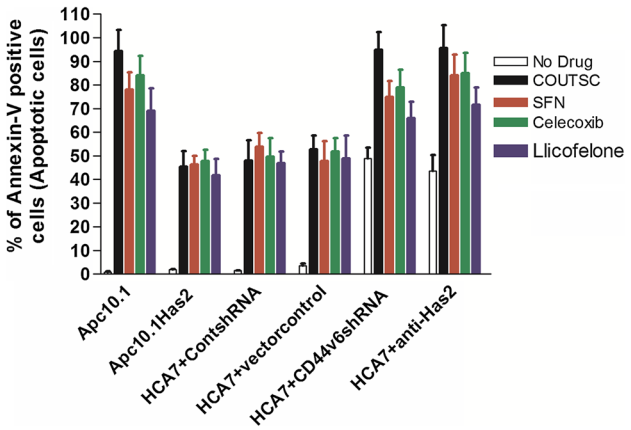

Fig. 8 ITSC-induced Apoptosis in colon cancer cells Analysis of ITSC-induced Apoptosis in HCA7, Apc10.1, and Apc10.1Has2 cells. Here we designate the cells apoptotic when they display Annexin V-positive staining. In each sample, at least 1,000 cells from five different fields were counted. The percentage of apoptotic cells calculated as Annexin V-positive cells/total number of cells. The values are presented as the mean ± SD. Statistical analysis was done using analysis of variance (ANOVA) as applicable when compared between the groups. $P \leq .05$ was considered statistically significant. When the group Apc10.1Has2 compared with Apc10.1, or when HCA7 + CD44v6shRNA compared to HCA7 + ControlshRNA, or HCA7 + anti-Has2 compared to HCA7 + vectorcontrol, results are considered statistically significant

special affinity toward CRC cells with respect to their COX-LOX status and which could be attributed to the presence of CD44v6 and HA expression in these cell lines (Kuhn *et al.*, 2007). Interestingly, the standard inhibitor for

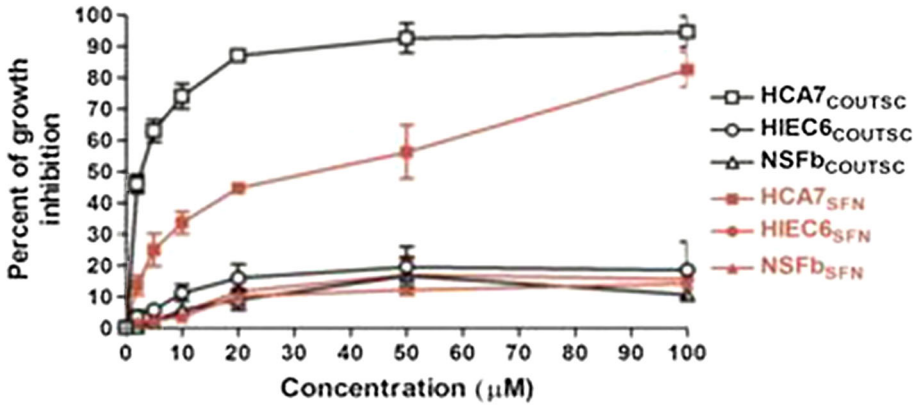

Fig. 7 COUITSC exhibited no pronounced cytotoxicity to normal cells. CA-7 cells, human normal epithelial cells, and human normal skin fibroblasts were left untreated, or treated with 16 h addition of various doses (as indicated in the Fig. 7) of COUITSC and 24 h later mitochondrial activity/viability was measured by MTT assay. The experiments were performed three times with identical outcome and the means ± SD are shown

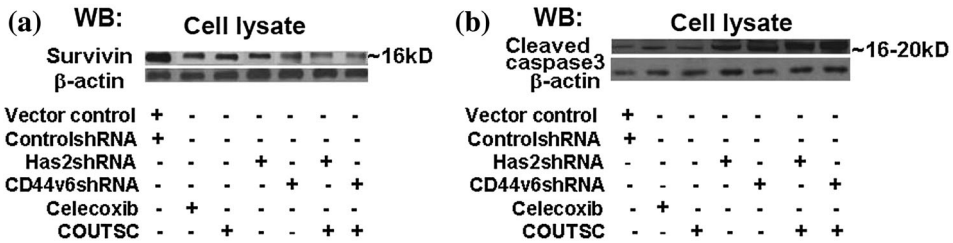

Fig. 9 ITSCs significantly decreased levels of chemo-resistant protein survivin, and increased apoptotic protein caspase-3 in HCA7 cells. Cell lysates isolated from HCA7 cells that express high level of HA, transfected with either vector control, or anti-Has2 cDNA, controlshRNA, CD44v6shRNAHA for 24 h, or treated with 16 h addition of celecoxib, or COUITSC, or first transfected with CD44v6shRNA followed by a 16 h addition of celecoxib, or COUITSC were processed for immunoblotting using anti-survivin antibody **a**, anti-caspase-3 antibody **b**, or anti-β-actin antibody (as a loading control). Data are representatives of three independent experiments

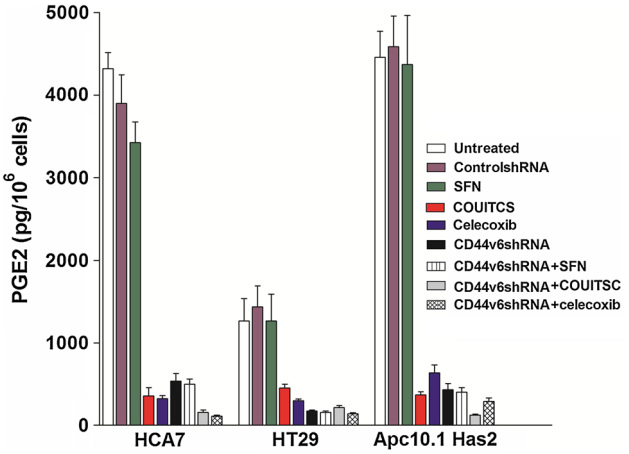

Fig. 10 Effect of ITSCs on PGE2 levels in colon cancer cells ITSCs target CD44v6/COX-2 to alter PGE2 level in colon tumor cells. Cell supernatants from HCA7, HT29, and Apc10.1Has2 cells that express high level of HA, transfected with either controlshRNA, or CD44v6shRNAHA for 24 h, or treated with 16 h addition of 25 μM SFN, or 2.5 μM COUTSC or 2.5 μM Celecoxib or first transfected with CD44v6shRNA followed by a 16 h addition of 25 μM SFN, or 2.5 μM COUTSC or 2.5 μM Celecoxib were processed for Enzyme-linked immunosorbent assay analysis (ELISA) analysis of the PGE2 level. Bars represent the means ± SE ($n = 3$) and the results were compared with untreated cells. Statistical analysis was done using analysis of variance (ANOVA) as applicable when compared between the groups. $P \leq .05$ was considered statistically significant. When the groups (CD44v6shRNA, CD44v6shRNA + SFN, CD44v6shRNA + COUITSC, CD44v6shRNA + celecoxib) compared with the corresponding control group (ControlshRNA), results are considered statistically significant. When the groups (COUITSC, celecoxib) compared with the corresponding untreated control group, results are considered statistically significant

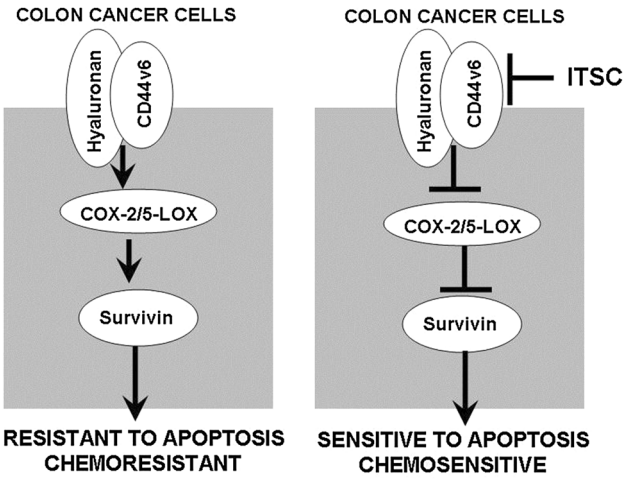

Fig. 11 HA-CD44v6-COX-2/5-LOX pathway modulates resistance to apoptosis of colon cancer cells. *Left panel* In HA-CD44v6-COX-2-5-LOX overexpressing cells, elevated survivin and lower caspase3 levels accounts for the elevated apoptosis resistance of colon cancer cells. *Right panel* ITSCs suppress HA-CD44v6-COX-2-5-LOX pathway leading to increased caspase-3 and inhibition of survivin level, thus portraying cells more sensitive to apoptosis induction

HA-synthase is 4-Methylumbelliferone, which in fact is a coumarin compound. Although these compounds have been shown to inhibit hyaluronan synthase-2 (Has2) mRNA level and cell surface HA levels to some extent in other cell type (Vigetti *et al.,* 2011; Lokeshwar *et al.*, 2010), results of genetic knock-out studies show that deletion of Has2 and CD44 genes has very different outcomes. Has2-null mice die in utero due to impair development of cushion mesenchymes (Camenisch *et al.*, 2000). On the other hand, CD44-null mice show increased inflammation because lack of CD44 impairs the clearing of HA from sites of inflammation (Teder *et al.*, 2002). Thus, we think that CD44 may be a much safer therapeutic target than Has2.

Conclusions

Specifically, our findings indicate that HA-CD44v6-mediated COX-2/5-LOX signaling facilitates survivin production, which in turn, exerts its influence on colon tumor cell-specific functions including anti-apoptosis and chemo-

resistance. This capacity of COX-2/5-LOX to enhance cell survival may help explain the COX-2-dependent resistance to radiation and chemotherapy in colon cancer. The work also demonstrates that over-expression of CD44v6shRNA as well as treatment with potent ITSC analog can significantly decrease the survival of colon tumor cells. Results described presently thus offer an opportunity to evolve potent inhibitors of HA synthesis and CD44v6 pathway and thus underscoring the importance of the ITSC analogs as chemopreventive agents for targeting HA/CD44v6 pathway.

Experimental Protocols

Materials

Apc 10.1, an intestinal cell line, was derived from *Apc* Min/+ mice and retains the host heterozygous *Apc* genotype. HCA7 clone 29 colon carcinoma cells were obtained from ECCA (United Kingdom). HT-29 cells were obtained from ATCC (Manassas, VA). RPMI, Dulbecco's modified Eagle's medium (DMEM) low glucose, glutamine, and pyruvate were from Life technology. Fetal bovine serum was from Atlanta Biological (GA); and L-glutamine, Gentamicin sulfate, and Amphotericin B were from Hyclone. HT29 cells were grown in RPMI media with 10 % FBS, and HCA7 and Apc10.1 cells were grown in RPMI media with 10 % FBS. Actinomycin D, cycloheximide, Nonidet P-40, ethylene glycol tetraacetic acid, sodium orthovanadate, glycerol, phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, Aprotinin, and hydroxyethyl piperazine ethane sulfate (HEPES) were purchased from Sigma (St. Louis, MO). The celecoxib, licofelone, sulforaphane (SFN), phenyl isothiocyanate (PITSC), antibodies against CD44v6, COX-2, 5-LOX, caspase 3, β-actin, horse radish peroxidase-linked anti-rabbit and anti-mouse antibodies, and Luminol reagent were purchased from commercial sources (Sigma, Santa Cruz Biotechnology, Abcam, Ebioscience, Thermo Fischer). CD44v6shrNA or controlshRNA are designed as described in our earlier studies (Misra *et al*., 2009).

Synthetic procedures

All reagents for synthesizing ITSCs were from Sigma-Aldrich of analytical grade (AR) and were used without further purification. Solvents employed were purified by standard procedures prior to use (Perrin and Armarego, 1988). The ITSC analogs were synthesized by the condensation reaction between thiosemicarbazide and respective aldehydes/acetophenones in methanolic solvent following a protocol described earlier (Scovill *et al*., 1982; Adsule *et al*., 2006; Doyle *et al*., 1956; Kapoor *et al*., 2011; Li *et al*., 2010). The synthesized compounds complied with the analytical and spectroscopic data reported by these workers. The FT-IR spectra of the compounds were recorded on JASCO FTIR-4100 spectrophotometer. The MASS spectra of synthesized compounds were performed on Shimadzu GC-17A, GCMS-QP5050A version 1.10 Gas Chromatograph Mass Spectrometer by GC–MS analysis method at Department of Chemistry, University of Pune, India. 1H-NMR spectra were recorded on a FT-NMR Varian Mercury YH-300 Spectrometer in Pune University, India. The analysis of C, H, and N contents in the compounds was performed on HOSLI CHN analyzer in the Microanalytical laboratory at Department of Chemistry, University of Pune (INDIA).

Cell culture

Epithelial cell culture

HT29, HCA7, and Apc10.1Has2 were cultured in DMEM with normal glucose, glutamine, and pyruvate (Life technology) supplemented with 10 % fetal bovine serum, 2 μM L-glutamine, gentamicin sulfate (50 μg/ml), and amphotericin B (5 μg/ml) at 37 °C in 10 % CO_2. The medium was changed every other day to remove dead and non-attached cells until colon cancer cells reached confluence. Monolayer cultures were maintained in the same medium. Colon cancer cells were used between the second and sixth passages in all experiments. All the treatments and transfection experiments were carried out in cells that were serum starved for 24 h.

Human Normal Epithelial Cells (HIEC6) and Human Normal Skin Fibroblasts (NSFbs) Cell Culture

The HIEC-6 human intestinal cell line (from J.F. Beaulieu, University of Sherbrook, Quebec, Canada) was maintained in DMEM (high glucose), 4 % fetal bovine serum, 20 mM HEPES buffer (pH 7.4), 50 units/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml insulin, and 5 ng/ml human recombinant and used between the 15th and 17th passage in this study as described earlier (Misra *et al*., 2008a, b). Cell lines were grown at 37 °C in 5 % CO_2.

The NSFbs were isolated and cultured as previously reported (Ghatak *et al*., 2013, 2014). Briefly, skin tissues were diced (approx. 0.5 mm × 0.5 mm pieces) and cultured in DMEM with normal glucose, glutamine, and pyruvate (Life technology) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, gentamicin sulfate (50 μg/ml), and amphotericin B (5 μg/ml) at 37 °C in 10 % CO_2. The medium was changed every 3 days to remove dead and non-attached cells until fibroblasts reached confluence. Monolayer cultures were maintained in the same medium. Skin fibroblasts were used between the second and fourth passages in all experiments.

Cell lysis and immunoblotting

Colon cancer cells were cultured until they were confluent. Cells were washed twice at 4 °C with PBS, harvested with 0.05 % Versene, and then washed in cold PBS again. The cells were pelleted by centrifugation at 5,000×*g* for 2 min at 4 °C. The pellet was treated with the lysis buffer containing 1 % Nonidet P-40, 0.5 mM EGTA, 5 mM sodium orthovanadate, 10 % (v/v) glycerol, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 50 mM HEPES, pH 7.5. The lysates were clarified by centrifugation at 12,000×*g* for 10 min at 4 °C and then stored at −80 °C as described previously (Misra *et al*., 1999, 1998, 2006; 2008a, b; 2003, 2005). Cell lysates (normalized for protein concentration) were analyzed by immunoblotting as described previously (Misra *et al*., 2006; 2008a, b). The proteins on the blots were analyzed with antibodies for CD44v6, COX-2, 5-LOX, surviving, and 5-LOX (β-tubulin and β-actin as internal standards), and detected by luminol reagent (Santa Cruz Biotechnology, CA) following treatment with horse radish peroxidase-linked anti-rabbit or anti-mouse antibody as secondary. Each protein was analyzed in samples from at least three independent experiments from each set of fibroblasts.

RNA Silencing

ControlsiRNA (scrambledsiRNA) and CD44v6shRNA were prepared as described previously (Cheng *et al*., 2006; Misra *et al*., 2009).

CD44v6shRNA Cloning in pSicoR Vectors

Double-stranded oligonucleotide cassettes for controlshRNA (scrambledshRNA), CD44v6shRNA were prepared. The linearized pSicoR vectors were ligated to the double-stranded oligonucleotide cassettes. The resulting pSicoR-CD44v6shRNA (CD44v6shRNA) transfectants constitutively silence CD44v6 genes in the cells. pSicoR-scrambledshRNA (controlshRNA) transfectants were used as control to the above shRNA transfectants.

Transient transfection using colon cancer cells

All transfections were done using Lipofectamine (Invitrogen) in cultures at ∼75 % confluence. After transfection, the cultures were grown for another 72–96 h for analyses.

Determination of PGE2 in the culture medium

Colon cancer cells (1×10^5 cells/well) were cultured in 6-well culture plates with appropriate media plus 10 % FBS. At confluence, culture medium was discarded, and each well was washed with PBS twice. Serum-free medium (QBSF-51; Sigma) was then added followed by incubation for various time points. The supernatants were collected and stored at −80 °C until use. PGE2 concentrations in culture supernatants were measured by an ELISA assay from (Elisa kit was from Cayman Chemicals).

Cell Proliferation Assay

Cell proliferation was measured by CellTiter 96® AQueous Assay (Promega). The reagent consists of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS). MTS is reduced by cells into a soluble formazan product in tissue culture medium. Growing cells were harvested, counted, and seeded at the 20 × 103 cells (200-μl volume) into 96-well microplates. After 18 h, culture medium was replaced by medium containing experimental agents (200 μl) as indicated in Fig. 2. MTS/PMS solution was freshly prepared at 2/0.92 mg/ml in DPBS. Ten micro liters of MTS/PMS reagent (excess MTS/PMS) was added to each well and then incubated at 5 % CO_2 atmosphere. Absorbance at 490 nm was recorded at 90 min. A blank experiment detecting cell-free background absorbance was also performed in parallel. Absorbance shown in the figures was obtained by subtracting the absorbance of cell-free equivalents. Trypan blue exclusion showed less than 1 % cell death both before and after the assays.

Cytotoxicity analysis

MTT was first prepared as a stock solution of 5 mg/ml in phosphate buffer (PBS, pH 7.2) and was filtered. At the end of the treatment period of our tested compounds (24 h), with four different concentrations in triplicate, 25 μl of MTT solution was added to each well. After incubation for 4 h at 37 °C, 100 μl of solubilizing buffer (10 % sodium dodecyl sulfate dissolved in 0.01 N HCl) was added to each well. After overnight incubation, the 96-well plate was read by an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm for absorbance density values to determine the cell viability. The viable cells produced a dark blue formazan product, whereas no such staining was formed in the dead cells.

IC_{50} of ITSC analogs, CD44v6shRNA, Celcoxib, Licofelone, SFN, and PITSC in colon cancer cell growth

Colon cancer cells were either untreated or treated with ITSCs, ITSC analogs, or celecoxib, or licofelone, or SFN, and

PITSC, in the presence or absence of HA, or controlshRNA, or CD44v6shRNA as above. These cells were then plated in 24-well culture plates in 0.2 ml of DMEM, or RPMI medium (chosen appropriately for each cell line) supplement (Invitrogen) containing no serum for 24 h at 37 °C in 5 % CO_2, 95 % air. In each experiment, a total of five plates (5wells/treatment) were used. Experiments were repeated 5 times. The in vitro growth of these cells was determined by measuring increases in cell number using Coulter counter. IC_{50} is designated as the concentration (nM) of chemotherapeutic drugs (e.g., ITSC analogs, CD44v6shRNA, celcoxib, licofelone, SFN, or PITSC) that causes 50 % inhibition of tumor cell growth. IC_{50} values are presented as the means ± SD. All assays consisted of at least 5 replicates and were performed on at least five different experiments analysis.

Analyses of ITSC analogs-induced apoptosis in colon cancer cells

Cells were designated apoptotic when displaying Annexin V-positive staining. In each sample, at least 1,000 cells from five different fields were counted, with the percentage of apoptotic cells calculated as Annexin V-positive cells/total number of cells (Annexin V-positive cells/total cells × 100 %). The values are presented as the mean ± SD.

Statistical analysis

Each experiment was repeated three times for each set of fibroblasts, which were considered as $n = 9$ and pooled for statistical analysis. Western blot analyses, proliferation experiments (MTS assay), and cytotoxic assay (MTT assay) for each separate experiment were repeated between 3 and 4 times, depending upon the particular study. Data are expressed as ±SD. Statistical analysis of the Western blots was performed using t test with Mann–Whitney modification or analysis of variance (ANOVA) as applicable. $P \leq .05$ was considered statistically significant.

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