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Synthesis of non-toxic anticancer active forskolin-indole-triazole conjugates along with their in silico succinate dehydrogenase inhibition studies

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Abstract

Biologically important three different pharmacophores, forskolin, indole and 1,2,3-triazoles are coupled to obtain a hybrid molecule. Here, we described the synthesis of novel series of forskolin-indole-triazole conjugates 5a-5l by using the Cu(I) catalyzed 1,3-dipolar cycloaddition reaction. Furthermore, the biological significance of the synthesized molecules was assessed by in silico and in vitro modes. All the synthesized compounds were evaluated for in vitro anticancer activity against PC-3, MCF-7, MDA-MB-231, COLO-205, HeLa, WRL-68, RAJI, CHANG and RAW-264.7 cell lines. Compound 5g was found to be the most potent in all the tested cell lines (IC_{50} range 9.6–21.66 μg/ml, except COLO-205), 5a, 5b and 5k were observed to exert its effect only against WRL-68 (IC_{50} range 27.69–48.18 μg/ml), when compared to parent 3 (IC_{50} > 100 μg/ml, tested concentrations 10–50 μg/ml) and standard Doxorubicin (IC_{50} range 0.42–3.16 μg/ml). The most potent compound 5g (MEF_{50} 0.57) was found non-toxic to human erythrocytes as compared to control (MEF_{50} 0.60) at tested concentration (50 μg/ml). In silico-based succinate dehydrogenase inhibition showed that the synthesized compounds were having potent binding affinity compared to forskolin. Predictive ADMET and toxicity risk assessment analysis revealed that most of the compounds were complying with the standard limit of Lipinski's rule of five for oral bioavailability.

KEYWORDS

anti-cancer activity, forskolin, indole, tsriazole

1 | INTRODUCTION

Natural products (NPs) and the molecules based on NP scaffolds, being the proven source of therapeutic agents, have increasingly attracted the interests of researchers involved in cancer therapy [1,2]. In fact, most anticancer drugs available today are either originated from nature or synthesized as natural identical. One of such important class of secondary metabolites is terpenoid which has pharmacologically active scaffold and provides privileged motifs for further modifications and structure activity

Devendar Ponnam and Niranjana Kumar Arigari have equal contribution and considered as first authors.

relationship analysis, etc. To date, several reports are available wherein simple or advanced modifications have been performed for bringing out the desired biological properties [3,4].

Although, several plant-derived anticancer drugs, such as Podophyllotoxin, Vincristine, Vinblastine, Ellipticine, Paclitaxel, Camptothecin, and Combretastatin A4, are used clinically, they failed to achieve the desired therapeutic effect due to multi-drug resistance, toxicity, or poor bioavailability. Therefore, it is necessary to identify and develop more effective and safe anticancer drugs [5,6].

Forskolin **3** is a labdane diterpenoid isolated from *Coleus forskohlii*. Forskolin increases intracellular levels of important signaling agent cyclic AMP (for biological response of cells to hormones and other extracellular signals) by activating adenylyl cyclase enzyme. Forskolin and its analogues have shown various biological activities, such as antihypertensive, positive inotropic [6–8], antiobesity [9,10], antidiabetic [11], and anticancer activities [12,13]. Experimental studies on different cancer cell lines have shown synergistic effects of forskolin in upregulating cellular responses [14–17]. A protective effect against ionizing radiation [18] and UBV-induced apoptosis [19] has been shown in in vitro. Forskolin has been demonstrated to have an angiogenic effect via cAMP-dependent protein kinase pathways [20]. However, the water-insoluble nature of forskolin limits its clinical usefulness.

Second pharmacophore, the indole and its various conjugates present in many natural and synthetic molecules exhibit a wide range of significant biological activities, such as antidiabetic [21], anti-inflammatory [22], antimalarial [23], antibacterial [24], antifungal [25], antiviral [26] and anticancer [27]. This moiety is very important for its medicinal and biological aspects, thus attracting a lot of scientific attention.

The third pharmacophore, 1,2,3-triazole has been shown to possess several desirable structural features in the context of medicinal and click chemistry. The synthetically prepared 1,2,3-triazole derivatives have shown diverse biological activities, such as anticancer [28], antibiotic [29], antifungal, antibacterial, antitubercular and antiviral [30–32]. Triazoles are stable to acid/base hydrolysis and reductive/oxidative conditions, indicative of a high aromatic stabilization and, also relatively resistant to metabolic degradation. At the same time, they readily associate with biological targets, through hydrogen bonding and dipole interactions [33,34]. NPs hooked with the 1,2,3-triazole moiety have also shown excellent anticancer activities, such as Podophyllotoxin (**A**) [35], Oleanolic acid (**B**) [36], betulinic acid (**C**) [37], dehydroabietic acid (**D**) [38], imbricatolic acid (**E**) [39] and 10β-artemisinin (**F**) derivatives [40] (Figure 1).

Our earlier studies on the cyclic acetal protection of hydroxyls of forskolins and andrographolides with suitable functional groups, significantly increased its cytotoxicity [41–43]. In view of the above finding, it is envisaged that synthesis of a hybrid of forskolin-indole-1,2,3-triazole (FIT) and its anlogues may result in high value, potent, selective and less toxic bioactive molecules. A series of novel FITs were generated by employing Cu(I)-catalyzed Huisgen [3 + 2] cycloaddition (click chemistry). The resulting derivatives were screened for their anticancer activity in in vitro mode using MTT assay against selected human cancer cell lines. MTT assay, catalyzed by succinate dehydrogenase, is an indicator of cell viability in terms of mitochondrial respiration. Assessment of molecular interactions shown by these analogues for succinate dehydrogenase inhibition (SDHI) and ADMET analysis in silico mode was also carried out to support the drawn in vitro inferences. The compounds showing potent anticancer activity were further tested for toxicity to human erythrocytes by performing osmotic fragility test.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

A three-step synthesis to obtain the target molecules is depicted in Schemes 1 and 2. In the first step, the precursor *N*-propargyl-indole-3-carbaldehyde **2** was synthesized by treating indole-3-carbaldehyde **1** with propargyl bromide/K_2CO_3 in DMF (92% yield). In the second step, aldehyde **2** was subjected to acetalization with forskolin **3** in presence of Amberlist-15 (wet) in CH_2Cl_2 at room temperature for 24 h, to give novel 3,19-*O*-(*N*-propargyl-3-indolylidene)forskolin **4** in 75% yield (Scheme 1). In the third step, terminal alkyne **4** and various substituted azides ***a-l*** (synthesized in situ from their halides/amine precursors) [44] in the presence of catalytic Cu(I) (CuSO4/sodium ascorbate), resulted in a series of 12 1,4-disubstituted triazoles **5a-5**l in high regioselectivity (Scheme 2). Aqueous conditions (H_2O/*t*-BuOH, 2:1) facilitated precipitation of products, which were isolated in high purity [45].

The structures of all the hybrids were elucidated by NMR, IR and mass spectral data. For example, structures of **4** and **5l** were assigned as follows. The compound **4** showed characteristic stretching in IR spectra at 2123 cm^{-1} (C≡C), confirmed the new triple bond. A molecular ion peak $[M + H]^+$ at *m/z* 576.3 was observed in ESI-MS spectra. In ^1H NMR spectrum, the presence of characteristic singlet at δ_H 5.74 (1H) corresponding to the newly formed acetal ring (*O*-CH-*O*). A triplet in up field at δ 2.41 ($^3J_{H,H}$ = 2.4 Hz, 1H) coupled with a doublet at δ

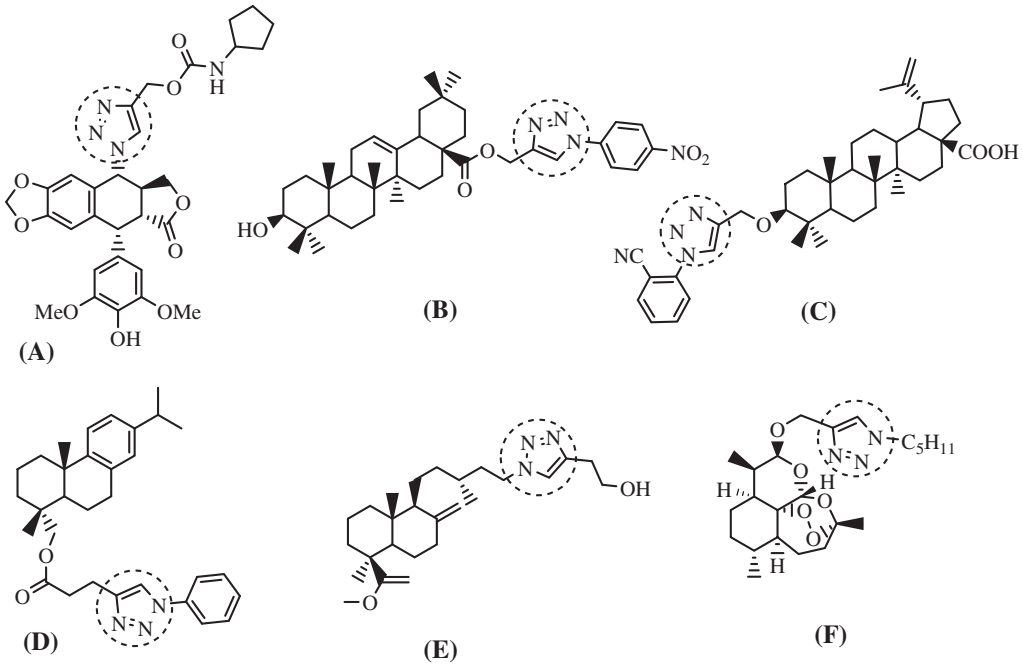
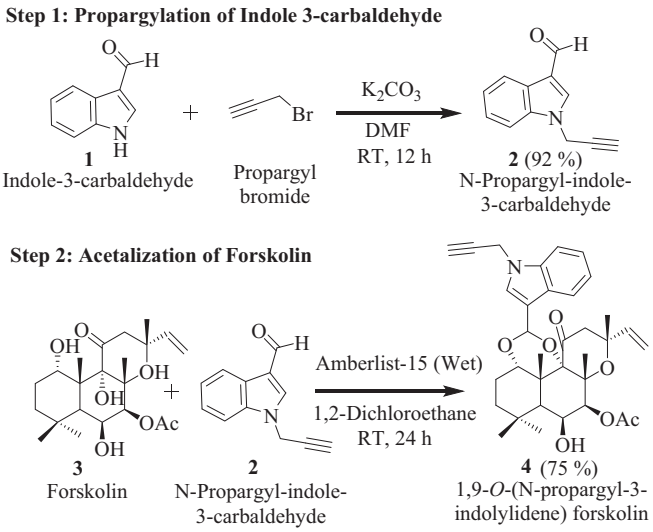


FIGURE 1 Semisynthetic cytotoxic 1,2,3-triazole conjugates of 10β-Podophyllotoxin (A), Oleanolic acid (B), Betulinic acid (C), Dehydroabietic acid (D), Imbricatolic acid (E) and 10β-Artemisinin (F)

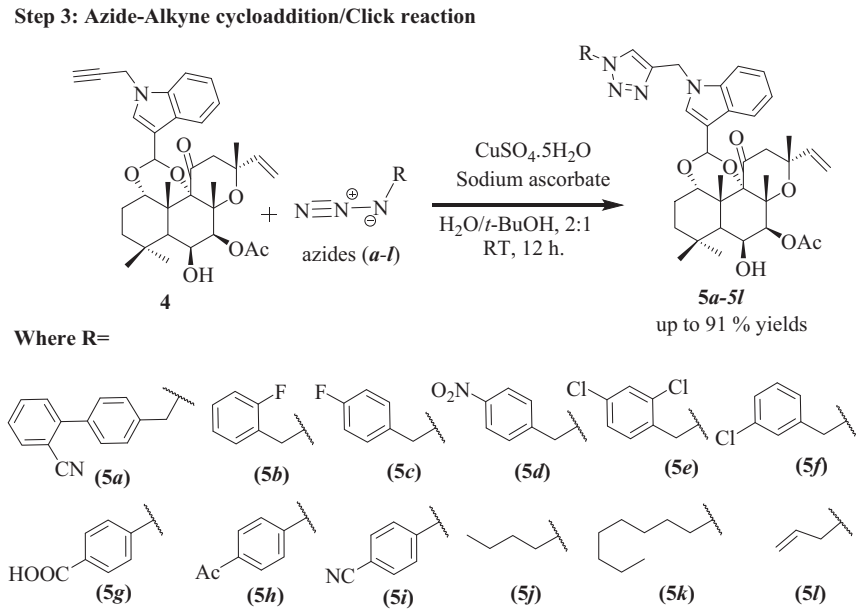


SCHEME 1 Synthesis of 1,9-O-(N-Propargyl-3-indolylidene) forskolin **4**

4.84 ($^3J_{H,H}$ = 2.4 Hz, 2H), confirms the presence of *N*-propargyl group (*N*–CH_2–C≡CH). In aromatic region, a characteristic singlet at δ_H 7.33 (1H, pyrrole ring H) along with two doubles (J = 8 Hz) at δ_H 7.35 (1H, Ar-H), 7.36 (1H, Ar-H) and two multiplets in the region of δ_H 7.11–7.25 (2H, Ar-H), due to the presence of 3-substituted indole. In ^{1}H NMR spectrum of **5l**, two characteristic singlets at δ_H 7.99 (1H), 7.60 (1H) corresponding to protons of triazole ring and pyrrole ring, respectively. A singlet at δ_H 5.65 (1H) confirmed the new cyclic indolylidene acetal ring (*O*-CH-*O*). The bridged indole N-CH_2 protons were showed as a singlet at δ_H 5.44 (2H). A characteristic doublet δ_H 4.96 (2H, J = 6 Hz, overlapped), multiplet δ_H 6.03–5.94 (1H) and two overlapped doublet of doublet at δ_H 5.22 (1H), 4.87 (1H) confirm the end allyl group ((N-CH_2-CH=CH_2) on triazole ring. Presence of two doublets (J = 8 Hz) at δ_H 7.75 (1H), 7.61 (1H) and two multiplets at δ_H 7.16 (1H), 7.04 (1H) confirmed the four phenyl ring protons of Indole. Also, a molecular ion peak $[M + H]^+$ observed at m/z 659.33 in ESI-MS spectrum, confirmed the assigned structure **5l**.

2.2 | In silico succinate dehydrogenase inhibition

Molecular docking of succinate dehydrogenase (1YQ3), a NAD (P) H-dependent cellular oxido-reductase enzyme known to catalyze the conversion of tetrazolium dye into its insoluble purple colored formazan crystals, is described here. Under the defined condition it reflects the number of viable cells, therefore, considered as a frontline target for cytotoxicity evaluation. Docking study was performed to understand the molecular interaction of forskolin derivatives with SDH and the obtained results are expressed in terms of binding energy (BE) and inhibition constant (Ki). All the compounds showed strong binding interaction with the SDH in terms of lowest



SCHEME 2 Synthesis of forskolin-indole-triazole conjugates 5a-5l

TABLE 1 Binding energy and inhibition constants for forskolin and its derivatives

Entry	Succinate dehydrogenase (PDB ID: 1YQ3) Docking score (kcal/mole)	Inhibitory constant (Ki)
3	−6.97	7.76 μM
4	−6.37	21.36 μM
5a	−7.49	3.24 μM
5b	−8.22	940.37 nM
5c	−8.51	581.01 nM
5d	−9.70	77.60 nM
5e	−9.14	198.06 nM
5f	−7.08	6.42 μM
5g	−9.02	245.32 nM
5h	−8.56	528.08 nM
5i	−8.04	1.27 μM
5j	−9.17	190.91 nM
5k	−6.75	11.29 μM
5l	−7.51	3.12 μM

binding energy ranged from −6.37 to −9.70 Kcal/mol and Ki from 77.60 nM to 21.36 μM (Table 1), when compared to forskolin and standard reference compound Malonate (−5.64 kcal/mol and 73.96 μM). Hence, it may be interpreted that these compounds have strong inhibitory activity for SDH and thus modulate the activity of this enzyme and affect the viability of the cells.

Further, to find out root mean square distance (RMSD), molecular geometry comparison was done either by superimposing one or more atoms in one structure on corresponding atom (s) of the other, or by ordered group in one structure over the other. The distance (error) RMS value in molecular geometry between two structures was calculated and showed (Table S1). The results indicate that RMSD between the compound **3** (forskolin) and the synthesized compounds were not significantly different, which indicated that perhaps most of the compounds are having potential cytotoxic activity.

In addition to calculate RMSD, the predictive ADMET, drug-likeness and toxicity levels (Tables S2–S4) were also evaluated. ADMET is helpful in deciding the acceptance of forskolin derivatives as potential anticancer compounds. The toxicity screening data can be used for (a) deciding safe concentration level to be used for animal assays, (b) in the reduction of animal usage during screening out of potentially toxic molecules and (c) in optimizing the therapeutic ratios of the potential anticancer forskolin derivatives. All derivatives showed compliance with the standard range. As per in silico studies, in comparison to forskolin **3** most of the synthesize compounds showed good binding affinity, low RMSD, low hepatotoxicity, genotoxicity, carcinogenicity and mutagenicity, and were following Lipinski's rule of five for oral bioavailability. Hence these results were validated using in vitro MTT assay.

2.3 | In vitro cytotoxic activity

All the compounds evaluated for in vitro cytotoxic activities against 10 cancer cell lines MCF-7 (human breast

adenocarcinoma), MDA-MB-231 (human breast adenocarcinoma), CHANG (human liver cell line), WRL-68 (human hepatocellular carcinoma), COLO-205 (human colon carcinoma), PC-3 (human prostrate carcinoma), K562 (human leukemia carcinoma), Hela (human cervical carcinoma), RAW-264.7 (mouse macrophages cell line) and RAJI (human Burrkit's lymphoma cell line) using MTT assay. Doxorubicin was used as a standard (Table 2). Among all the synthesized derivatives, **5g** (*p*-COOH on phenyl) possessed excellent cytotoxicity (IC_{50} 9.6–21.66 μg/ml) in most of the tested cell lines (except COLO-205) and **5a**, **5b** and **5k** displayed moderate cytotoxicity against WRL-68 cell line (IC_{50} 27.69–48.18 μg/ml) and remaining compounds **5c-5f**, **5h-5j** and **5l** were found inactive ($IC_{50} > 100$ μg/ml), when compared to parent (in all the cell lines $IC_{50} > 100$ μg/ml, tested concentrations 10–50 μg/ml) and standard Doxorubicin (IC_{50} range 0.42–3.16 μg/ml). From Table 2, it clearly indicates that FIT hybrids having *p*-flurobenzyl, *p*-nitrobenzyl *o*,*p*-dichlorobenzyl, *m*-chlorobenzyl, *p*-acetylphenyl, *p*-cyanophenyl, n-butyl, allyl group substitutions were found non-toxic against all the tested cell lines at tested concentration. Whereas substitutions like *n*-octyl, biphenyl, *o*-flurobenzyl substitutions moderate active against only WRL-68 cell line, and with a suitable polar *p*-carboxyphenyl on triazole ring was found to be highly potential against eight cell lines.

Since compound **5g** showed better cytotoxicity profiles, its interaction with the SDH receptor was further studied in depth and found that the interacting amino acids residues at the binding site were ARG 464, SER 57, GLU 46, GLU 34, LYS 52,THR 475, GLY 217, GLN 477, TYR 220, ASP 92, LYS 87, THR 86, GLY 256, THR 367, THR 228 for compound **5g** with four H-bonds (Figure 2).

2.4 | Erythrocyte osmotic fragility assay

Erythrocyte tensile strength is responsible for normal function of erythrocytes. Determination of osmotic fragility patterns is a good index of membrane integrity, so it is frequently used for the measurement of erythrocyte tensile strength and resistance. Compound **5g** possessed significant cytotoxicity was further evaluated for osmotic fragility. Compound **5g** has exerted a pronounced protective effect on erythrocyte osmotic fragility which is evident by a significant shift of the curve to the right and a decrease in mean erythrocyte fragility (MEF), representing a resistance to hemolysis (Figure 3), when exposed to an environment of hypotonic solutions. At higher tested concentration (50 μg/ml) protective effect of both compounds on osmotic fragility of erythrocyte is shown (Table S5) which gives the MEF of erythrocyte incubated for 30 min at 370°C.

A significant ($p < 0.01$) decrease in MEF, compared with control was observed when erythrocytes are incubated with respective compounds. Hence, the tested concentration of **5g** may be considered as non-toxic toward human erythrocytes.

3 | CONCLUSION

In conclusion, synthesis of molecular hybrids with multiple pharmacophores is an emerging structural modification tool toward designing potential medicinal drugs. In the present synthesis, the introduced N-propargyl group on 1,9-*O*-(3-indolylidene) forskolin provided a uniquely reactive handle within the structure and was subsequently reacted with a series of aryl, benzyl, aliphatic and allylic azides to afford 12 forskolin-indole-triazole (FIT)

TABLE 2 Cytotoxic potential of forskolin and their conjugates against various human cancer cell lines by MTT assay

	Cytotoxic activity (IC_{50} μg/ml)[a]					
Entry	**3**	**5a**	**5b**	**5g**	**5k**	**Doxo**
PC-3	>100	>100	>100	9.89	>100	3.16
MCF-7	>100	>100	>100	9.93	>100	1.65
MDA-MB-231	>100	>100	>100	6.00	>100	1.97
COLO-205	>100	>100	>100	>100	>100	0.49
HeLa	>100	>100	>100	12.86	>100	0.51
WRL-68	>100	46.46	27.69	9.39	48.18	1.92
RAJI	>100	>100	>100	21.66	>100	3.06
CHANG	>100	>100	>100	15.30	>100	0.42
RAW-264.7	>100	>100	>100	9.20	>100	2.96

[a]IC_{50} values depicted are mean of three experiments ($n = 3$) in duplicate.

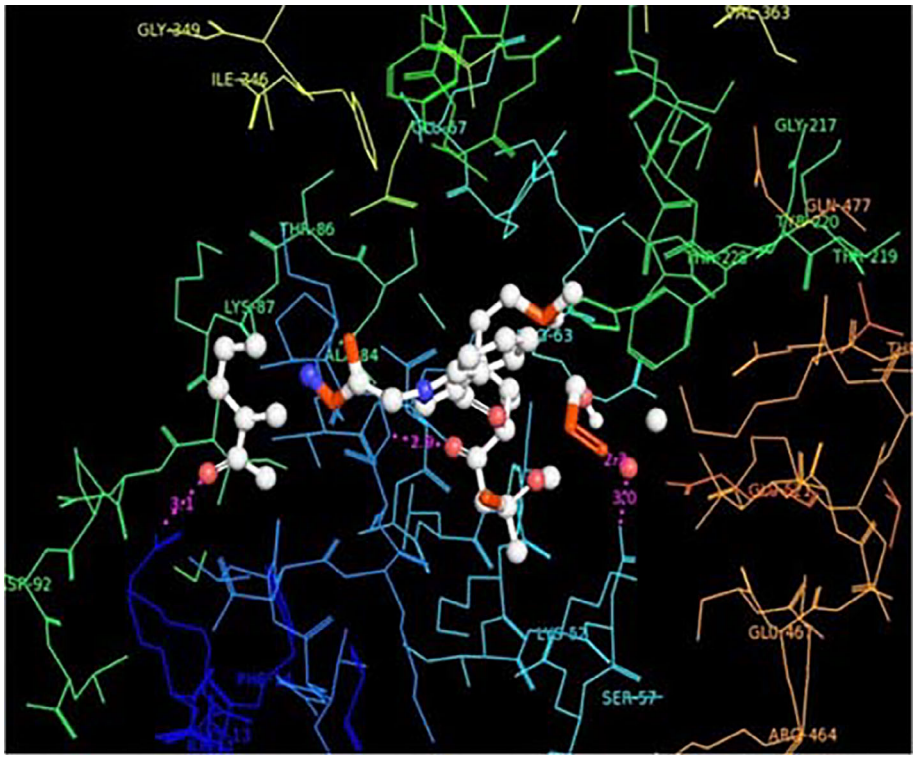


FIGURE 2 Binding pocket for compound 5g within the succinate dehydrogenase (PDB ID: 1YQ3). Hydrogen bonds formed between the compound 5g and various amino acid residues within the pockets are represented by dashed lines (magenta color)

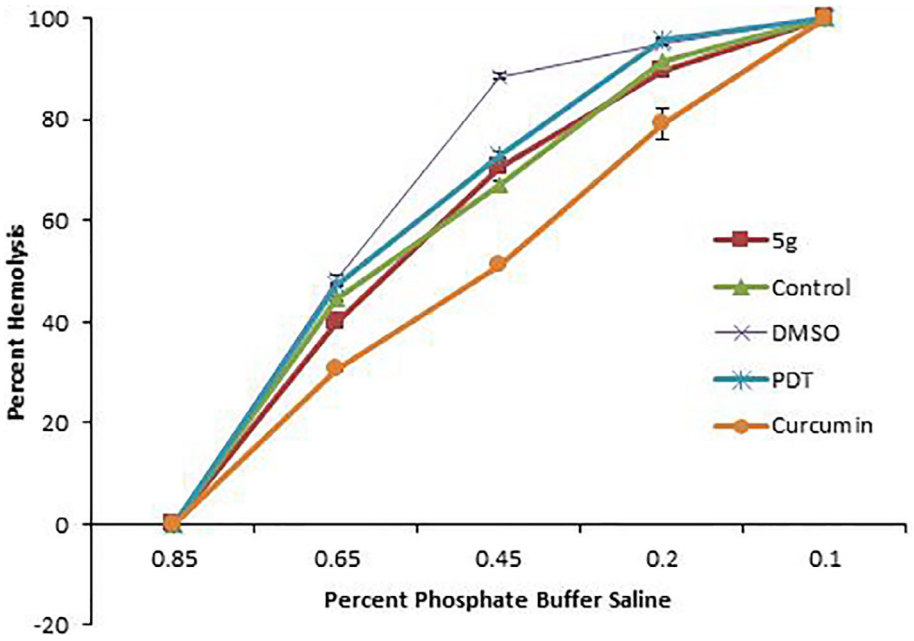


FIGURE 3 Osmotic fragility curve of compound **5g**

library via Cu (I)-catalyzed 1,3-dipolar cycloaddition. Novel derivatives, clean reaction profiles, greater selectivity, simple experimental/product isolation procedures, makes it a useful and attractive strategy for the synthesis of forskolin-indole-triazole hybrids.

The derivatives were evaluated for anticancer activity against PC-3, MCF-7, K562, MDA-MB-231, COLO-205, HeLa, WRL-68, RAJI, CHANG and RAW-264.7 cell lines. Compound **5g** has shown significant cytotoxicity $IC_{50} < 21.66$ μg/ml against all the tested cell lines except COLO-205. To validate the above MTT assay results, an in silico-based molecular docking of the derivatives was performed against SDH to understand binding interactions. Most of the compounds showed strong inhibitory activity for SDH when compared to parent forskolin. Mean erythrocyte fragility (MEF_{50}) assay revealed compound **5g** was nontoxic to human erythrocytes at 50 μg/ml. All the experimental results prompt us to

consider the designed scaffold as a starting point for the development of the novel and more potent forskolin anticancer agents in the future.

4 | EXPERIMENTAL SECTION

4.1 | Chemistry

All other chemicals and reagents purchased from Aldrich (India), AVRA Chemicals Pvt. Ltd. (India) and were used without further purification. TLC silica gel 60 F_{254} (Merck, Germany) was used for TLC. Visualization of the developed TLC was performed by UV light or 5% H_2SO_4 in MeOH stain. Melting points were measured using A. KRUSS OPTRONIC (Germany) melting point apparatus and are uncorrected. IR spectra were recorded on a Bruker Optics (Tensor 27 model, Germany) FT-IR spectrophotometer (using KBr pellets) and reported in wave number (cm^{-1}). ^1H NMR (300/400 MHz) and ^{13}C NMR (75/100 MHz) spectra were measured in $CDCl_3$ at room temperature on a Bruker Avance-III 300/400 MHz (Switzerland) instruments. The chemical shifts are reported as δ parts per million (ppm) in $CDCl_3$ ($\delta_H = 7.26$; $\delta_C = 77.0$) using tetramethylsilane as an internal standard. Data are reported as follows: chemical shift, multiplicity (s: singlet, d: doublet, t: triplet, dd: doublet of doublet, q: quartet, br: broad, m: multiplet), coupling constants (*J* in Hz) and integration.

4.2 | General procedure and spectral data of synthesized compounds

4.2.1 | Synthesis of *N*-propargyl-indole-3-carbaldehyde 2 (Step 1)

To a solution of indole-3-carbaldehyde **1** (0.73 g, 5 mmol) and potassium carbonate (1.04 g, 7.5 mmol) in DMF (20 ml), propargyl bromide (0.7 ml g, 7.5 mmol) was added drop wise and the resulting mixture was allowed to stir overnight at temperature. After completion of reaction monitored by TLC, the reaction mixture was partitioned between CH_2Cl_2 and water, and the CH_2Cl_2 layer was collected. The aqueous layer was extracted two times with CH_2Cl_2, the combined organic extracts was dried over anhydrous Na_2SO_4 and concentrated under vacuum. It was crystallized from *i*-propanol-water (1:9) to obtain the desired *N*-propargyl-Indole-3-carbaldehyde **2** in pure form. Yield: 0.85 g (92%), white solid, m.p. 104–106°C; IR (KBr): 3196, 2121, 1740, 1641 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ_H (ppm) 10.02 (s, 1H), 8.32 (dd, $J = 6.8$ and 2.0 Hz, 1H), 7.90 (s, 1H), 7.42 (m, 1H), 7.39–7.34 (m, 2H), 4.94 (d, $J = 2.4$ Hz, 2H), 2.56 (t, $J = 2.4$ Hz, 1H); ESI-MS: positive ion mode: $m/z = 183.96$ $[M + H]^+$, calculated mass (M) for $C_{12}H_9NO$ is 183.07.

4.2.2 | Synthesis of 1,9-O-(*N*-propargyl-3-indolylidene)forskolin 4 (Step 2)

To a solution of forskolin **3** (0.5 g, 1.22 mmol) and *N*-propargyl-indole-3-carbaldehyde **2** (0.33 g, 1.83 mmol) in 1,2-dichloroethane (DCE), Amberlist-15 wet (1 g) was added and the reaction mixture was further stirred at rt for 24 h. After completion of reaction, reaction mixture was filtered and washed with DCE and evaporated off under reduced pressure. The obtained crude product was subjected to neutral Al_2O_3 column chromatography using 50% ethyl acetate in *n*-hexane as mobile phase to give pure **4**. Yield: 0.53 g (75%), white solid, m.p. 190–192°C; IR (KBr): 3443, 2931, 2123, 1737, 1697, 1370, 1238, 1053 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.75 (d, $J = 8$ Hz, 1H), 7.37 (d, $J = 8$ Hz, 1H), 7.33 (s, 1H), 7.25–7.22 (m, 1H), 7.16–7.12 (m, 1H), 5.75 (dd, $J = 17.2$ and 10.8 Hz, 1H), 5.74 (s, 1H), 5.51 (d, $J = 3.6$ Hz, 1 H), 5.18 (dd, $J = 17.2$ and 1.2 Hz, 1H), 4.84 (d, $J = 2.8$ Hz, 2H), 4.74 (dd, $J = 10.8$ and 1.2 Hz, 1H), 4.51 (brs, 1H), 4.27 (brs, 1H), 2.93 (d, $J = 16.4$ Hz, 1H), 2.54 (d, $J = 2$ Hz, 1H), 2.45 (d, $J = 16.4$ Hz, 1H), 2.41 (t, $J = 2.4$ Hz, 1H), 2.16 (m, 1H), 2.15 (s, 3H), 1.83–1.70 (m, 2H), 1.75 (s, 3H), 1.64–1.61 (m, 1H), 1.60 (s, 3H), 1.34 (s, 3H), 1.29 (s, 3H), 1.08–1.05 (m, 1H), 1.03 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 206.20, 169.62, 146.10, 136.05, 126.18, 122.17, 120.60, 120.14, 113.96, 110.30, 109.41, 94.37, 85.48, 80.86, 77.50, 76.61, 76.48, 75.42, 73.76, 70.21, 50.20, 43.11, 39.39, 36.53, 35.88, 34.53, 33.05, 31.26, 24.81, 23.73, 23.38, 21.20, 17.58; ESI-MS: positive ion mode: $m/z = 576.32$ $[M + H]^+$, 598.27 $[M + Na]^+$, 614.24 $[M + K]^+$, calculated mass (M) for $C_{34}H_{41}NO_7$ is 575.29.

4.2.3 | Synthesis of forskolin-indole-triazole conjugates **5a-5l** (Step 3)

To a solution of **4** (100 mg, 0.174 mmol), aryl azide (***a-l***, 0.34 mmol) were suspended in 2:1 mixture of water and *tert*-butyl alcohol (10 ml total volume). Sodium ascorbate (10 mol%, 1 M) and $CuSO_4.5H_2O$ (5 mol%, 0.1M) were added sequentially to the reaction mixture and the mixture was allowed to room temperature for next 12 h. After completion of reaction (TLC monitoring), the reaction mixture was diluted with water (10 ml) and cooled on ice. The precipitate was isolated by vacuum filtration and washed with cold water (3 × 5 ml) and cold *n*-hexane (3 × 5 ml) to afford crude compound. It was

purified using flash column chromatography to afford pure compounds **5a**-**5l**.

4.2.4 | 1,9-O-{*N*-[(1-Biphenyl-1H-1,2,3-triazol-4-yl)methyl]-3 indolylidene} forskolin (**5a**)

Yield: 102 mg (72%), brown solid, m.p. 108–110°C; IR (KBr): 3463, 2965, 2226, 1739, 1370, 1223, 1023 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.76 (d, J = 8 Hz, 2H), 7.63 (td, J = 8 and 1.2 Hz, 1H), 7.52 (d, J = 8.4 Hz, 2H), 7.46 (d, J = 7.6 Hz, 2H), 7.29 (m, 3H), 7.29 (d, J = 7.6 Hz, 2H), 7.21–7.09 (m, 2H), 5.72 (s, 1H), 5.67 (dd, J = 17.2 and 10.4 Hz, 1H), 5.52 (s, 2H), 5.48 (d, J = 3.6 Hz, 1H), 5.42 (d, J = 1.6 Hz, 2H), 5.07 (dd, J = 17.2 and 1.6 Hz, 1H), 4.53 (dd, J = 10.4 and 1.2 Hz, 1H), 4.49 (brs, 1H), 4.25 (brs, 1H), 2.90 (d, J = 16.4 Hz, 1H), 2.52 (brs, 1H), 2.44 (d, J = 16.4 Hz, 1H), 2.14 (m, 3H), 2.13 (s, 3H), 1.86–1.76 (m, 2H), 1.74 (s, 3H), 1.70–1.63 (m, 1H), 1.58 (s, 3H), 1.32 (s, 3H), 1.29 (s, 3H), 1.09–1.06 (m, 1H), 1.02 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ_H (ppm) 206.11, 169.70, 146.15, 144.99, 145.53, 138.53, 136.18, 135.11, 133.80, 132.92, 130.04, 129.48, 129.48, 128.35, 128.35, 127.90, 126.64, 126.0, 122.23, 122.15, 120.51, 120.06, 118.51, 113.95, 111.20, 110.09, 109.74, 94.36, 85.40, 80.85, 77.3, 76.5, 75.37, 70.17, 53.74, 50.13, 43.12, 42.32, 39.34, 36.55, 34.53, 33.06, 31.24, 24.79, 23.71, 23.34, 21.24, 17.57; ESI-MS: positive ion mode: m/z = 810 $[M + H]^+$, 832.34 $[M + Na]^+$, 848.31 $[M + K]^+$, calculated mass (M) for $C_{48}H_{51}N_5O_7$ is 809.38.

4.2.5 | 1,9-O-{*N*-[(1-[*o*-flurobenzyl]-1H-1,2,3-triazol-4-yl)methyl]-3-indolylidene} forskolin (**5b**)

Yield: 105 mg (83%), yellow solid, m.p. 118–120°C; IR (KBr): 3463, 2964, 1739, 1370, 1226, 1099 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.74 (d, J = 7.5 Hz, 1H), 7.33 (d, J = 8.1 Hz, 1H), 7.29 (s, 1H), 7.26–7.03 (m, 7H), 5.71 (s, 1H), 5.67 (dd, J = 17.1 and 10.5 Hz, 1H), 5.51 (s, 2H), 5.48 (d, J = 3.9 Hz, 1H), 5.39 (brs, 1H), 5.07 (d, J = 17.1 Hz, 1H), 4.53 (d, J = 11.1 Hz, 1H), 4.50 (brs, 1H), 4.25 (brs, 1H), 2.89 (d, J = 16.2 Hz, 1H), 2.52 (brs, 1H), 2.44 (d, J = 16.2 Hz, 1H), 2.14 (m, 4H), 1.84–1.79 (m, 2H), 1.74 (s, 3H), 1.63 (m, 1H), 1.59 (s, 3H), 1.33 (s, 3H), 1.29 (s, 3H), 1.09 (m, 1H), 1.03 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 206.47, 170.00, 146.53, 145.22, 136.57, 134.5, 131.16, 130.85, 126.99, 126.42, 125.16, 122.58, 122.50, 120.89, 120.42, 116.34, 116.06, 114.33, 110.45, 110.10, 94.78, 85.85, 81.24, 77.8, 76.9, 75.76, 70.60, 50.54, 48.04, 43.53, 42.68, 39.77, 36.93, 34.91, 33.43, 31.67, 25.18, 24.10, 23.72, 21.56, 17.96; ESI-MS: positive ion mode: m/z = 727.31 $[M + H]^+$, 749.33 $[M + Na]^+$, calculated mass (M) for $C_{41}H_{47}FN_4O_7$ is 726.34.

4.2.6 | 1,9-O-{*N*-((1-[*p*-flurobenzyl]-1H-1,2,3-triazol-4-yl)methyl)-3-indolylidene} forskolin (**5c**)

Yield: 115 mg (91%), yellow solid, m.p. 120–122°C; IR (KBr): 3461, 2964, 1738, 1370, 1224, 1021 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.75 (d, J = 7.6 Hz, 1H), 7.30 (d, J = 8 Hz, 1H), 7.25 (s, 1H), 7.23–7.18 (m, 4H), 7.13–7.09 (m, 1H), 7.00 (t, J = 8.4 Hz, 2H), 5.71 (s, 1H), 5.64 (dd, J = 17.2 and 10.8 Hz, 1H), 5.47 (d, J = 4 Hz, 1H), 5.41 (s, 2H), 5.39 (d, J = 2.8 Hz, 1H), 5.05 (dd, J = 17.2 and 1.6 Hz, 1H), 4.49 (brs, 1H), 4.48 (dd, J = 10.8 and 1.2 Hz, 1H), 4.51 (brs, 1H), 4.26 (brs, 1H), 2.88 (d, J = 16.4 Hz, 1H), 2.51 (d, J = 2 Hz, 1H), 2.44 (d, J = 16.4 Hz, 1H), 2.17–2.10 (m, 1H), 2.14 (s, 3H), 1.84–1.79 (m, 2H), 1.74 (s, 3H), 1.68–1.63 (m, 1H), 1.59 (s, 3H), 1.33 (s, 3H), 1.30 (s, 3H), 1.10–1.06 (m, 1H), 1.03 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 205.97, 169.69, 164.07, 146.17, 145.04, 136.16, 130.38, 130.02, 129.94, 126.58, 126.01, 122.21, 121.77, 120.50, 120.07, 116.19, 115.97, 113.98, 110.0, 109.68, 94.34, 85.42, 80.83, 77.3, 76.5, 75.33, 70.20, 53.41, 50.11, 43.14, 42.33, 39.36, 36.54, 34.52, 33.06, 31.27, 24.78, 23.71, 23.33, 21.21, 17.57; ESI-MS: positive ion mode: m/z = 727.38 $[M + H]^+$, 749.33 $[M + Na]^+$, calculated mass (M) for $C_{41}H_{47}FN_4O_7$ is 726.34.

4.2.7 | 1,9-O-{*N*-((1-[*p*-nitrobenzyl]-1H-1,2,3-triazol-4-yl)methyl)-3-indolylidene} forskolin (**5d**)

Yield: 118 mg (90%), yellow solid, m.p. 108–110°C; IR (KBr): 3464, 2963, 1738, 1350, 1224, 1020 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.18 (d, J = 8.4 Hz, 1H), 7.75 (d, J = 7.8 Hz, 1H), 7.38–7.28 (m, 3H), 7.24–7.09 (m, 4H), 5.72 (s, 1H), 5.65 (dd, J = 16.8 and 10.4 Hz, 1H), 5.56 (s, 2H), 5.49–5.36 (m, 2H), 5.04 (d, J = 16.8 Hz, 1H), 4.45 (m, 2H), 4.26 (brs, 1H), 2.88 (d, J = 16.4 Hz, 1H), 2.49–2.44 (m, 2H), 2.14 (s, 3H), 2.04 (m, 1H), 1.78–1.68 (m, 2H), 1.74 (s, 3H), 1.58 (s, 3H), 1.32 (s, 3H), 1.30 (s, 3H), 1.10–1.06 (m, 1H), 1.01 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 205.92, 169.87, 148.02, 146.23, 145.52, 141.47, 136.07, 128.74, 128.74, 126.54, 126.01, 124.30, 124.30, 122.30, 122.22, 120.54, 120.17, 114.14, 109.92, 109.63, 94.26, 85.36, 80.83, 76.52, 76.44, 75.31, 70.18, 53.10, 50.04, 43.13, 42.32, 39.32, 36.54, 34.53, 33.04, 31.24, 24.76, 23.71, 23.32, 21.27, 17.59; ESI-MS: positive ion mode: m/z = 754.32 $[M + H]^+$, 776.27 $[M + Na]^+$, calculated mass (M) for $C_{41}H_{47}N_5O_9$ is 753.34.

4.2.8 | 1,9-O-{*N*-((1-[*o*,*p*-dichlorobenzyl]-1H-1,2,3-triazol-4-yl)methyl)-3-indolylidene} forskolin (**5e**)

Yield: 98 mg (72%), white solid, m.p. 98–100°C; IR (KBr): 3435, 2970, 1741, 1369, 1222 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.74 (d, J = 7.6 Hz, 1H), 7.40 (d, J = 2 Hz, 1H), 7.32 (d, J = 8 Hz, 1H), 7.29 (s, 1H), 7.26 (s, 1H), 7.22–7.16 (m, 2H), 7.13–7.09 (m, 2H), 7.05 (d, J = 8 Hz, 1H), 5.72 (s, 1H), 5.66 (dd, J = 17.2 and 10.8 Hz, 1H), 5.54 (s, 2H), 5.48 (d, J = 3.6 Hz, 1H), 5.41 (d, J = 2 Hz, 2H), 5.08 (dd, J = 17.2 and 1.6 Hz, 1H), 4.53 (d, J = 10.8 and 1.6 Hz, 1H), 4.49 (t, J = 3.2 Hz, 1H), 4.25 (brs, 1H), 2.88 (d, J = 16.4 Hz, 1H), 2.52 (d, J = 2.4 Hz, 1H), 2.44 (d, J = 16.4 Hz, 1H), 2.16 (m, 1H), 2.14 (s, 1H), 1.82–1.79 (m, 2H), 1.74 (s, 3H), 1.71–1.63 (m, 1H), 1.59 (s, 3H), 1.33 (s, 3H), 1.29 (s, 3H), 1.09–1.05 (m, 1H), 1.03 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 206.06, 169.69, 146.16, 144.93, 136.15, 135.49, 134.07, 130.99, 130.96, 129.75, 127.93, 126.58, 126.02, 122.32, 122.23, 120.50, 120.09, 113.99, 110.04, 109.70, 94.32, 85.42, 80.84, 77.3, 76.50, 75.35, 70.19, 50.85, 50.11, 43.13, 42.30, 39.35, 36.54, 34.53, 33.07, 31.25, 24.78, 23.71, 23.33, 21.20, 17.57; ESI-MS: positive ion mode: m/z = 777.68 $[M + H]^+$, 779.22 $[M + Na]^+$, calculated mass (M) for $C_{41}H_{46}Cl_2N_4O_7$ is 776.27.

4.2.9 | 1,9-O-{*N*-((1-[*m*-chlorobenzyl]-1H-1,2,3-triazol-4-yl)methyl)-3-indolylidene} forskolin (**5f**)

Yield: 102 mg (79%), white solid, m.p. 118–120°C; IR (KBr): 3464, 2959, 1738, 1370, 1222, 1021 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.75 (d, J = 8.0 Hz, 1H), 7.31 (d, J = 8.4 Hz, 1H), 7.28 (d, J = 2.4 Hz, 1H), 7.26 (s, 1H), 7.24–7.09 (m, 5H), 5.72 (s, 1H), 5.65 (dd, J = 17.2 and 10.8 Hz, 1H), 5.48 (d, J = 4.0 Hz, 1H), 5.42 (s, 2H), 5.41 (d, J = 2.4 Hz, 2H), 5.07 (dd, J = 17.2 and 1.2 Hz, 1H), 4.53–4.48 (m, 2H), 4.25 (brs, 1H), 2.88 (d, J = 16.4 Hz, 1H), 2.52 (d, J = 2.0 Hz, 1H), 2.44 (d, J = 16.4 Hz, 1H), 2.18 (m, 1H), 2.15 (s, 1H), 1.84–1.79 (m, 2H), 1.74 (s, 3H), 1.67–1.61 (m, 1H), 1.59 (s, 3H), 1.33 (s, 3H), 1.30 (s, 3H), 1.15–1.10 (m, 1H), 1.02 (s, 3H); ESI-MS: positive ion mode: m/z = 765.36 $[M + Na]^+$ calculated mass (M) for $C_{41}H_{47}ClN_4O_7$ is 742.31.

4.2.10 | 1,9-O-{*N*-((1-[*p*-carboxyphenyl]-1H-1,2,3-triazol-4-yl)methyl)-3-indolylidene} forskolin (**5g**)

Yield: 107 mg (83%), white solid, m.p. 139–141°C; IR (KBr): 2967, 2929, 1739, 1370, 1223, 1047 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.33 (s, 1H), 8.11 (d, J = 8.0 Hz, 1H), 7.95 (dd, J = 8.0 and 1.2 Hz, 1H), 7.78 (d, J = 8.0 Hz, 1H), 7.56 (t, J = 8 Hz, 1H), 7.40 (d, J = 8.0 Hz, 1H), 7.37 (s, 1H), 7.22 (t, J = 7.2 Hz, 1H), 7.13 (t, J = 7.2 Hz, 1H), 5.76 (s, 1H), 5.75 (dd, J = 17.2 and 10.4 Hz, 1H), 5.48 (d, J = 4.0 Hz, 1H), 5.42 (s, 2H), 5.41 (d, J = 2.4 Hz, 2H), 5.07 (dd, J = 17.2 and 1.2 Hz, 1H), 4.53–4.48 (m, 2H), 4.25 (brs, 1H), 2.88 (d, J = 16.4 Hz, 1H), 2.52 (d, J = 2.0 Hz, 1H), 2.44 (d, J = 16.4 Hz, 1H), 2.18 (m, 1H), 2.15 (s, 1H), 1.84–1.79 (m, 2H), 1.74 (s, 3H), 1.67–1.61 (m, 1H), 1.59 (s, 3H), 1.33 (s, 3H), 1.30 (s, 3H), 1.15–1.10 (m, 1H), 1.02 (s, 3H); ESI-MS: positive ion mode: m/z = 761.30 $[M + Na]^+$, calculated mass (M) for $C_{41}H_{46}N_4O_9$ is 738.32.

4.2.11 | 1,9-O-{*N*-((1-[*p*-acetylphenyl]-1H-1,2,3-triazol-4-yl)methyl)-3-indolylidene} forskolin (**5h**)

Yield: 111 mg (87%), white solid, m.p. 144–146°C; IR (KBr): 3466, 2963, 1738, 1685, 1367, 1228, 1020 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.02 (d, J = 8.4 Hz, 1H), 7.34 (d, J = 8.1 Hz, 1H), 7.78–7.69 (m, 3H), 7.20–7.07 (m, 3H), 5.72 (s, 1H), 5.67 (dd, J = 17.1 and 10.5 Hz, 1H), 5.48–5.46(m, 3H), 5.07 (d, J = 17.1 Hz, 1H), 4.53 (d, J = 17.1 Hz, 1H), 4.46 (brs, 1H), 4.23 (brs, 1H), 2.88 (d, J = 16.5 Hz, 1H), 2.58 (s, 3H), 2.44 (m, 2H), 2.10 (s, 3H), 2.0 (m, 1H), 1.71–1.66 (m, 2H), 1.71 (s, 3H), 1.67–1.61 (m, 1H), 1.55 (s, 3H), 1.26 (s, 3H), 1.29 (s, 3H), 1.10 (m, 1H), 1.0 (s, 3H); ESI-MS: positive ion mode: m/z = 759.39 $[M + Na]^+$, calculated mass (M) for $C_{42}H_{48}N_4O_8$ is 736.34.

4.2.12 | 1,9-O-{*N*-((1-[*p*-cyanophenyl]-1H-1,2,3-triazol-4-yl)methyl)-3-indolylidene} forskolin (**5i**)

Yield: 114 mg (91%), yellow solid, m.p. 140–142°C; IR (KBr): 3463, 2959, 2231, 1737, 1370, 1235, 1042 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.75 (d, J = 8.0 Hz, 1H), 7.31 (d, J = 8.4 Hz, 1H), 7.28 (d, J = 2.4 Hz, 1H), 7.26 (s, 1H), 7.24–7.09 (m, 5H), 5.72 (s, 1H), 5.65 (dd, J = 17.2 and 10.8 Hz, 1H), 5.48 (d, J = 4.0 Hz, 1H), 5.42 (s, 2H), 5.41 (d, J = 2.4 Hz, 2H), 5.07 (dd, J = 17.2 and 1.2 Hz, 1H), 4.53–4.48 (m, 2H), 4.25 (brs, 1H), 2.88 (d, J = 16.4 Hz, 1H), 2.52 (d, J = 2.0 Hz, 1H), 2.44 (d, J = 16.4 Hz, 1H), 2.18 (m, 1H), 2.15 (s, 1H), 1.84–1.79 (m, 2H), 1.74 (s, 3H), 1.67–1.61 (m, 1H), 1.59 (s, 3H), 1.33 (s, 3H), 1.30 (s, 3H), 1.15–1.10 (m, 1H), 1.02 (s, 3H); ESI-MS: positive ion mode: m/z = 720.33 $[M + H]^+$, 742.35 $[M + Na]^+$, 758.31 $[M + K]^+$, calculated mass (M) for $C_{41}H_{45}N_5O_7$ is 719.33.

4.2.13 | 1,9-O-{*N*-((1-[*n*-butyl]-1H-1,2,3-triazol-4-yl)methyl)-3-indolylidene} forskolin (**5j**)

Yield: 93 mg (79%), white solid, m.p. 100–102°C; IR (KBr): 3461, 2928, 1739, 1370, 1223, 1021 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.77 (d, J = 7.8 Hz, 1H), 7.34–7.26 (m, 2H), 7.20–7.09 (m, 3H), 5.73 (s, 1H), 5.72 (dd, J = 17.1 and 10.5 Hz, 1H), 5.48 (d, J = 3.9 Hz, 1H), 5.42 (brs, 2H), 5.10 (d, J = 16.8 Hz, 1H), 4.58 (d, J = 10.5 Hz, 1H), 4.50 (brs, 1H), 4.28–4.23 (m, 3H), 2.91 (d, J = 16.5 Hz, 1H), 2.54 (brs, 1H), 2.46 (d, J = 16.5 Hz, 1H), 2.17 (m, 1H), 2.14 (s, 3H), 1.83–1.78 (m, 5H), 1.75 (s, 3H), 1.59 (s, 3H), 1.33 (m, 4H), 1.30 (s, 3H), 1.10 (m, 1H), 1.04 (s, 3H), 0.91 (t, J = 7.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 206.40, 170.02, 146.64, 144.85, 136.60, 127.07, 126.44, 122.58, 122.08, 120.91, 120.42, 114.31, 110.46, 110.13, 94.80, 85.84, 81.23, 77.8, 76.9, 75.75, 70.58, 50.54, 50.54, 43.54, 42.84, 39.76, 36.95, 34.92, 33.46, 32.53, 31.63, 25.18, 24.11, 23.74, 21.57, 20.07, 17.96, 13.78; ESI-MS: positive ion mode: m/z = 675.36 $[M + H]^+$, 697.31 $[M + Na]^+$, 713.28 $[M + K]^+$, calculated mass (M) for $C_{38}H_{50}N_4O_7$ is 674.37.

4.2.14 | 1,9-O-{*N*-((1-[*n*-octyl]-1H-1,2,3-triazol-4-yl)methyl)-3-indolylidene} forskolin (**5k**)

Yield: 102 mg (80%), white solid, m.p. 75–77°C; IR (KBr): 2968, 1740, 1370, 1223 cm^{-1}; ^1H NMR (400 MHz, CDCl3): δ (ppm) 7.77 (d, J = 8.0 Hz, 1H), 7.33 (d, J = 8 Hz, 1H), 7.28 (s, 1H), 7.30–7.10 (m, 3H), 5.73 (s, 1H), 5.72 (dd, J = 17.2 and 10.8 Hz, 1H), 5.49 (d, J = 4 Hz, 1H), 5.42 (d, J = 3.2 Hz, 2H), 5.11 (dd, J = 17.2 and 1.6 Hz, 1H), 4.59 (dd, J = 10.8 and 1.2 Hz, 1H), 4.51 (brs, 1H), 4.26–4.22 (m, 3H), 2.91 (d, J = 16.4 Hz, 1H), 2.55 (d, J = 2.4 Hz, 1H), 2.46 (d, J = 16.4 Hz, 1H), 2.19–2.11 (m, 1H), 2.14 (s, 3H), 1.84–1.80 (m, 4H), 1.75 (s, 3H), 1.70–1.64 (m, 1H), 1.59 (s, 3H), 1.34 (s, 3H), 1.30 (s, 3H), 1.26–1.23 (m, 10H), 1.10–1.06 (m, 1H), 1.04 (s, 3H), 0.86 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 206.03, 169.63, 146.23, 144.45, 136.20, 126.71, 126.04, 122.19, 121.67, 120.52, 120.04, 113.88, 110.08, 109.75, 94.41, 85.45, 80.83, 77.0, 76.54, 75.35, 70.18, 50.45, 50.15, 43.14, 42.49, 39.36, 36.56, 34.53, 33.08, 31.67, 31.25, 30.20, 29.0, 28.9, 26.46, 24.79, 23.72, 23.36, 22.57, 21.19, 17.57, 14.04; ESI-MS: positive ion mode: m/z = 731.44 $[M + H]^+$, 753.45 $[M + Na]^+$, calculated mass (M) for $C_{42}H_{58}N_4O_7$ is 730.43.

4.2.15 | 1,9-O-{*N*-[(1-allyl-1H-1,2,3-triazol-4-yl)methyl]-3-indolylidene}forskolin (**5l**)

Yield: 86 mg (75%), white solid, m.p. 95–97°C; IR (KBr): 3462, 2948, 1737, 1370, 1224, 1021 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.99 (s, 1H), 7.75 (d, J = 8 Hz, 1H), 7.61 (d, J = 8 Hz, 1H), 7.51 (s, 1H), 7.16 (m, 1H), 7.04 (m, 1H), 6.03–5.94 (m, 1H), 5.70 (dd, J = 17.2, 10.4 Hz, 1H), 5.65 (s, 1H), 5.44 (s, 2H), 5.22 (dd, J = 10.4 and 1.2 Hz, 1H), 5.15 (dd, J = 17.2 and 1.2 Hz, 1H), 5.02–4.92 (m, 4H), 4.54 (dd, J = 10.8 and 0.8 Hz, 1H), 4.41 (brs, 1H), 4.16 (s, 1H), 2.93 (d, J = 17.2 Hz, 1H), 2.55 (d, J = 17.2 Hz, 1H), 2.43 (s, 1H), 2.10–2.06 (m, 1H), 2.02 (s, 3H), 1.76–1.73 (m, 1H), 1.69 (s, 3H), 1.49 (s, 3H), 1.47–1.39 (m, 2H), 1.27 (s, 6H), 1.13–1.11 (m, 1H), 1.04 (s, 3H); ESI-MS: positive ion mode: m/z = 659.33 $[M + H]^+$, 681.28 $[M + Na]^+$, calculated mass (M) for $C_{37}H_{46}N_4O_9$ is 658.34.

4.3 | In silico studies

The 2D chemical structures of synthesized compounds were drown, modeled and later optimized through molecular mechanics (MM2/MM3) force field for conversion into 3D structure using Chem Bio Office Software v6 (Cambridge-Soft Inc.) [46]. X-ray crystallographic 3D structures of succinate dehydrogenase (PDB ID: 1YQ3) [47] was retrieved from Brookhaven Protein Data Bank (http://www.rcsb.org). Binding energy (BE) and inhibition constant (Ki) values were calculated by using Auto dock version 4.2 (autodock.scripps.edu) [48]. Polar hydrogen atoms were added to the macromolecules, and all rotatable bonds present in the ligands were set as rotatable bond for experiments. A grid box was created for macromolecule (dimensions of the grid box was 126 × 126 × 126 with grid spacing of 0.381). A GA was used to run Auto dock. Population sizes of 150 and 10 million energy were used for the 100 times search, with 126 × 126 × 126 dimensions of grid box size and 0.381 Å grid spacing around the catalytic triad. The best conformation was selected from the lowest docking energy identified among the different pockets of the macromolecule for each ligand, with not more than 1.5 Å RMSD. The formula for calculation is as follows: Ki = exp (ΔG/RT; http:// www.autodock.scripps.edu.org). The RMSD values were calculated using the PyMOL Molecular Graphics System, Version 1.level.

4.4 | ADMET screening

Various pharmacokinetics parameters related with absorption, distribution, metabolism, excretion and toxicity (ADMET) were calculated by using Discovery Studio v3.5 [49]. The ADMET properties calculated were blood brain barrier penetration, hepatotoxicity, cytochrome P450 2D6 binding, aqueous solubility, plasma protein

binding and intestinal absorption. For toxicity levels, also TOPKAT protocols of Discovery studio v3.5 was used [50].

4.5 | In vitro anticancer activity

4.5.1 | Chemicals

Dulbecco's modified essential eagle medium (DMEM) and fetal bovine serum (FBS) were taken from Gibco, India. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye, HEPES, trypsin–EDTA, antibiotic/antimitotic (Ab/Am), phosphate buffer saline (PBS) were purchased from Sigma Aldrich. Sodium bicarbonate ($NaHCO_3$) was purchased from Himedia, India while dimethyl sulphoxide (DMSO), ethanol and isopropanol were purchased from Merck India, Ltd.

4.5.2 | Cytotoxicity

MCF-7 (human breast adenocarcinoma), MDA-MB-231 (human breast adenocarcinoma), CHANG (human liver cell line), WRL-68 (human hepatocellular carcinoma), COLO-205 (human colon carcinoma), PC-3 (human prostrate carcinoma), HeLa (human cervical carcinoma), RAW-264.7 (mouse macrophages cell line) and RAJI (human Burrkit's lymphoma cell line) were grown in DMEM supplemented with 10% FBS and Ab/Am, under 5% CO_2, 95% humidity and 37°C temperature. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was done by Mosmann method reported previously [51,52]. Seeding in 96 well plates was done with approximately 2×10^3cells/well and treatment of the compound was given for 24 h then MTT dye (500 μg/ml) was added to the media in each well and plate was incubated at 37°C for 4 h. After the incubation period, media was discarded and DMSO was added to dissolve the formazan crystal and absorbance was taken at 570 nm.

4.6 | Erythrocyte osmotic fragility

The erythrocyte osmotic fragility assay was performed in vitro as per previously reported procedure. The osmotic fragility of human erythrocyte was determined by measuring the release of hemoglobin from erythrocytes exposed to an environment of hypotonic solutions of varying concentrations of phosphate buffered saline (0.1–0.85%) followed by incubation at 37°C for 60 min with mild shaking and recording the extent of hemolysis Spectro photo metrically at 540 nm. Likewise, heparinized blood was incubated with the effective concentration of compound 50 and 100 mg/ml at 37°C for 60 min and then transferred to the tubes containing decreasing concentrations of PBS. The cell suspensions were left to equilibrate for 30 min and then centrifuged. The absorbance of the supernatants was read at 540 nm and the percent lysis was calculated by dividing the optical density (OD) of the supernatant obtained from a particular saline concentration by the OD of the standard representing 100% hemolysis. Osmotic fragility curves were constructed by plotting the lysis percentage against the concentration of the saline solution. The results are expressed in terms of MEF_{50}, which is the level of hemolysis at 50% PBS. The MEF_{50} values at standard pH and temperature were then obtained from the standard curve. Curcumin, podophyllotoxin and DMSO were used as a positive control, standard drug control and vehicle control, respectively.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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