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PAPER

Design and synthesis of colchicine derivatives with potent *in vitro* and *in vivo* anticancer activity and reduced p-glycoprotein induction liability

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Colchicine (1), a nature-derived microtubule polymerization inhibitor develops multi-drug resistance in tumor cells due to its P-gp substrate and induction activity, which in turn leads to its rapid efflux from tumor cells. This auto-induction of the efflux of colchicine remains a major challenge to medicinal chemists. Based on the structure-based molecular modeling, a series of new colchicine derivatives were designed and synthesized with a potential of reduced P-gp induction liability. Screening of prepared derivatives for P-gp induction activity revealed that number of derivatives possess remarkably lower P-gp-induction activity (>90% intracellular accumulation of rhodamine 123 in LS-180 cells) compared to the parent natural product colchicine (62% Rh123 accumulation in LS-180 cells). The reduced P-gp-induction activity of new derivatives may be due to their reduced ability to interact and change the conformation of P-gp. The synthesized derivatives were then screened for antiproliferative activity against two colon cancer cell lines including HCT-116 and Colo-205. The derivative 4o showed potent cytotoxicity in HCT-116 cells with IC_{50} of 0.04 µM with significantly reduced P-gp induction liability. Compound 4o also inhibited microtubule assembly and induced expression of pro-apoptotic protein p21. In Ehrlich solid tumor mice model, the compound 4o at 2 mg/kg dose (oral) showed 38% TGI with no mortality. Compound 4o possessing potent *in vitro* and *in vivo* anticancer activity, significantly reduced P-gp-induction activity and excellent physicochemical and pharmacokinetic properties opens up a new opportunity for colchicine scaffold.

Introduction

Colchicine (**1**), a natural product isolated from *Colchicum autumnale* (Meadow Saffron) has been used for the treatment of rheumatic condition 'gout' and also for the treatment of familial Mediterranean fever, pericarditis, and Behcet's disease.[1-3] Colchicine exhibits potent antiproliferative activity by binding to tubulin, inhibiting it's self-assembly and microtubule polymerization and finally arresting cell division at metaphase. Colchicine interacts mainly with three known proteins *viz.* tubulin, cytochrome P450 3A4 (CYP3A4), and p-glycoprotein (P-gp). CYP3A4 is the major protein found mainly in liver and intestine which metabolizes colchicine by demethylation at C-2 and C-3 positions. The development of tumor resistance to colchicine mainly occurs via its P-gp induction activity causing its active efflux from tumor cells, and increase in the expression of β III tubulin isotype.[2, 4, 5]

P-gp induction is responsible for the multidrug resistance (MDR) particularly in the case of cancer cells and makes the currently available chemotherapy ineffective.[6, 7] Therefore, the search for cytotoxic compounds which are devoid of MDR is of great importance.[8, 9] Colchicine (**1**) is a substrate of P-gp efflux pump and it induces its activity by inducing conformational change.[4] The P-gp substrate and induction liability of colchicine (**1**) restricts its use in combination with other P-gp substrate cytotoxic drugs like vinblastine, doxorubicin and paclitaxel.[10, 11] To overcome the P-gp-mediated resistance of colchicine, Cosentino *et al.*[12] demonstrated that its *N*-benzylated derivative **2** maintained similar level of cytotoxic activity in the resistant A2780AD ovarian carcinoma cells which are known to overexpress the ABCB1 (P-gp) drug transporter. The structures of colchicine (**1**) and derivative **2** are shown in Figure 1.

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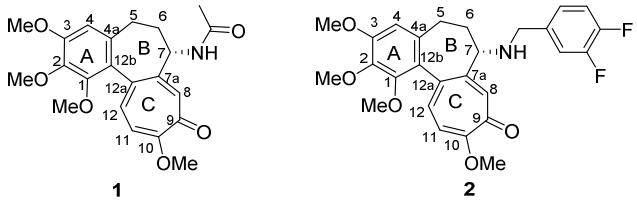


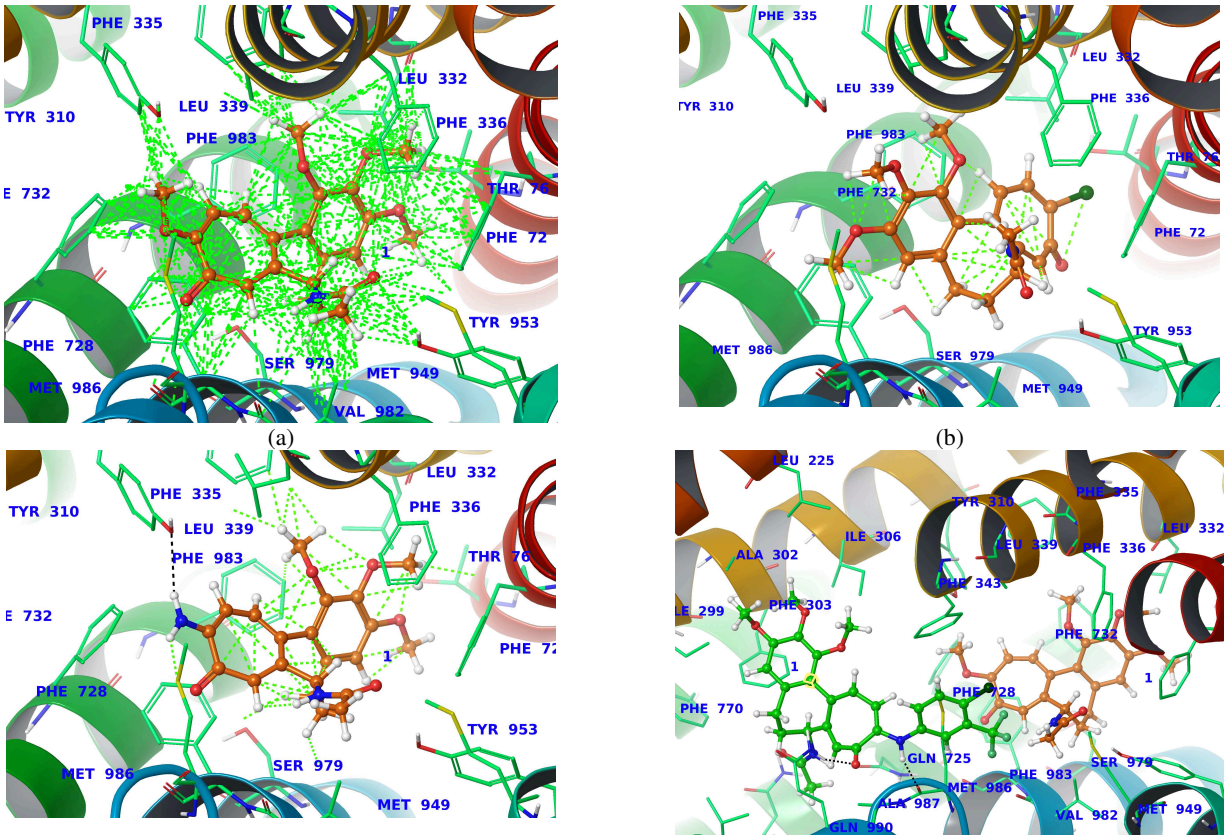
1 2

Figure 1. Chemical structures of colchicine (**1**) and its derivative **2**.

The discovery of *Caenorhabditis elegans* P-gp 3D-structure,[7, 13-15] encouraged us to tailor colchicine structure based on its interactions with P-gp to specifically address its P-gp induction liability without compromising cytotoxicity. Towards the search for anticancer compounds with minimum P-gp induction liability and in continuation to our efforts on the discovery of natural product-based P-gp modulators,[16-18] herein we report our efforts on reducing P-gp induction liability of anticancer natural product colchicine. The structure-activity relationship (SAR) of colchicine as anti-tubulin agent[12, 19-25] is available in the literature, however it's SAR for P-gp-induction activity has not been examined. Herein, our objective was to address the P-gp-induction liability of colchicine with significantly maintaining its antiproliferative activity. Based on the structure-based molecular docking, a series of C- and B-ring substituted derivatives were designed, synthesized and evaluated for P-gp-induction and antiproliferative activity. Identified lead derivative was then studied in detail for its target activity, physicochemical properties, other liabilities, pharmacokinetics, and *in vivo* efficacy.

Results and discussion

Designing colchicine derivatives. Since our aim was to identify colchicine derivatives with reduced P-gp-induction liability without affecting its antiproliferative activity, firstly, the established colchicine SAR was examined, which indicated that A- and C-rings of colchicine scaffold are the minimum structural features necessary for high affinity drug–tubulin binding.[19] Although, it has been reported that inter-changing the position of carbonyl and methoxy of the C-ring leads to completely inactive compound (isocolchicine), but recently it has been observed that isocolchicine analog efficiently inhibited microtubulin assembly.[20, 21] Based on the literature reports,[12, 22-25] it was observed that C-ring modifications and B-ring substitutions (particularly, on acetamido group) are well tolerated for its anti-tubulin activity. Therefore, in order to maintain anti-tubulin activity while modulating P-gp-induction activity, we planned to design derivatives by modifications on B and C rings using P-gp structure guided information. P-gp is a 170 kD large transmembrane ATPase protein whose structure and mechanism of substrate translocation is very complex and not yet fully understood.[26] Available reports suggest that the vacuum cleaner hypothesis of P-gp is highly accepted model.[10, 27, 28] The



(a) (b) (c) (d)

Figure 2. Interaction of colchicine (Figure 2a, orange), 10-chloro **3** (Figure 2b, orange), 10-amino **4k** (Figure 2c, orange) and 10-NH-substituted **4o** (Figure 2d, orange colchicine and green **4o**) derivatives with P-gp.

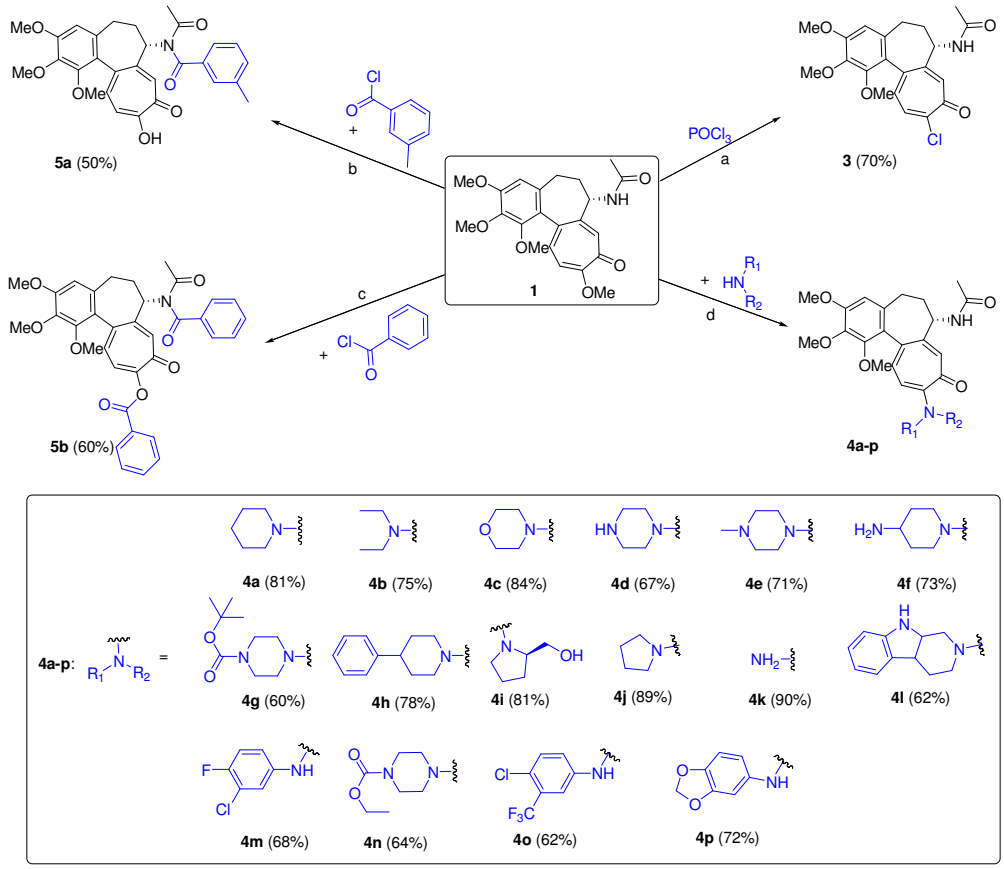


Figure 3. Synthesis of colchicine derivatives 3, 4a-p and 5a-b. Reagents and conditions: (a) $POCl_3$ (4.0 equiv.), DMF (2 mL), rt, 24 h, 70%; (b) *m*-methyl-benzoyl chloride (1.5 equiv.), triethylamine (2.5 equiv.), CH_2Cl_2 (3 mL), rt, 24 h, 50%; (c) benzoyl chloride (1.5 equiv.), triethylamine (2.5 equiv.), CH_2Cl_2 (3 mL), rt, 24 h, 60%; (d) NH-R_1R_2 or liquid ammonia (4.0 equiv.), MeOH (3 mL), rt, 24-72h, 60-90%.

interaction of colchicine at substrate-binding site of P-gp was studied using human P-gp homology model. The mechanism of interaction of colchicine and its exact binding site to P-gp is not yet fully understood by crystallographic studies. However, few reports are available based on predictions[11, 29, 30] which state that colchicine binds to substrate binding pocket of P-gp, where it induces conformational changes in the transmembrane helical domains and facilitate the transport behavior of other ligands as well as colchicine itself,[4] as large binding cavity of P-gp can accommodate two or more ligands simultaneously.[31-33] As reported earlier,[29] colchicine displayed strong hydrophobic interaction with the Ile-306, Phe-335, Phe-728 residues of ligand binding cavity. The interaction map of colchicine with P-gp is depicted in Figure 2a where all the interactions as reported previously were observed except Ile-306. Analysis of the P-gp substrate binding cavity, substrate translocation mechanism and colchicine P-gp binding interactions reveals potential role of hydrophobic interactions in colchicine efflux by P-gp in resistant tumors. Therefore, in order to reduce P-gp efflux liability, it was decided to introduce polar substituents at C-10 position of colchicine by replacing 10-OMe group with OH, Cl, NH_2 or NH-substituted groups, to dodge hydrophobic interactions. The interaction maps of designed derivatives in the P-gp binding cavity showed less hydrophobic interactions compared with the colchicine (Figure 2). Based on the loss of hydrophobic interactions and gain of polar H-bonding in newly designed colchicine derivatives, we propose that designed compounds would be less liable to get effluxed by hydrophobic transmembrane domains of P-gp compared to colchicine (which mainly shows hydrophobic interactions with P-gp). Therefore, a series of 10-amino substituted derivatives was planned for synthesis.

Chemistry. The preparation of semisynthetic derivatives of colchicine was directed on the substitution of *NH*-acetyl side chain and replacement of 10-OMe group with various *N*-linked functionalities. Treatment of colchicine (**1**) with phosphoryl chloride directly produced 10-chloro-10-demethoxy colchicine (**3**)[34, 35] in 70% yield. Earlier the preparation of **3** was reported in two steps, wherein the first step was replacement of OMe group with OTs followed by treatment with HCl gas.[35] As 10-OMe group of colchicine was found to be highly labile for removal, we decided to replace it with various amine functionalities. Treatment of colchicine (**1**) with different amines (HN-R_1R_2) in methanol produced corresponding 10-amino-linked colchicine derivatives **4a-p** in 60-90% yield (Figure 3). The treatment of colchicine with liquid ammonia produced product **4k**. The 10-OMe group was found to be more labile for removal as it is located adjacent to the carbonyl group, which facilitates the nucleophilic attack by amines. The possible mechanism for

Table 1 Antiproliferative and P-gp induction activities of colchicine and its derivatives

Entry	P-gp induction activity (% intracellular accumulation of Rh123 inside LS-180 cells at 100 nM)[a]	Cytotoxicity (IC_{50}, μM)	
		HCT-116	Colo-205
1	62.91 ± 9.5[@]	0.05 ± 0.001	0.032 ± 0.001
3	106.51 ± 4.9***	6.0 ± 0.009	4.0 ± 0.007
4a	119.01 ± 3.7***	3.0 ± 0.008	1.8 ± 0.005
4b	95.38 ± 1.67**	0.800 ± 0.003	0.430 ± 0.009
4c	95.97 ± 3.1**	5.0 ± 0.009	1.2 ± 0.004
4d	95.52 ± 9.1**	>10 ± 0.027	8.0 ± 0.008
4e	102.82 ± 9.2***	3.0 ± 0.008	1.0 ± 0.006
4f	102.04 ± 4.0***	10 ± 0.024	5.0 ± 0.006
4g	90.94 ± 2.6**	4.0 ± 0.008	3.0 ± 0.007
4h	101.03 ± 8.3***	1.0 ± 0.006	0.800 ± 0.009
4i	86.53 ± 6.7	10 ± 0.025	8.0 ± 0.008
4j	107.12 ± 7.3***	0.300 ± 0.002	0.240 ± 0.002
4k	107.41 ± 4.1***	0.150 ± 0.002	0.120 ± 0.002
4l	119.70 ± 1.0***	3.0 ± 0.008	0.800± 0.003
4m	90.05 ± 7.0**	0.700 ± 0.003	0.500 ± 0.003
4n	95.49 ± 2.1**	>10 ± 0.026	8.0 ± 0.008
4o	90.29 ± 7.3**	0.040 ± 0.001	0.030 ± 0.001
4p	102.66 ± 5.0***	4.0 ± 0.008	3.0 ± 0.008
5a	101.88 ± 3.0***	1.2 ± 0.004	0.9 ± 0.002
5b	88.08 ± 7.1	>10 ± 0.023	8.0 ± 0.009
Rifampicin	69.01 ± 9.5	nd	nd

[a] The % intracellular accumulation of Rh123/protein in LS-180 cells. The decrease in % intracellular accumulation (compared to control) of Rh123 indicates induction of P-gp. Rifampicin (10 μM) was used as a reference P-gp-inducer. Statistical comparisons were made between control vs **1** and **1** vs other compounds. The p value <0.5 was considered to be significant. P value *< 0.5, **<0.01, ***<0.001. Comparison between control and **1** are represented by @. Data are expressed as mean ± SD; nd, not determined.

formation of 10-amino linked compounds is depicted in Figure S97 of ESI.

The ^{1}H NMR of compounds **4a-p** showed the absence of one –OCH_3 group at δ 4.1 ppm suggesting the substitution at 10th position which was further confirmed by the 2D NMR analysis of compound **4a**. The reaction of colchicine (**1**) with secondary amines proceeds faster as compared to primary amines. Compounds **5a** and **5b** were prepared by routine benzoylation protocol. Treatment of colchicine with 3-methyl benzoyl chloride produced 10-demethyl *N*-benzoyl derivative **5a** in 50% yield. In this case, one more product (possibly a di-substituted product) was also formed, however could not be isolated. Similarly, when colchicine was reacted with benzoyl chloride, two products were formed, however we could isolate only dibenzoylated product **5b** (60% yield). The synthesis of colchicine derivatives **3-5** is depicted in Figure 3.

SAR of colchicine and synthesized derivatives for P-gp-induction and antiproliferative activity. All synthesized compounds along with colchicine (**1**) were screened for their ability to induce P-gp in P-gp-overexpressing LS-180 cells and antiproliferative activity in two colon cancer cell lines including HCT-116 and Colo-205. The P-gp induction activity was determined in P-gp-overexpressing LS-180 cells using rhodamine123 (Rh123) cell exclusion method. Rifampicin (69% intracellular accumulation of Rh123) was used as positive control in this study.

Colchicine (**1**) showed potent cytotoxicity in HCT-116 and Colo-205 cells with IC_{50} values of 0.05 and 0.032 μM. However, it showed P-gp induction as indicated by the decrease in % intracellular accumulation of Rh123 in LS-180 cells (62%) compared to the control (100%). The colchicine derivative substituted with chloro group at C-10 (compound **3**) showed loss of P-gp induction liability (106% intracellular accumulation of Rh123) due to loss of hydrophobic interactions at C-10 position (Figure 2b) but the cytotoxicity has been reduced drastically (IC_{50} values 6.0 and 4.0 μM). Further, introduction of –OH group at C-10 position and a benzoyl group at C-7 acetamido position(compound **5a**) showed slightly better cytotoxic activity than **3** with IC_{50} values of 1.2 (HCT-116) and 0.9 (Colo-205) μM with loss of P-gp induction (101% intracellular accumulation of Rh123) liability. However, disubstituted benzoyl compound **5b** showed complete loss of cytotoxicity. Further, the introduction of amino group (compound **4k**) at C-10 position showed improved cytotoxicity (0.150 μM in HCT-116 and 0.120 μM in Colo-205) with no P-gp induction liability (107% intracellular accumulation of Rh123) due to loss of hydrophobic interactions and gain of polar hydrogen bonding and ionic interactions with Tyr-310 and Met-986 (Figure 2c). This suggests that colchicine modifications at C-10 position with amines can be useful in reducing P-gp induction liability. The loss of P-gp induction liability in these compounds is most probably due to the inability of P-gp to translocate polar amino functionality bearing colchicine derivatives. Furthermore, substitution of different substituted primary and secondary amines at C-10 position resulted in the identification of compounds **4b**, **4h**, **4j**, **4m** and **4o** with potent cytotoxicity ($IC_{50} \leq 1.0$ μM in HCT-116 cells) and devoid of P-gp induction liability. The compound **4o** was found to display slightly better cytotoxicity (IC_{50} 0.04 μM in HCT-116 and 0.03 μM in Colo-205 cells) than colchicine.

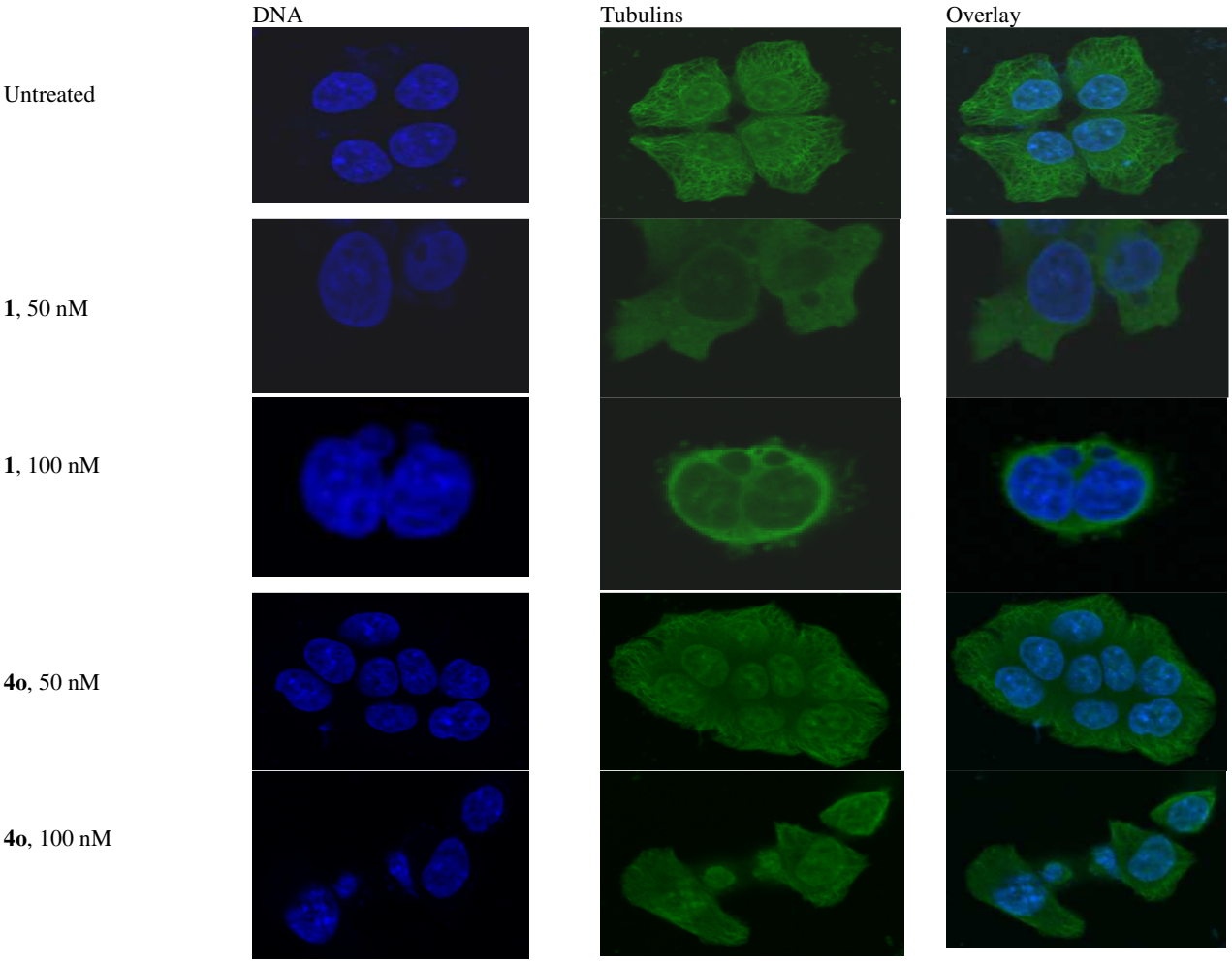


Figure 6 Effect of colchicine (**1**) and its derivative **4o** on microtubules in HCT-116 cells. Cells were cultured on coverslips. After 24 h, the cells were treated with different concentrations of the compound. Immuno-cytochemical staining was conducted using anti-α-tubulin antibody and Alexa Flour-488-labeled secondary antibody. Nuclei were stained with DAPI (left panel). The data are representative of three separate sets of experiments.

Compound **4o** also showed significantly reduced P-gp induction liability (90% intracellular accumulation of Rh123). The loss of P-gp induction liability of compound **4o** is possibly be due to its altered binding orientation and position with P-gp in comparison to colchicine (**1**). Compound **4o** does not bind deeper in the substrate binding pocket of P-gp, and stays on the outer side of the cavity and display H-bonding and ionic interactions with the Ala-987 and Gln-725 residues (Figure 2d). Based on the P-gp-induction and antiproliferative activity results (Table 1), the derivative **4o** was selected for further studies.

The cytotoxicity of compound **4o** and colchicine (**1**) to the normal epithelial tissue (fR2) was evaluated to estimate their selectivity towards cancer cells. As a result, we found that **4o** possess 1.75 fold selectivity towards HCT-116 cells compared with normal fR2 cells (Table 2).

The EC_{50} for P-gp induction was determined and the compound **4o** has shown 5-times less P-gp induction liability measured in terms of EC_{50} value (69 nM) compared to that of colchicine (14.4 nM) (Table 2). Further, in order to relate the altered P-gp-induction activity with its change in expression, we analyzed the expression of P-gp in LS-180 cells treated with colchicine (**1**) and derivative **4o** by western blotting. As depicted in Figure 4, the results indicated that neither colchicine nor derivative **4o** showed effect on the expression of P-gp in the cells. Colchicine is a substrate and known to induce P-gp activity by change in its conformation,[4] however, the reduced induction activity of new derivatives may be due to their reduced ability to interact and change the conformation of P-gp.

Table 2 P-gp induction liability and selectivity of compound **4o** and colchicine (**1**) *versus* epithelial tissue (fR2)

Entry	**1**	**4o**
P-gp induction (EC_{50}, nM)	14.4	69
Cytotoxicity (IC_{50}, μM)		
HCT-116	0.05 ± 0.001	0.04 ± 0.001
fR2	0.05 ± 0.001	0.07 ± 0.002

Selectivity (fR2/HCT-116)	1.0	1.75

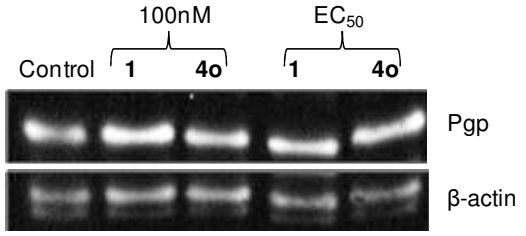


Figure 4. Western-blot analysis of colchicine (**1**) and derivative **4o** for P-gp activity

Mechanistic evaluation. Tubulin is a dynamic protein that undergoes different post-translational modifications that are important for regulation of the microtubule cytoskeleton.[36] Colchicine (**1**) along with selected derivative **4o** was tested at three different concentrations viz. 25, 50 (~ IC_{50} value) and 100 nM for their effect on α-tubulin and β-tubulin expression in HCT-116 cells. Both compounds effectively decreased the expression of α-tubulin in a concentration-dependent manner in HCT-116 cells. Results are depicted in Figure 5.

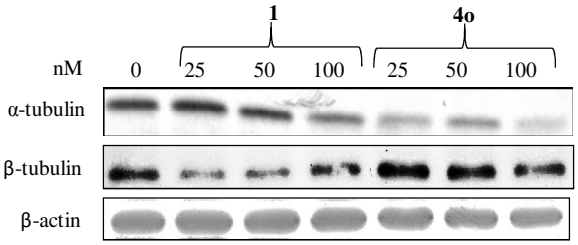


Figure 5 The effect of compounds **1** and **4o** on α-tubulin and β-tubulin expression in HCT-116 cells. Equal amount of protein was loaded on SDS-PAGE gel for western blot analysis. β-Actin was used as an internal control. Data are representative of one of three similar experiments

Colchicine (**1**) and 10-amino linked derivative **4o** were tested for their ability to show disruption of microtubule assembly using confocal microscopy. The compounds were tested at two concentrations viz. 50 (~ IC_{50} value) and 100 nM. As shown in Figure 6, the cells treated with colchicine (**1**) and derivative **4o** showed a remarkable disruption and loss of microtubules compared to the control. Thus, the fluorescent microscopic data indicated that these compounds possess potent antitumor activity in HCT-116 cells via disruption of microtubule assembly.

Next, the effect of colchicine (**1**) and derivative **4o** on the expression of Skp2, p21 and p27 proteins was evaluated at three concentrations viz. 25, 50 (~ IC_{50} value) and 100 nM. As shown in Figure 7, both colchicine (**1**) and compound **4o** reduced the expression of Skp2 and increased the expression of p21 in a concentration dependent manner. Colchicine is reported to increase the expression of p21.[37] Further, this effect on p21 and p27 expression was confirmed by confocal microscopy. As depicted in Figure 8, both the colchicine (**1**) and compound **4o** increased the expression of p21 in a concentration dependent manner. However, there was no effect on expression of p27 (Images are shown in Figure S96 of ESI).

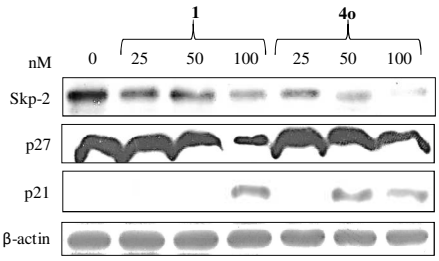


Figure 7. The effect of compounds **1** and **4o** on Skp2, p27 and p21 expression in HCT-116 cells. Equal amount of protein was loaded on SDS-PAGE gel for western blot analysis. β-Actin was used as an internal control. Data are representative of one of three similar experiments. Skp2 is an oncogenic protein which is overexpressed in certain cancers including colorectal cancer.[38, 39]

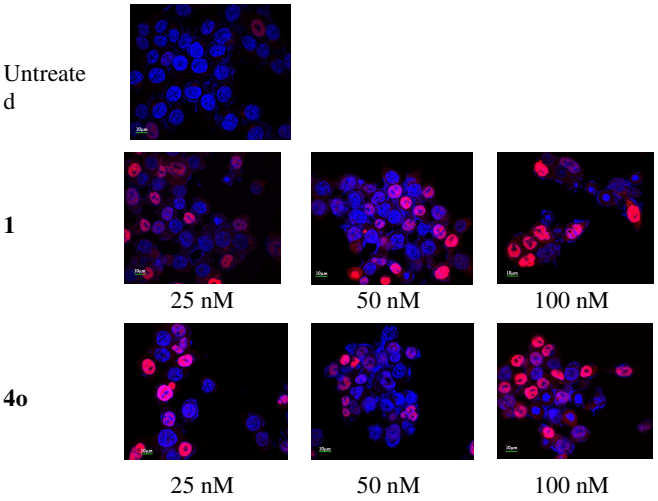
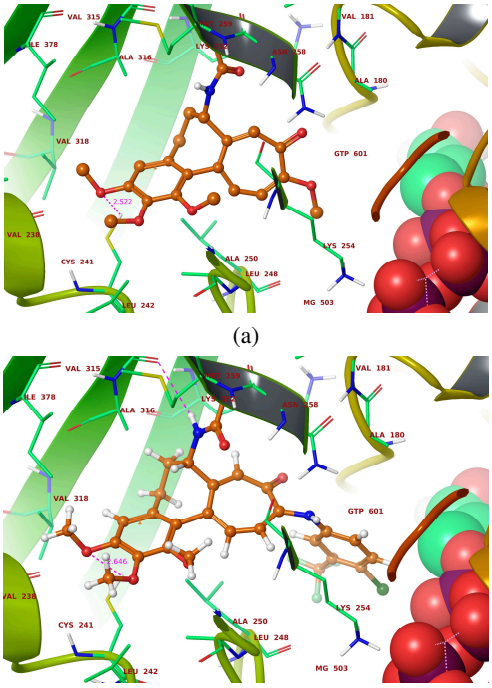


Figure 8. The effect of colchicine (**1**) and its derivative **4o** on p21 expression in HCT-116 cells. Cells were cultured on coverslips. After 24 h, the cells were treated with different concentrations of the compound. Immunocytochemical staining was conducted using p21 antibody and Alexa Flour-488-labeled secondary antibody. Staining was done by using DAPI. The data are representative of three separate sets of experiments.

Molecular docking for tubulin. Tubulin binding agents including colchicinoids, vinca alkaloids, taxanes and depsipeptides, exercise their cytotoxic effects by disrupting α, β-tubulin dimerization process.[40-42] These agents alter dynamic instability of cellular microtubules, disrupt spindle microtubules and inhibit microtubule assembly by inhibiting tubulin-dependent GTP hydrolysis as well as nucleotide exchange on beta-tubulin and, as a consequence, inhibit further addition of new tubulin units.[43, 44] X-ray crystallographic analysis of colchicine-tubulin complex structure reveals that, colchicine binds at interphase of α and β tubulin by H-bonding (with Cys241 residue of beta-tubulin) and hydrophobic interactions.[45] Furthermore, molecular modeling

studies were carried out in order to envisage the mode of interactions of colchicine and **4o** with tubulin. Our studies infer the possibility that **4o** might interact with Cys241 and Val315 residues by polar H-bonding and Val238, Leu248, Ala250, Lys254, Val318 and Lys358 residue by H-bonding in vicinity of nucleotide, GTP binding site and Mg^{2+} ion as cofactor. Colchicine (**1**) and its derivative **4o**, induce conformational changes in tubulin-polymeric structure, which further perturb GTP hydrolysis and nucleotide exchange in tubulin polymerization. The interaction of colchicine (**1**) and its derivative **4o** with the colchicine-binding site of tubulin are shown in Figure 9.



(a)

(b)

Figure 9. Interactions of colchicine (a) and derivative **4o** (b) with the colchicine-binding site of tubulin.

Physicochemical properties and CYP P450 inhibition of best compounds. The solubility of best compound **4o** and colchicine (**1**) was determined in water, PBS, SGF and SIF. Both compounds displayed high solubility in water as well as biological fluids phosphate buffer saline (PBS), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) (Table 3). Experimental lipophilicity (Log Po/w) of colchicine (**1**) and **4o** was determined using capacity factor as per 'OECD guidelines for testing of chemicals 117'. The Log P of colchicine and **4o** was found to be 0.77 and 3.39, respectively. In general, the compounds with LogP of > 0.8 and < 4 have a favourable ADMET profile,[46] indiating that compound **4o** falls in desired range.

Table 3 Physicochemical properties of colchicine (**1**) and its derivative **4o**

Entry	Thermodynamic equilibrium solubility (µg/mL)[a]				Exp. log Po/w[b]
	Water	PBS (pH 7.4)	SGF (pH 1.2)	SIF (pH 6.8)	
1	>1500	800	200	800	0.77
4o	>1500	>1500	>1500	>1500	3.39

[a] The thermodynamic aqueous solubility was determined by 96-well plate assay; [b] Experimental log Po/w values were determined as per 'OECD guidelines for testing of chemicals 117'

Compound **4o** was then tested for inhibition of five major CYP450 isoenzymes; CYP3A4, CYP2D6, CYP2C9, CYP1A2 and CYP2C19 at 10 µM[47] and it showed 45, 11, 8, 20 and 22% inhibition, respectively of these enzymes. The results indicated that compound **4o** showed moderate or very low inhibition of CYP3A4, CYP2C9 and CYP2C19 enzymes, and thus do not have CYP liability. Whereas, colchicine is reported to downregulate the expression of different isoforms of CYPs including CYP2C9 and CYP3A4.[48-50]

Pharmacokinetics. Colchicine is promptly absorbed by the jejunum and ileum.[51] So, increase in solubility in SIF fluid of compound **4o** can be considered to improve the oral bioavailability. On the other hand, P-gp induction is one of the key reason for lower bioavailability of oral compounds.[52] Since, our synthesized compound **4o** has shown lower P-gp induction activity and better solubility in SIF fluid compared to colchicine, we hypothesized that compound **4o** will have good oral pharmacokinetic properties. Therefore, we studied the pharmacokinetics of compound **4o** in BALB/c mice. The pharmacokinetic parameters of compound **4o** following IV and oral route are summarized in Table 4. The time vs. plasma concentration profile of **4o** is shown in Figure 10. After i.v. injection of **4o** (1 mg/kg), the $AUC_{0-\infty}$ was 2691 ng.h/ml with C_{max} of 698 ng/ml and the terminal half-life (t½) is 2.78 h. After oral administration of **4o** (10 mg/kg), compound **4o** showed much better parameters. $AUC_{0-\infty}$ was 17334 ng.h/ml and C_{max} was 3031 ng/ml with the better terminal half-life (t½), 5.43 h. The oral bioavailability (%F) was found to be 64.4%, which envisioned a good possibility for oral administration.

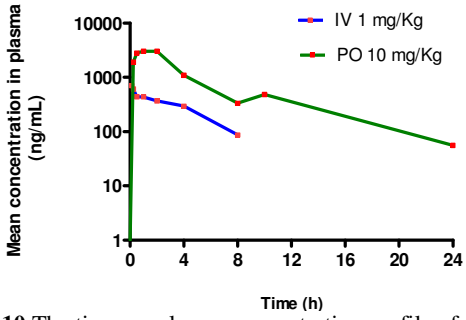


Figure 10 The time vs. plasma concentration profile of **4o**.

Table 4 Pharmacokinetic parameters of **4o** post IV dose at 1 mg/kg and PO dose at 10 mg/kg to BALB/c mice

Parameter	Unit	Value	
		IV (1 mg/kg)	PO (10 mg/kg)
$t_{1/2, ß}$	h	2.78	5.43

C_{max}	ng/mL	698	3031
t_{max}	h	--	2.0
C_0	ng/mL	819	--
AUC_{0-t}	ng·h/mL	2343	16899
$AUC_{0-\infty}$	ng·h/mL	2691	17334
CL	mL/min/kg	6.19	--
V_d	L/kg	1.49	--
V_{dss}	L/kg	1.47	--
T_{last}	h	8.00	--
Bioavailability	% F	--	64.4
Time points considered for $t_{1/2, \beta}$ calculation		2 – 8 h	8 – 24 h

***In vivo* activity in Ehrlich solid tumor model.** Ehrlich carcinoma resembles human tumors which are the most sensitive to chemotherapy due to the fact that it is undifferentiated and has a rapid growth rate.[53 54] The compound **4o** was evaluated for anticancer activity in Ehrlich solid tumor model in mice. The results are summarized in Table 5. The compound **4o** has shown promising activity at 1 mg/kg (i.p.), 1.25 mg/kg (p.o.) and 2 mg/kg (p.o.) dose with 47.4%, 29.9% and 38.1% inhibition respectively in tumor size compared to control. There is no mortality observed in the group treated with **4o**. On the other hand, colchicine (**1**) has shown promising activity with 52.3%, 24.9% and 29.9% TGI at 0.75 mg/kg (i.p.), 0.75 mg/kg (p.o.) and 1.25 mg/kg (p.o.) dose, respectively. However, mortality was observed in 2 animals in the group of 7 animals treated with colchicine (**1**) at the dose of 0.75 mg/kg, i.p.

Conclusion

In summary, the present study has identified a new colchicine-based lead **4o** with potent *in vitro* and *in vivo* anticancer activity and no P-gp-induction liability, which is associated with the parent natural product colchicine. The lead compound **4o** showed inhibition of microtubule assembly and also led to increase in the expression of pro-apoptotic protein p21. The excellent physicochemical parameters (solubility and lipophilicity), good pharmacokinetic profile and *in vivo* efficacy in solid tumor model indicate its promise for development as an anticancer agent.

Experimental Section

General information. All chemicals and reagents were obtained from Sigma-Aldrich Company and used as received. All HPLC solvents were obtained from Merck India Limited. The ^1H, ^{13}C and DEPT NMR spectra were recorded on Brucker-Avance DPX FT-NMR 500 and 400 MHz instruments. Chemical data for protons are reported in parts per million (ppm) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent ($CDCl_3$, 7.26 and CD_3OD, 3.31 ppm). Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded at 125 MHz or 100 MHz: chemical data for carbons are reported in parts per million (ppm, δ scale) downfield from tetramethylsilane and are referenced to the carbon resonance of the solvent ($CDCl_3$, 77 and CD_3OD, 49 ppm). ESI-MS and HRMS spectra were recorded on Agilent 1100 LC-Q-TOF and HRMS-6540-UHD machines. IR spectra were recorded on Perkin-Elmer IR spectrophotometer. Melting points were recorded on digital melting point apparatus. LC-ESI-MS/MS analysis was carried out on Agilent Triple-Quad LC-MS/MS system (model 6410). The purity of compounds was determined by using HPLC (Agilent 1260 infinity). The HPLC conditions for Method I was: Chromolith C-18 column: 50 × 4.6 mm; flow rate: 1.0 ml/min.; PDA detector; binary mobile phase consisted of A (aqueous) and B (acetonitrile) with gradient programme was used as follows: 0–2 min. 5% B, 2–10 min. 5-100% B, 10–12 min. 100% B, 12–13 min. 100-5% B; 13–15 min. 100% B;. The HPLC conditions for Method II was: Lichrospher C-18 column: 125 × 3 mm, 5 µ; flow rate: 0.8 ml/min.; PDA detector; binary mobile phase consisted of A (aqueous) and B (acetonitrile) with gradient programme was used as follows: 0–10 min. 2–100% B, 10–12 min. 100% B, 13–14 min. 100-2% B, 14–15 min. 2% B. The animals used in the

Table 5. In-vivo activity of compounds **1** and **4o** in Ehrlich solid tumor model

Sr No	Groups	ABW (g) of animals on days			Day 13		% TGI	Mortality
		1	5	9	ABW (g)	Avg. tumor weights (mg)		
1	**1** (0.75 mg/kg, i.p.)[a]	22.28	21.42	20.2	19.25	705.0 ± 190.01*	52.29	2/7
2	**4o** (1 mg/kg, i.p.)	22.28	22.0	21.28	21.66	778.0 ± 101.12**	47.35	0/7
3	Normal control NS (0.2 ml, i.p.)	23.40	24.4	25.4	25.33	1477.94 ± 225.96	0	0/10
4	**1** (0.75 mg/kg, p.o.)	20.42	21.14	21.57	21.57	1128.28 ± 102.83***	24.86	0/7
5	**1** (1.25 mg/kg, p.o.)	20.42	20.57	19.57	19.85	1189.26 ± 57.57***	29.91	0/7
6	**4o** (1.25 mg/kg, p.o.)	20.42	21.42	21.28	22.0	989.92 ± 114.27***	34.10	0/7
7	**4o** (2 mg/kg, p.o.)	20.42	21.28	21.32	21.42	1149.92 ± 82.99***	38.14	0/7
8	Normal Control NS (0.2 ml, p.o.)	20.82	21.9	22.3	23.40	1697.50 ± 103.58	0	0/10
9	5-Fluorouracil (22 mg/kg, i.p.)	22.42	22.28	19.4	19.40	668.20 ± 82.10**	54.78	0/7

[a] Colchicine (**1**) was toxic at 1 mg/kg with mortality of 5 animals in the group of 7 animals. %TGI is not determined.
Asterisks *, **, *** indicated significant difference as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively. ABW: Average body weight; TGI: Tumor growth inhibition.

study were breed in-house and used. The animal facility is registered with CPCSEA vide registration no. 67/99/CPCSEA. All of the biologically tested compounds have met the 95% purity requirement.

Designing colchicine derivatives. The P-gp homology model constructed based on crystal of P-gp from *C. elegans* and was kindly provided by the Prof. Jue Chen.[14] The hypothetical colchicine binding sites in human closed nucleotide free P-gp homology model was first identified by the Becker J. P. *et al* in 2009,[29] where they found that in major poses of colchicine-P-gp complex, colchicine interact with the Ile-306, Phe-335, Phe-728 residues. Therefore, grid was prepared at the centroid of these residues. Colchicine and other ligands were sketched using Maestro, hydrogens added, stereochemistry was defined and structures were minimized using Macromodel minimization tool of Schrodinger using using MMFFs force field and Polak-ribiere conjugate gradient (PRCG) method in default setting. All molecules were used as such in neutral form because of the better correlation of molecular modeling studies of P-gp substrate/inhibitors in neutral form. Previous theories on P-gp mechanism, also states that P-gp substrates enters P-gp binding cavity via membrane where they are assumed to be in neutral form rather than their presence as charged form in cytoplasm.[55] All minimized molecules were docked to the colchicine binding site using XP docking method. The loss of hydrophobic interactions and gain of polar interactions including ionic and H-bonding were taken into consideration in newly designed derivatives in terms of Glide docking score.

General method for preparation of 10-chloro-colchicine (3). To the solution of colchicine (**1**, 0.25 mmol) in DMF (2 mL) was added $POCl_3$ (1.0 mmol)**.** The reaction mixture was stirred at room temp. for 24 h, and then the mixture was diluted with cold water and extracted with ethyl acetate followed by purification by silica gel column chromatography (MeOH: $CHCl_3$) to yield compound **3** (70% yield). Yellow solid; HPLC, t_R = 6.27 min (>99% purity; Method: I); m.p. 120-123 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.97 (1H, s, C8-H), 7.42 (1H, d, *J* = 12.8 Hz, C12-H), 7.16 (1H, d, *J* = 12.8 Hz, C11-H), 6.65 (1H, d, *J* = 6.8 Hz, NH), 6.56 (1H, s, C4-H), 4.58-4.52 (1H, m, C7-H), 3.92 (3H, s, Ar-OCH_3), 3.90 (3H, s, Ar-OCH_3), 3.69 (3H, s, Ar-OCH_3), 2.55-2.50 (1H, m, C5-Ha), 2.35-2.28 (2H, m, C5-Hb, C6-Ha), 2.05 (3H, s, C14-CH_3) 2.03-2.01 (1H, m, C6-Hb); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 179.33 (C9), 170.38 (C13), 154.37 (C3), 151.02 (C1), 147.12 (C7a), 143.36 (C2), 141.65 (C10), 141.30 (C12a), 141.25 (C11), 135.12 (C4a), 135.05 (C12), 133.33 (C8), 125.08 (C12b), 107.71 (C4), 61.72 (C1-OCH_3), 61.39 (C2-OCH_3), 56.19 (C3-OCH_3), 52.20 (C7), 38.33 (C6), 29.91 (C5), 22.94 (C14); HR-MS: *m/z* calcd 404.1256 for $C_{21}H_{22}ClNO_5$+H^+ (404.1259); IR ($CHCl_3$): ν_{max} 3304, 3003, 2936, 2855, 1659, 1583, 1488, 1458, 1405, 1322, 1260, 1195, 1095, 1037 cm^{-1}.

General method for synthesis of 10-amino linked colchicine derivatives 4a-p. To the solution of colchicine (**1**, 0.25 mmol) in MeOH (3 mL) was added corresponding amines (1.0 mmol). The amines used for the preparation of compounds **4a-p** were piperidine, diethylamine, morpholine, piperazine, N-methyl piperazine, 4-amino piperidine, 1-Boc-piperazine, 4-phenyl piperidine, L-prolinol, pyrrolidine, ammonium hydroxide solution (28.0-30.0% NH_3), tryptoline, 3-chloro-4-fluoroaniline, ethyl piperazine-1-carboxylate, 4-chloro-3-(trifluoromethyl)aniline and 3,4-(methylenedioxy)aniline respectively. The reaction mixture was stirred at room temperature for 24-72 h, and then the mixture was diluted with cold water and extracted with ethyl acetate followed by purification with silica gel column chromatography (MeOH: $CHCl_3$) to yield compounds **4a-p** (60-90 % yield).

*N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(piperidin-1-yl)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (**4a**).* Yield: 81%; yellow solid; HPLC, t_R = 8.16 min (99% purity; Method: II); m.p. 162-164 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.70 (1H, d, *J* = 6.8 Hz, NH), 7.34 (1H, s, C8-H), 7.26 (1H, d, *J* = 11.2 Hz, C12-H), 6.80 (1H, d, *J* = 11.2 Hz, C11-H), 6.50 (1H, s, C4-H), 4.64-4.61 (1H, m, C7-H), 3.93 (3H, s, Ar-OCH_3), 3.88 (3H, s, Ar-OCH_3), 3.64 (3H, s, Ar-OCH_3), 3.54-3.47 (2H, m, C2′-Ha, C6′-Ha), 3.39-3.34 (2H, m, C2′-Hb, C6′-Hb), 2.48-2.35 (2H, m, C6-Ha, C5-Ha), 2.26-2.18 (1H, m, C6-Hb), 2.01 (3H, s, C14-CH_3), 1.89-1.84 (1H, m, C5-Hb), 1.74-1.70 (6H, m, C3′-H, C4′-H, C5′-H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 181.30 (C9), 169.95 (C13), 158.76 (C10), 153.02 (C3), 151.32 (C1), 149.65 (C7a), 141.61 (C2), 136.33 (C12), 134.48 (C4a), 133.15 (C12a), 128.40 (C8), 126.25 (C12b), 117.62 (C11), 107.35 (C4), 61.43 (C1-OCH_3), 61.39 (C2-OCH_3), 56.12 (C3-OCH_3), 51.83 (C7), 50.20 (C2′), 50.20 (C6′), 37.04 (C6), 30.15 (C5), 26.00 (C3′), 26.00 (C5′), 24.62 (C4′), 22.95 (C14); HR-MS: *m/z* calcd 453.2381 for $C_{26}H_{32}N_2O_5$+H^+ (453.2384); IR ($CHCl_3$): ν_{max} 3399, 2932, 2854, 1602, 1543, 1349, 1321, 1220, 1195, 1095, 1021 cm^{-1}.

*N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(diethylamino)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (**4b**).* Yield: 75%; yellow solid; HPLC, t_R = 7.90 min (97% purity; Method: II); m.p. 168-171 ºC. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.19 (1H, d, *J* = 11.4 Hz, C12-H), 7.08 (1H, s, C8-H), 6.57 (1H, d, *J* = 11.4 Hz, C11-H), 6.50 (1H, s, C4-H), 4.62-4.60 (1H, m, C7-H), 3.93 (3H, s, Ar-OCH_3), 3.88 (3H, s, Ar-OCH_3), 3.64 (3H, s, Ar-OCH_3), 3.59-3.56 (2H, m, C2′-Ha, C4′-Ha), 2.44-2.41 (2H, m, C6-Ha, C5-Ha), 2.26-2.20 (1H, m, C6-Hb), 2.01 (3H, s, C14-CH_3), 1.81-1.80 (3H, m, C5-Hb, C2′-Hb, C4′-Hb), 1.30-1.25 (6H, m, C3′-H, C5′-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 179.45 (C9), 169.98 (C13), 156.05 (C10), 152.70 (C3), 151.36 (C1), 149.23 (C7a), 141.49 (C2), 136.48 (C12), 134.60 (C4a), 129.49 (C12a), 126.50 (C12b), 124.97 (C8), 111.99 (C11), 107.23 (C4), 61.43 (C1-OCH_3), 61.32 (C2-OCH_3), 56.08 (C3-OCH_3), 51.64 (C7), 46.10 (C2′), 46.10 (C4′), 37.26 (C6), 30.21 (C5), 22.95 (C14), 12.66 (C3′), 12.66 (C5′); HR-MS: *m/z* calcd 441.2372 for $C_{25}H_{32}N_2O_5$+H^+ (441.2384); IR ($CHCl_3$): ν_{max} 3270, 2932, 2854, 1661, 1600, 1538, 1487, 1400, 1348, 1278, 1236, 1158, 1137, 1096, 1080 cm^{-1}.

*N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(morpholin-4-yl)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (**4c**).*Yield: 84%; yellow solid; HPLC, t_R = 6.77 min (99% purity; Method: II); m.p. 152-155 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.29 (1H, s, C8-H), 7.28 (1H, d, *J* = 11.2 Hz, C12-H), 6.77 (1H, d, *J* = 11.2 Hz, C11-H), 6.52 (1H, s, C4-H), 4.63-4.60 (1H, m, C7-H), 3.93 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 3.64 (3H, s, Ar-OCH_3), 3.59-3.54 (2H, m, C3′-Ha, C5′-Ha), 3.33-3.27 (2H, m, C3′-Hb, C5′-Hb), 2.89-2.87 (4H, m, C2′-H, C6′-H), 2.55-2.50 (1H, m, C5-Ha), 2.35-2.28 (2H, m, C5-Hb, C6-Ha), 2.05 (3H, s, C14-CH_3) 1.85-

1.83 (1H, m, C6-Hb); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 181.27 (C9), 169.73 (C13), 157.94 (C10), 153.21 (C3), 151.29 (C1), 149.70 (C7a), 141.61 (C2), 136.03 (C12), 134.74 (C12a), 134.31 (C4a), 129.47 (C8), 125.86 (C12b), 118.15 (C11), 107.30 (C4), 67.85 (C3′), 66.76 (C5′), 61.46 (C1-OCH_3), 61.41 (C2-OCH_3), 56.09 (C3-OCH_3), 51.92 (C7), 49.00 (C2′), 46.27 (C6′), 36.94 (C6), 30.03 (C5), 23.02 (C14); HR-MS: *m/z* calcd 455.2161 for $C_{25}H_{30}N_2O_6$+H^+ (455.2177); IR ($CHCl_3$): ν_{max} 3279, 2955, 2933, 2855, 1660, 1605, 1544, 1305, 1264, 1231, 1116, 1050, 1033 cm^{-1}.

*N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(piperazin-1-yl)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (**4d**).* Yield: 67%; yellow solid; HPLC, t_R = 7.63 min (97% purity; Method: II); m.p. 158-161 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.44 (1H, s, br, NH), 7.28 (1H, s, C8-H), 7.25 (1H, d, *J* = 10.8 Hz, C12-H), 6.79 (1H, d, *J* = 10.8 Hz, C11-H), 6.52 (1H, s, C4-H), 4.64-4.61 (1H, m, C7-H), 3.93 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 3.64 (3H, s, Ar-OCH_3), 3.56-3.53 (2H, m, C2′-Ha, C6′-Ha), 3.35-3.32 (2H, m, C2′-Hb, C6′-Hb), 3.14-3.04 (4H, m, C3′-H, C5′-H), 2.50-2.39 (2H, m, C5-Ha, C6-Ha), 2.25-2.18 (1H, m, C5-Hb), 2.01 (3H, s, C14-CH_3), 1.87-1.85 (1H, m, C6-Hb); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 181.35 (C9), 169.78 (C13), 158.35 (C10), 153.15 (C3), 151.29 (C1), 149.63 (C7a), 141.57 (C2), 136.11 (C12), 134.36 (C12a), 134.27 (C4a), 129.22 (C8), 125.96 (C12b), 118.26 (C11), 107.29 (C4), 61.46 (C1-OCH_3), 61.41 (C2-OCH_3), 56.09 (C3-OCH_3), 51.87 (C7), 49.74 (C2′), 49.74 (C6′), 45.85 (C3′), 45.85 (C5′), 36.99 (C6), 30.05 (C5), 23.03 (C14); HR-MS: *m/z* calcd 454.2317 for $C_{25}H_{31}N_3O_5$+H^+ (454.2336); IR ($CHCl_3$): ν_{max} 3283, 2926, 2852, 1660, 1544, 1462, 1349, 1234, 1195, 1118, 1033 cm^{-1}.

*N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(N-methyl-piperazin-1-yl)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (**4e**).* Yield: 71%; yellow solid; HPLC, t_R = 7.83 min (99% purity; Method: II); m.p. 154-155 ºC. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.25 (1H, d, *J* = 11.0 Hz, C12-H), δ 7.20 (1H, s, C8-H), 6.77 (1H, d, *J* = 11.0 Hz, C11-H), 6.52 (1H, s, C4-H), 4.64-4.60 (1H, m, C7-H), 3.93 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 3.64 (3H, s, Ar-OCH_3), 3.59-3.58 (2H, m, C2′-Ha, C6′-Ha), 3.39-3.37 (2H, m, C2′-Hb, C6′-Hb), 2.67-2.64 (4H, m, C3′-H, C5′-H), 2.50-2.41 (2H, m, C5-Ha, C6-Ha), 2.39 (3H, N-CH_3), 2.24-2.22 (1H, m, C5-Hb), 2.01 (3H, s, C14-CH_3) 1.83-1.81 (1H, m, C6-Hb); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 181.31 (C9), 169.70 (C13), 158.03 (C10), 153.13 (C3), 151.30 (C1), 149.50 (C7a), 141.58 (C2), 136.05 (C12), 134.32 (C12a), 134.26 (C4a), 129.23 (C8), 125.96 (C12b), 118.34 (C11), 107.26 (C4), 61.45 (C1-OCH_3), 61.42 (C2-OCH_3), 56.09 (C3-OCH_3), 54.80 (C3′), 54.80 (C5′), 51.86 (C7), 48.30 (C2′), 45.91 (C4′-CH_3), 37.00 (C6), 30.03 (C5), 29.69 (C6′), 23.05 (C14); HR-MS: *m/z* calcd 468.2448 for $C_{26}H_{33}N_3O_5$+H^+ (468.2493); IR ($CHCl_3$): ν_{max} 3274, 2997, 2935, 2851, 2802, 1661, 1548, 1400, 1292, 1237, 1196, 1175, 1036 cm^{-1}.

*N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(4-amino-piperidin-1-yl)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (**4f**).* Yield: 73%; yellow solid; HPLC, t_R = 6.23 min (99% purity; Method: II); m.p. 154-155 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.40 (1H, d, *J* = 11.6 Hz, C12-H), 7.39 (1H, s, C8-H), 7.22 (1H, d, *J* = 8 Hz, NH), 6.63 (1H, d, *J* = 11.6 Hz, C11-H), 6.51 (1H, s, C4-H), 4.64-4.61 (1H, m, C7-H), 3.92 (3H, s, Ar-OCH_3), 3.88 (3H, s, Ar-OCH_3), 3.60 (3H, s, Ar-OCH_3), 3.08-3.03 (4H, m, C2′-H, C6′-H), 2.81-2.70 (1H, m, C2′-H), 2.68-2.55 (1H, m, C4′-H), 2.48-2.35 (2H, m, C6-Ha, C5-Ha), 2.26-2.18 (1H, m, C6-Hb), 2.01 (3H, s, C14-CH_3), 1.89-1.84 (1H, m, C5-Hb), 1.74-1.70 (4H, m, C3′-H, C5′-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 175.08 (C9), 169.90 (C13), 153.01 (C10), 152.84 (C3), 151.06 (C1), 150.08 (C7a), 141.48 (C2), 139.07 (C12), 134.53 (C4a), 130.17 (C12a), 126.85 (C12b), 122.83 (C8), 108.53 (C11), 107.15 (C4), 61.43 (C1-OCH_3), 61.35 (C2-OCH_3), 56.09 (C3-OCH_3), 52.52 (C7), 49.03 (C4'), 45.48 (C2′), 45.13 (C6′), 37.28 (C3′), 37.20 (C6), 32.67 (C5′), 30.07 (C5), 22.94 (C14); HR-MS: *m/z* calcd 468.2448 for $C_{26}H_{33}N_3O_5$+H^+ (468.2493); IR ($CHCl_3$): ν_{max} 3305, 2927, 2853, 2802, 1654, 1579, 1488, 1463, 1427, 1283, 1194, 1095, 1045 cm^{-1}.

*N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(N-boc-piperazin-1-yl)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (**4g**).* Yield: 60%; yellow solid; HPLC, t_R = 8.43 min (98% purity; Method: II); m.p. 158-160 ºC. ^{1}H NMR (CD_3OD, 500 MHz): δ 7.32 (1H, d, *J* = 11.0 Hz, C12-H), 7.18 (1H, s, C8-H), 7.05 (1H, d, *J* = 11.0 Hz, C11-H), 6.72 (1H, s, C4-H), 4.48-4.46 (1H, m, C7-H), 3.91-3.81 (2H, m, C2′-Ha, C6′-Ha), 3.89 (3H, s, Ar-OCH_3), 3.58 (3H, s, Ar-OCH_3), 3.45-3.42 (2H, m, C2′-Hb, C6′-Hb), 3.30 (3H, s, Ar-OCH_3), 2.65-2.57 (2H, m, C3′-Ha, C5′-Ha). 2.39-2.31 (2H, m, C3′-Hb, C5′-Hb), 2.20-2.12 (2H, m, C6-Ha, C5-Ha), 1.98 (3H, s, C14-CH_3), 1.95-1.94 (2H, m, C5-Hb, C6-Hb), 1.48 (9H, -CH_3×3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 181.28 (C9), 169.43 (C13), 157.98 (C10), 154.73 (C7'), 153.24 (C3), 151.37 (C1), 149.15 (C7a), 141.74 (C2), 135.93 (C12), 134.34 (C4a), 134.24 (C12a), 129.41 (C8), 125.96 (C12b), 118.03 (C11), 107.39 (C4), 80.07 (C8'), 61.40 (C1-OCH_3), 61.40 (C2-OCH_3), 56.13 (C3-OCH_3), 51.88 (C7), 48.53 (C2′), 48.53 (C6′), 37.24 (C6), 30.02 (C5), 29.70 (C3'), 29.70 (C5′), 28.45 (C9′), 28.45 (C10′), 28.45 (C11′), 22.69 (C14); HR-MS: *m/z* calcd 554.2885 for $C_{30}H_{39}N_3O_7$+H^+ (554.2861); IR ($CHCl_3$): ν_{max} 3390, 2924, 2854, 1653, 1422, 1322, 1232, 1160, 1044 cm^{-1}.

*N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(4-phenyl-piperidin -1-yl)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (**4h**).* Yield: 78%; yellow solid; HPLC, t_R = 9.49 min (99% purity; Method: II); m.p. 169-172 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.35-7.31 (m, 3H, C9′-H, C10′-H, C11′-H) 7.28-7.20 (2H, C8′-H, C12′-H), 7.26 (1H, s, C8-H), 7.23 (1H, d, *J* = 11.2 Hz, C12-H), 6.84 (1H, d, *J* = 11.2 Hz, C11-H), 6.51 (1H, s, C4-H), 4.65-4.62 (1H, m, C7-H), 4.27-4.24 (1H, m, C2′-Ha), 4.11-4.08 (1H, m, C6′-Ha), 3.94 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 3.66 (3H, s, Ar-OCH_3), 3.06-3.05 (1H, m, C4′-H), 2.86-2.80 (2H, m, C2′-Hb, C6′-Hb), 2.48-2.42 (2H, m, C6-Ha, C5-Ha), 2.22-2.17 (1H, m, C6-Hb), 2.04 (3H, s, C14-CH_3), 2.03-1.92 (3H, m, C5-Hb, C3′-Ha, C5′-Ha). 1.85-1.84 (2H, m, C3′-Hb, C5′-Hb); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 181.51 (C9), 169.79 (C13), 158.62 (C10), 153.06 (C3), 151.31 (C1), 149.10 (C7a), 145.60 (C7'), 141.57 (C2), 136.19 (C12), 134.41 (C4a), 134.30 (C12a), 128.80 (C8), 128.86 (C9'), 128.86 (C11'), 126.86 (C8'), 126.86 (C12'), 128.41 (C10'), 126.11 (C12b), 118.03 (C11), 107.27 (C4), 61.47 (C1-OCH_3), 61.42 (C2-OCH_3), 56.09 (C3-OCH_3), 51.87 (C7), 49.76 (C2′), 49.68 (C6′), 42.84 (C3'), 36.99 (C6), 33.55 (C3'), 31.14 (C5′), 30.09 (C5), 23.05 (C14); HR-MS: *m/z* calcd 529.2696 for

$C_{32}H_{36}N_2O_5$+H^+ (529.2697); IR ($CHCl_3$): ν_{max} 3291, 2932, 2850, 1654, 1601, 1543, 1486, 1399, 1321, 1216, 1095, 1007 cm^{-1}.

N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(L-prolinol-1-yl)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (***4i***). Yield: 81%; yellow solid; HPLC, t_R = 6.73 min (99% purity; Method: II); m.p. 164-166 ºC. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.91 (1H, d, *J* = 6.2 Hz, NH), 7.33 (1H, d, *J* = 11.8 Hz, C12-H), 7.25 (1H, s, C8-H), 6.53 (1H, s, C4-H), 6.48 (1H, d, *J* = 11.8 Hz, C11-H), 6.2 (1H, s, br, C5′-OH), 5.35-5.33 (1H, m, C3′-H), 4.55-4.53 (1H, m, C7-H), 3.93 (3H, s, Ar-OCH_3), 3.88 (3H, s, Ar-OCH_3), 3.73-3.69 (2H, m, C6′-H), 3.62 (3H, s, Ar-OCH_3), 3.55-3.52 (1H, m, C2′-Ha), 3.47-3.41 (1H, m, C5′-Ha), 2.44-2.42 (1H, m, C6-Ha), 2.34-2.25 (2H, m, C6-Hb, C5-Ha), 2.12-2.11 (2H, m, C2′-Hb, C5′-Hb), 2.01 (3H, s, C14-CH_3), 2.01-1.97 (2H, m, C4′-H), 1.97-1.87 (1H, m, C6-Hb); ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 176.77 (C9), 169.85 (C13), 156.25 (C10), 152.92 (C3), 151.34 (C1), 149.89 (C7a), 141.49 (C2), 138.27 (C12), 134.69 (C4a), 129.65 (C12a), 126.20 (C12b), 123.54 (C8), 113.10 (C11), 107.35 (C4), 64.15 (C6'), 62.20 (C2'), 61.43 (C1-OCH_3), 61.27 (C2-OCH_3), 56.08 (C3-OCH_3), 52.04 (C7), 50.58 (C5′), 37.47 (C6), 30.15 (C5), 28.51 (C5′), 23.01 (C4′), 22.45 (C14); HR-MS: *m/z* calcd 469.2336 for $C_{26}H_{32}N_2O_6$+H^+ (469.2333); IR ($CHCl_3$): ν_{max} 3350, 2928, 2853, 1653, 1598, 1527, 1486, 1455, 1401, 1382, 1349, 1237, 1145, 1096, 1019 cm^{-1}.

N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(pyrrolidin-1-yl)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (***4j***). Yield: 89%; yellow solid; HPLC, t_R = 7.52 min (99% purity; Method: II); m.p. 152-156 ºC. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.34 (1H, d, *J* = 11.0 Hz, C12-H), 7.07 (1H, d, *J* = 6.0 Hz, NH), 7.07 (1H, s, C8-H), 7.05 (1H, s, C4-H), 6.39 (1H, d, *J* = 11.0 Hz, C11-H), 4.61-4.58 (1H, m, C7-H), 3.93 (3H, s, Ar-OCH_3), 3.88 (3H, s, Ar-OCH_3), 3.63 (3H, s, Ar-OCH_3), 3.55-3.52 (2H, m, C2′-Ha, C5′-Ha), 2.46-2.44 (2H, m, C6-Ha, C5-Ha), 2.23-2.20 (2H, m, C2′-Hb, C5′-Hb), 2.05 (3H, s, C14-CH_3), 1.98-1.80 (6H, m, C5-Hb, C6-Hb, C3′-H, C4′-H); ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 178.38 (C9), 169.93 (C13), 155.31 (C10), 152.67 (C3), 151.35 (C1), 149.83 (C7a), 141.49 (C2), 137.22 (C12), 134.69 (C4a), 128.45 (C12a), 126.66 (C12b), 124.42 (C8), 111.36 (C11), 107.25 (C4), 61.44 (C1-OCH_3), 61.26 (C2-OCH_3), 56.09 (C3-OCH_3), 51.92 (C7), 50.81 (C2′), 50.81 (C5′), 37.42 (C6), 30.22 (C5), 25.45 (C3′), 25.45 (C4′), 23.07 (C14); HR-MS: *m/z* calcd 439.2231 for $C_{25}H_{30}N_2O_5$+H^+ (439.2227); IR ($CHCl_3$): ν_{max} 3261, 3047, 2934, 2874, 1724, 1660, 1599, 1532, 1485, 1459, 1399, 1386, 1347, 1321, 1282, 1235, 1194, 1144, 1042, 1017 cm^{-1}.

N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-amino-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (***4k***).[56] Yield: 90%; yellow solid; HPLC, t_R = 6.19 min (99% purity; Method: II); m.p. 138-140 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.48 (1H, d, *J* = 3.6 Hz, NH), 7.47 (1H, s, C8-H), 7.35 (1H, d, *J* = 11.0 Hz, C12-H), 6.89 (1H, d, *J* = 11.0 Hz, C11-H), 6.53 (1H, s, C4-H), 5.96 (2H, s, br, NH_2), 4.73-4.66 (1H, m, C7-H), 3.93 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 3.62 (3H, s, Ar-OCH_3), 2.51-2.46 (1H, m, C6-Ha), 2.42-2.34 (1H, m, C5-Ha), 2.30-2.22 (1H, m, C6-Hb), 2.00 (3H, s, C14-CH_3), 1.92-1.85 (1H, m, C5-Hb); ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 175.16 (C9), 170.04 (C13), 155.64 (C10), 153.03 (C3), 151.05 (C1), 150.96 (C7a), 141.51 (C2), 139.05 (C12), 134.52 (C4a), 132.28 (C12a), 126.64 (C12b), 125.24 (C8), 112.41 (C11), 107.23 (C4), 61.43 (C1-OCH_3), 61.43 (C2-OCH_3), 56.13 (C3-OCH_3), 52.58 (C7), 37.13 (C6), 30.06 (C5), 22.86 (C14); HR-MS: *m/z* calcd 385.1764 for $C_{21}H_{24}N_2O_5$+H^+ (385.1758); IR ($CHCl_3$): ν_{max} 3305, 2924, 2853, 1745, 1658, 1598, 1505, 1484, 1400, 1348, 1321, 1283, 1219, 1195, 1142, 1017 cm^{-1}.

N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-2-yl)-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (***4l***). Yield: 62%; yellow solid; HPLC, t_R = 8.70 min (99% purity; Method: II); m.p. 158-160 ºC. ^{1}H NMR (CD_3OD, 500 MHz): δ 7.44 (1H, d, *J* = 10.0 Hz, C12-H), 7.35 (1H, d, *J* = 10.0 Hz, C11-H), 7.30 (1H, m, C9'-H), 7.18 (1H, s, C8-H), 7.15-6.98 (2H, m, C10'-H, C11'-H,), 6.75-6.73 (1H, m, C8'-H), 6.72 (1H, s, C4-H), 4.75-4.69 (1H, m, C7-H), 4.51-4.43 (1H, m, C6'-Ha), 4.10-4.01 (1H, m, C6'-Hb), 3.89 (3H, s, Ar-OCH_3), 3.87 (3H, s, Ar-OCH_3), 3.63-3.61 (1H, m, C2'-Ha), 3.57 (3H, s, Ar-OCH_3), 3.10-2.90 (2H, m, C2'-Hb, C3'-Ha), 2.61-2.51 (1H, m, C6-Ha), 2.45-2.25 (2H, m, C3'-Hb, C6-Hb), 2.24-2.10 (1H, m, C5-Ha), 1.99 (3H, s, C14-CH_3), 1.99-1.97 (1H, m, C5-Hb); ^{13}C NMR (CD_3OD, 100 MHz): *δ* 171.65 (C9), 167.16 (C13), 159.69 (C10), 158.44 (C3), 154.83 (C1), 151.92 (C7a), 142.69 (C2), 138.47 (C12), 138.07 (C12'), 136.23 (C4a), 132.09 (C12a), 130.38 (C8), 128.83 (C7'), 128.32 (C12b), 127.26 (C5'), 122.16 (C9'), 120.47 (C4'), 119.86 (C10'), 118.60 (C8'), 116.20 (C11'), 111.92 (C11), 108.83 (C4), 61.67 (C1-OCH_3), 61.67 (C2-OCH_3), 56.65 (C3-OCH_3), 53.39 (C7), 48.66 (C2'), 44.54 (C6'), 37.71 (C6), 30.73 (C5), 22.50 (C3'), 22.45 (C14); HR-MS: *m/z* calcd 540.2493 for $C_{32}H_{33}N_3O_5$+H^+ (540.2493); IR ($CHCl_3$): ν_{max} 3392, 2923, 2852, 1649, 1602, 1537, 1487, 1454, 1399, 1349, 1321, 1219, 1095, 1043, 1019 cm^{-1}.

N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(3-chloro-4-fluoro-phenylamino)-5,6,7,9-tetrahydrobenzo[a] heptalen-7-yl]acetamide (***4m***). Yield: 68%; yellow solid; HPLC, t_R = 6.80 min (99% purity; Method: II); m.p. 171-173 ºC. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.62 (1H, s, C10-NH), 7.56 (1H, s, C8-H), 7.41 (1H, d, *J* = 4 Hz, C5'-H), 7.37 (1H, d, *J* = 12.0 Hz, C12-H), 7.23 (1H, d, *J* = 4 Hz, C6'-H), 7.21 (1H, s, C2'-H), 7.13 (1H, d, *J* = 12.0 Hz, C11-H), 6.54 (1H, s, C4-H), 4.71-4.69 (1H, m, C7-H), 3.93 (3H, s, Ar-OCH_3), 3.90 (3H, s, Ar-OCH_3), 3.64 (3H, s, Ar-OCH_3), 2.54-2.49 (1H, m, C5-Ha), 2.42-2.29 (2H, m, C5-Hb, C6-Ha), 2.02 (3H, s, C14-CH_3) 1.93-1.91 (1H, m, C6-Hb); ^{13}C NMR ($CDCl_3$, 100 MHz): *δ* 175.57 (C9), 169.77 (C13), 157.25 (C4'), 154.78 (C10), 153.25 (C3), 151.79 (C1), 151.13 (C7a), 141.71 (C2), 138.67 (C12), 134.98 (C1'), 134.95 (C4a), 134.28 (C12a), 133.68 (C12b), 126.43 (C8), 125.44 (C2'), 124.12 (C5'), 122.21 (C3'), 117.68 (C6'), 110.20 (C11), 107.32 (C4), 61.43 (C1-OCH_3), 61.39 (C2-OCH_3), 56.14 (C3-OCH_3), 52.65 (C7), 37.31 (C6), 29.99 (C5), 23.00 (C14); HR-MS: *m/z* calcd 513.1597 for $C_{27}H_{26}ClFN_2O_5$+H^+ (513.1587); IR ($CHCl_3$): ν_{max} 3272, 2927, 2853, 1658, 1599, 1545, 1503, 1462, 1428, 1401, 1349, 1322, 1257, 1205, 1143, 1095, 1046, 1017 cm^{-1}.

N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-(4-ethoxycarbonyl-piperazin-1-yl)-5,6,7,9-tetrahydrobenzo[a] heptalen-7-yl]acetamide (***4n***). Yield: 64%; yellow solid; HPLC, t_R = 6.50 min (98% purity; Method: II); m.p. 170-171 ºC. ^{1}H NMR ($CDCl_3$, 500 MHz): *δ* 7.79 (1H, br, NH), 7.32 (1H, s, C8-H), 7.25 (1H, d, *J* = 12.0 Hz, C12-H), 6.76 (1H, d, *J* = 12.0 Hz, C11-H),

6.50 (1H, s, C4-H), 4.66-4.59 (1H, m, C7-H), 4.16-4.13 (2H, m, C8'-H), 3.90 (3H, s, Ar-OCH_3), 3.86 (3H, s, Ar-OCH_3), 3.62 (3H, s, Ar-OCH_3), 3.62-3.41 (6H, m, C3′-Ha, C3′-Hb, C5′-Ha, C5′-Hb, C2′-Ha, C6′-Ha), 3.31-3.25 (2H, m, C2′-Hb, C6′-Hb), 2.47-2.44 (1H, m, C5-Ha), 2.39-2.32 (1H, m, C6-Ha), 2.26-2.21 (1H, m, C5-Hb), 1.97 (3H, s, C14-CH_3) 1.85-1.83 (1H, m, C6-Hb), 1.29-125 (3H, m, C9'-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 181.26 (C9), 169.97 (C13), 157.90 (C10), 155.53 (C7'), 153.29 (C3), 151.26 (C1), 150.47 (C7a), 141.64 (C2), 136.12 (C12), 135.19 (C12a), 134.36 (C4a), 129.79 (C8), 125.90 (C12b), 118.80 (C11), 107.39 (C4), 61.64 (C8'), 61.48 (C1-OCH_3), 61.38 (C2-OCH_3), 56.12 (C3-OCH_3), 52.03 (C7), 48.57 (C3′), 48.57 (C5′), 43.55 (C2′), 43.55 (C6′), 36.70 (C6), 30.06 (C5), 22.87 (C14), 14.70 (C9'); HR-MS: *m/z* calcd 526.2558 for $C_{28}H_{35}N_3O_7$+H^+ (526.2548); IR ($CHCl_3$): ν_{max} 3287, 2933, 2859, 1697, 1605, 1545, 1486, 1462, 1431, 1400, 1382, 1350, 1285, 1226, 1123, 1095, 1076, 1027 cm^{-1}.

*N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-[3-(trifluoromethyl)-4-chloro-phenylamino]-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (**4o**).* Yield: 62%; yellow solid; HPLC, t_R = 6.23 min (99% purity; Method: II); m.p. 168-171 ºC. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.71 (1H, s, C10-NH), 7.66 (1H, s, C8-H), 7.59 (1H, d, *J* = 4 Hz, C5'-H), 7.56 (1H, s, C2'-H), 7.50 (1H, d, *J* = 4 Hz, C6'-H), 7.40 (1H, d, *J* = 12.0 Hz, C12-H), 7.23 (1H, d, *J* = 12.0 Hz, C11-H), 6.54 (1H, s, C4-H), 4.71-4.69 (1H, m, C7-H), 3.93 (3H, s, Ar-OCH_3), 3.90 (3H, s, Ar-OCH_3), 3.65 (3H, s, Ar-OCH_3), 2.54-2.51 (1H, m, C5-Ha), 2.44-2.29 (2H, m, C5-Hb, C6-Ha), 2.02 (3H, s, C14-CH_3) 1.94-1.92 (1H, m, C6-Hb); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 175.83 (C9), 169.73 (C13), 153.35 (C10), 152.14 (C3), 151.12 (C1), 150.79 (C7a), 141.58 (C2), 138.46 (C12), 137.40 (C3'), 134.53 (C1'), 134.26 (C4a), 132.79 (C8), 132.79 (C7'), 128.41 (C12a), 127.21 (C6'), 126.27 (C12b), 125.90 (C5'), 122.58 (C4'), 122.54 (C2'), 110.45 (C11), 107.27 (C4), 61.47 (C1-OCH_3), 61.41 (C2-OCH_3), 56.13 (C3-OCH_3), 52.70 (C7), 37.21 (C6), 29.97 (C5), 22.99 (C14); HR-MS: *m/z* calcd 563.1556 for $C_{28}H_{26}ClF_3N_2O_5$+H^+ (563.1555); IR ($CHCl_3$): ν_{max} 3270, 3001, 2929, 2855, 1660, 1601, 1582, 1543, 1502, 1487, 1463, 1402, 1349, 1322, 1236, 1194, 1142, 1096, 1047, 1015 cm^{-1}.

*N-[(7S)-1,2,3-Trimethoxy-9-oxo-10-[3,4-(methylenedioxy)-phenylamino]-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide (**4p**).* Yield: 72%; yellow solid; HPLC, t_R = 6.50 min (98% purity; Method: II); m.p. 158-160 ºC. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.59 (1H, s, C10-NH), 7.49 (1H, s, C8-H), 7.34 (1H, d, *J* = 12.0 Hz, C12-H), 7.08 (1H, d, *J* = 8 Hz, C5'-H), 6.87 (1H, d, *J* = 8 Hz, C6'-H), 6.83 (1H, s, C2'-H), 6.82 (1H, d, *J* = 12.0 Hz, C11-H), 6.53 (1H, s, C4-H), 6.03 (2H, s, C7'-H), 4.73-4.67 (1H, m, C7-H), 3.92 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 3.63 (3H, s, Ar-OCH_3), 2.52-2.48 (1H, m, C5-Ha), 2.44-2.24 (2H, m, C5-Hb, C6-Ha), 2.02 (3H, s, C14-CH_3) 1.93-1.86 (1H, m, C6-Hb); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 175.25 (C9), 169.93 (C13), 153.07 (C10), 153.00 (C3), 151.48 (C1), 151.12 (C7a), 148.56 (C3'), 146.06 (C4'), 141.63 (C2), 138.95 (C12), 134.43 (C4a), 132.58 (C12a), 131.92 (C1'), 126.70 (C12b), 124.57 (C8), 118.18 (C5'), 110.28 (C11), 108.86 (C6'), 107.27 (C4), 106.20 (C2'), 101.71 (C7'), 61.39 (C1-OCH_3), 61.39 (C2-OCH_3), 56.13 (C3-OCH_3), 52.64 (C7), 37.30 (C6), 30.06 (C5), 22.94 (C14); HR-MS: *m/z* calcd 505.1975 for $C_{28}H_{28}N_2O_7$+H^+ (505.1969); IR ($CHCl_3$): ν_{max} 3271, 2928, 2849, 1658, 1599, 1579, 1502, 1487, 1426, 1382, 1322, 1302, 1283, 1239, 1191, 1143, 1096, 1037 cm^{-1}.

General method for preparation of compounds 5a and 5b. To the solution of colchicine (**1**, 0.25 mmol) in dichloromethane (3 mL) was added benzoyl chlorides (0.40 mmol) and triethylamine (0.60 mmol). The reaction mixture was stirred at room temperature for 24 h, and then the mixture was diluted with cold water and extracted with ethyl acetate followed by purification by silica gel column chromatography (MeOH: $CHCl_3$) to yield compounds **5a** and **5b** (50-60 % yield). In both these reactions, we observed formation of two products; however, we could isolate only **5a** (mono- product in first reaction) and **5b** (di-product in second reaction).

*(10S)-10-[N-Acetyl(m-toluoyl)amino]-14-hydroxy-3,4,5-trimethoxytricyclo[9.5.0.0^{2,7}]hexadeca-1(16),2(7),3,5,11,14-hexaen-13-one (**5a**).* Yield: 50%; yellow solid; HPLC, t_R = 8.37 min (98% purity; Method: II); m.p. 148-151 ºC. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.02 (1H, d, *J* = 11.2 Hz, C6′-H), 7.83 (1H, s, C2′-H), 7.60 (1H, d, *J* = 19.7 Hz, C4′-H), 7.44 (1H, d, *J* = 8.5 Hz, C12-H), 7.39 (1H, dd, *J* = 15.0, 15.0 Hz, C5′-H), 7.27 (1H, s, C8-H), 6.80 (1H, d, *J* = 10.6 Hz, C11-H), 6.55 (1H, s, C4-H), 5.50-5.40 (1H, m, C7-H), 3.97 (3H, s, Ar-OCH_3), 3.91 (3H, s, Ar-OCH_3), 3.71 (3H, s, Ar-OCH_3), 2.58-2.51 (3H, m, C6-Ha, C6-Hb, C5-Ha), 2.43 (3H, s, Ar-CH3), 2.18 (3H, s, C13-H), 1.98-1.85 (1H, m, C5-Hb); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 179.57 (C9), 174.25 (C1'), 171.39 (C13), 164.20 (C10), 153.55 (C3), 151.44 (C1), 149.56 (C7a), 141.95 (C2), 139.54 (C6'), 138.48 (C12a), 136.58 (C2'), 136.03 (C4a), 134.76 (C5'), 134.35 (C12b), 133.40 (C8), 131.13 (C12), 130.11 (C4'), 128.59 (C7'), 127.81 (C3'), 111.96 (C11), 107.48 (C4), 61.65 (C1-OCH_3), 61.51 (C2-OCH_3), 59.83 (C3-OCH_3), 56.54 (C7), 32.40 (C6), 30.26 (C5), 26.93 (C8'), 21.39 (C14); HR-MS: *m/z* calcd 504.2022 for $C_{29}H_{29}NO_7$+H^+ (504.2017); IR ($CHCl_3$): ν_{max} 3368, 2925, 2853, 1740, 1705, 1665, 1619, 1586, 1488, 1456, 1404, 1322, 1254, 1188, 1139, 1093, 1046, 1021 cm^{-1}.

*(10S)-10-[N-Acetyl(benzoyl)amino]-3,4,5-trimethoxy-13-oxotricyclo[9.5.0.0^{2,7}]hexadeca-1(16),2(7),3,5,11,14-hexaen-14-yl benzoate (**5b**).* Yield: 60%; yellow solid; HPLC, t_R = 10.61 min (98% purity; Method: II); m.p. 154-157 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.24 (2H, d, *J* = 7.6, C2″-H, C6″-H), 8.05 (1H, d, *J* = 12.4 Hz, C12-H), 8.03 (1H, s, C8-H), 7.79 (2H, d, *J* = 7.6, C2′-H, C6′-H), 7.65-7.42 (6H, m, C3″-H, C4″-H, C5″-H, C3′-H, C4′-H, C5′-H), 7.24 (1H, d, *J* = 12.4 Hz, C11-H), 5.37-5.32 (1H, m, C7-H)1, 3.97 (3H, s, Ar-OCH_3), 3.92 (3H, s, Ar-OCH_3), 3.78 (3H, s, Ar-OCH_3), 2.68-2.52 (3H, m, C6-Ha, C6-Hb, C5-Ha), 1.98-1.85 (1H, m, C5-Hb), 1.82 (3H, s, C13-H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 179.57 (C9), 174.25 (C1'), 171.39 (C13), 169.91 (C1"), 164.20 (C10), 153.55 (C3), 151.44 (C1), 149.56 (C7a), 141.95 (C2), 139.54 (C12a), 136.58 (C2'), 136.03 (C4a), 134.86 (C8), 134.76 (C5"), 134.76 (C5'), 134.65 (C3'), 134.35 (C12b), 133.40 (C3"), 133.40 (C7"), 131.13 (C12), 130.11 (C4'), 129.23 (C4"), 129.07 (C2"), 128.59 (C7'), 127.81 (C6"), 126.99 (C6'), 111.96 (C11), 107.48 (C4), 61.65 (C1-OCH_3), 61.51 (C2-OCH_3), 59.83 (C3-OCH_3), 56.54 (C7), 32.40 (C6), 30.26 (C5), 21.39 (C14); HR-MS: *m/z* calcd 594.2130 for $C_{35}H_{31}NO_8$+H^+ (594.2129); IR

($CHCl_3$): ν_{max} 3377, 2924, 2853, 1693, 1602, 1584, 1490, 1452, 1427, 1321, 1288, 1176, 1096, 1045, 1021 cm^{-1}.

P-gp-induction assay. Colchicine along with all synthesized compounds were screened for their ability to induce P-gp using rhodamine123 (Rh123) cell exclusion method. In this method, the P-gp function was evaluated in terms of rhodamine 123 (Rh123) accumulation and efflux.[57] Briefly, the protocol used is as follows: Colorectal LS-180 cells were seeded at a density of 2×10^4 per well of 96 well plate and were allowed to grow for next 24 h. Cells were further incubated with the test compounds, and were diluted to a final concentration of 100 nM and rifampicin (standard) to a final concentration of 10 µM in complete media for 48 h. The final concentration of DMSO was kept at 0.1%. Drugs were removed and cells were incubated with HANKS buffer for 40 minutes before further incubation with HANKS buffer (containing 10 µM of Rh123 as a P-gp substrate) for 90 minutes. At the end of Rh123 treatment cells were washed four times with cold PBS followed by cell lysis for 1 h by using 200 µl of lysis buffer (0.2% triton X 100 and 0.2N NaOH). A total of 100 µl of lysate was used for reading fluorescence of Rh123 at 485 nm/529 nm. Samples were normalized by dividing fluorescence of each sample with total protein present in the lysate.

Antiproliferative activity. Human colon carcinoma cell lines HCT-116 and Colo-205 were procured from National Cancer Institute, USA and normal epithelial cell line fR2 was purchased from Sigma Aldrich (ECACC type). HCT-116 cells were grown in McCoy's 5A and Colo-205 and fR2 cells were grown in RPMI growth medium respectively containing 10% FCS, 100U penicillin and 100 mg streptomycin per mL medium. Cells were grown in CO_2 incubator (Thermocon Electron Corporation, Houston, TX) at 37 ºC with 95% humidity and 5% CO_2 gas environment. Cells treated with tested materials were dissolved in DMSO while the untreated control cultures received only the vehicle (DMSO < 0.2%). Cells were seeded in 96 well plates and exposed to tested compounds at various concentrations for 48 h time interval. MTT dye (2.5 mg/ml in PBS) was added 4 hrs priors to experiment termination. The plates were then centrifuged at 1500 rpm for 15 min and the supernatant was discarded, and MTT formazan crystals were dissolved in 150 µl of DMSO. The OD measured at 570 nm with reference wavelength of 620 nm.[58] The percentages of cell viability and growth inhibition were calculated using formulas:

$$\text{The \% of cell viability} = \frac{\text{Absorbance of treated cells - Absorbance of Blank}}{\text{Absorbance of control cells - Absorbance of Blank}} \times 100$$

$$\text{\% Growth inhibition} = 100 - \text{\% of cell viability}$$

Effect of compounds on microtubules, p21 and p27 using confocal microscopy. For detection of tubulins, HCT-116 cells (1 x 10^5 cells/well) were seeded on to 18 mm square cover slips in six well plates in complete medium. Cells were allowed to adhere for 24 h and were treated with respective concentrations of compounds **1**, and **4o** for 24 h. After the treatment period, cells were washed twice with PBS and fixed in 4% paraformaldehyde for 10-15 min at room temperature and permeabilized using Triton-X (0.5% for tubulin and 0.1% for p21, p27) in PBS for few min. (5 min. for tubulin and 15 min. for p21, p27). Nonspecific binding sites were blocked by incubating the cells in 10% BSA. Cells were then incubated with a monoclonal α-tubulin antibody (Sigma) or p21 and p27 antibodies (CST) diluted 1:100 in 0.1% Triton X-100 in PBS for 1 h at room temperature and Alexa Fluor 488 conjugated secondary antibody (Invitrogen) diluted 1:500 in PBS for 1 h at room temperature. Cells were then washed three times in PBS and stained with 4',6-diamidino-2-phenylindole (DAPI 1 µg/ml in PBS). The cover slips were mounted over glass slides and cells were imaged by a laser scanning confocal microscope (Olympus Fluoview FV1000).

Western-blot analysis. Protein was measured employing Bio-Rad protein assay kit using bovine serum albumin as standard. Proteins aliquots (70 µg) were resolved on SDS-PAGE and then electro transferred to PVDF membrane overnight at 4 °C at 30V. Nonspecific binding was blocked by incubation with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The blots were probed with respective primary antibodies (Sigma and CST) for 2-4 h and washed three times with TBST. The blots were then incubated with horseradish peroxidase conjugated antimouse secondary antibodies for 1 h, washed again three times with TBST and signals detected by using ECL plus chemiluminescence's kit on X-ray film.

Molecular docking for tubulin. The tubulin-colchicine complex (PDB ID 1SA0) was retrieved from the Protein data bank.[45] In this complex Protein is heterodimeric in nature, consisting two, tubulin α chains (451 residues), two tubulin β chains (452 residues) and Stathmin like domain (142 residues). Protein is prepared using protein preparation wizard of Schrodinger in default mode and grid file constructed considering colchicines like ligand as centroid of grid at interphase of α, β-tubulin chains. Ligands were sketched in maestro, prepared using ligprep and docked by Glide molecular docking software.[59]

Human cytochrome P450 (CYP450) isoenzymes assay. The Cytochrome P450 isoenzymes were aliquoted as per the total concentration required to conduct the study and stored at -70 °C until use. Total assay volume was adjusted to 200 µl containing three components: cofactors, inhibitor/vehicle and enzyme substrate (ES) mix. The 50 µl of working cofactor stock solution was dispensed to all the specified wells in a black nunc microtiter polypropylene plate. The 50 µl of diluted working concentrations of 4o/ positive control inhibitors/vehicle were dispensed in triplicates to the specified wells as per the plate map design. Reaction plate with cofactor and test item was pre incubated at 37°C ± 1 °C shaking incubator for 10 minutes. Simultaneously, ES mix was prepared by mixing the CYP P450 isoenzymes. Remaining volume was made up with the buffer and pre-incubated for 10 minutes at 37 °C ± 1 °C. 100 µL of ES mix was dispensed per well as per the plate map design and incubated at 37 °C ± 1 °C shaking incubator for predetermined time. A set of controls were incubated with CYP P450 isoenzymes and substrate without test or reference item. A set of blanks were incubated with substrate and test or reference item, in the absence of CYP P450 isoenzymes. Reaction was terminated by adding specific quenching solutions (For CYP2C19 and CYP3A4 – 75 µl of 100% acetonitrile; For CYP2C9 – 20 µl of 0.25 M Tris in 60% methanol; For CYP2D6 – 75 µl of 0.25 M Tris in 60% methanol). The reaction was quenched by thoroughly mixing the final

contents of the wells by repeated pipetting using multichannel pipette. The product fluorescence per well was measured using a fluorimeter at excitation and emission wavelength for respective CYP P450 isoenzyme fluorogenic metabolites. Data was analyzed by using Excel spreadsheet and the % inhibition was calculated.[47]

Determination of solubility and partition coefficient. The thermodynamic equilibrium solubility in water, PBS, SGF and SIF was determined using a 96-well plate protocol.[60] The partition coefficient (Log Po/w) was determined using capacity factor method as per 'OECD guidelines for testing of chemicals 117'.

Pharmacokinetics studies. Oral and intravenous (IV) pharmacokinetic studies of compound **4o** were carried out in BALB/c male mice of age 4-6 weeks, by administering **4o** orally and IV at dose of 10 mg/kg for oral and 1 mg/kg for IV. Plasma samples were collected at appropriate time points between the range of 0-24 hrs and analyzed by LC-MS-MS. Mean plasma concentration calculated and data was further analyzed to determine PK parameters using WinNonlin 5.3 software package.

This study was carried out at Jubilant Biosys Bangalore on commercial basis. These experiments were approved by the Jubilant Biosys Institutional Animal Ethics Committee, Bangalore, India (IAEC/JDC/2012/27) and were in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Environment, Government of India.

***In vivo* activity in Ehrlich solid tumor model.** Ehrlich ascites carcinoma (EAC) cells were collected from the peritoneal cavity of the swiss mice weighing 18-23 gm, harbouring 8-10 days old ascitic tumor. $1x10^7$ EAC cells were injected intramuscularly in the right thigh of swiss male mice selected for the experiment on day 0. The next day, animals were randomized and divided into 9 groups. Eight treatment groups contained 7 animals each and two control groups contained 10 animals each. Treatment was given as follows:

Group I: **1** (0.75 mg/kg, i.p.) from day 1-9.

Group II: **4o** (1 mg/kg, i.p.) from day 1-9.

Group III: **1** (0.75 mg/kg, p.o.) from day 1-9.

Group IV: **4o** (1 mg/kg, p.o.) from day 1-9.

Group V: **1** (1.25 mg/kg, p.o.) from day 1-9.

Group VI: **4o** (2 mg/kg, p.o.) from day 1-9.

The seventh treatment group was treated with 5-fluorouracil (22 mg/kg, i.p.) from day 1-9 and it served as positive control. The control group was similarly administered normal saline (0.2 ml, i.p. and p.o.) from day 1-9. On day 9 & 13, tumor bearing thigh of each animal was shaved and longest and shortest diameters of the tumor were measured with the help of vernier caliper. Tumor weight of each animal was calculated using the following formula.

$$\text{Tumor weight (mg)} = \frac{\text{Length (mm)} \times [\text{width (mm)}]^2}{2}$$

The percent tumor growth inhibition was calculated on day 13 by comparing the average values of treated groups with that of control group. Tumor growth in saline treated control animals was taken to be 100 %.

The experimental procedures employed in this study were approved by the Institutional Animal Ethics Committee, Indian Institute of Integrative Medicine, Jammu, India.

Statistical analysis. Data expressed as mean ± SD or representative of one of three similar experiments unless otherwise indicated. Comparisons were made between control and treated groups or the entire intra group using one way ANOVA with post Bonferroni test through GraphPad Prism 5.00.288 statistical analysis software. p -values *<0.5 were considered significant.

Abbreviations

CYP3A4, cytochrome P450 3A4; CYP2D6, cytochrome P450 2D6; CYP2C9 cytochrome P450 2C9; CYP2C19, cytochrome P450 2C19; $CHCl_3$, chloroform; Colo-205, human colon cancer cell line; DAPI, 4',6-diamidino-2-phenylindole; DEPT, Distortionless Enhancement by Polarization Transfer; DMF, dimethylformamide; DMSO, Dimethyl sulfoxide; DNA, Deoxyribonucleic acid; ESI-MS, electrospray ionization-mass spectroscopy; fR2, normal epithelial tissue; HCT-116, human colon carcinoma cell lines; HRMS, high resolution mass spectroscopy; IR, Infrared spectroscopy; LS-180, Intestinal human colon adenocarcinoma; IP, Intraperitoneal; IV, intravenous; MDR, multi-drug resistance; MeOH, methanol; MM/GBSA, molecular mechanics/generalized born surface area; mp, melting point; NMR, Nuclear magnetic resonance; OTs, O-tosyl; PARP, Poly (ADP-ribose) polymerase; PBS, phosphate buffered saline; Pgp, p-glycoprotein; PO, oral route; $POCl_3$, Phosphoryl chloride; Rh123, Rhodamine 123; SAR, structure activity relationship; SD, standard deviation; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

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