Chemical Synthesis of Rationally Design 9-(n-arylmethylamino) Noscapinoids and Cellular Evaluation as Potent Tubulin Binding Anticancer Agent Using Breast Cancer Cell Lines

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Abstract:

A safe antitussive agent, Noscapine has been discovered as tubulin-binding anticancer agent. To increase its anticancer activity, we have designed new congeners of 9-(N-arylmethylamino) noscapinoids by eliciting the N-aryl methyl pharmacophore at the C-9 position of noscapine scaffold. We have screened out three promising derivatives based on molecular docking, 6-8 with higher binding affinity with tubulin than noscapine. The selected 9-(N-arylmethylamino) noscapinoids, ***6-8*** *were chemically synthesized and were investigated experimentally using MCF-7 and MDAMB-231 breast cancer cells. All three noscapinoids inhibit the proliferation of both cancer cells at a lower concentration in comparison to noscapine. The IC_{50} value was in the range of 13.0 to 45.6 μM for the three derivatives against both the cell lines, which was 3.2 to 1.4-fold lower than noscapine. It was also demonstrated that these noscapinoids arrest the cancer cells at the G2/M phase and induce apoptosis more effectively than noscapine.*

Keywords: **Noscapine, 9-(N-arylmethylamino) noscapinoids, Tubulin binding, Anticancer agents, Breast cancer**

1. INTRODUCTION

Noscapine, an opium alkaloid, has been administered in clinics as an antitussive medication for over 40 years. It is non-narcotic, non-sedative, has no respiratory-depressant properties, and does not promote elation or dependency [1]. It was demonstrated to bind tubulin dimer with a 1:1 stoichiometry and suppress its dynamic instability [2]. Because of its interference with microtubule dynamics, it inhibits cellular proliferation and induces apoptosis selectively to cancer cells without hampering normal healthy cells [3]. The normal cells do not undergo apoptosis because they have active cell cycle checkpoints and arrest themselves in the mitotic phases in the presence of noscapine, until it is removed from the site. In contrast, the cancer cells are compromised with cell cycle checkpoints and thus undergo apoptosis due to the polyploidy nature of

chromosomal DNA. Because of its unique mechanism of action, many researchers have looked into its therapeutic effectiveness as a robust chemotherapeutic agent [2-5]. Its antiproliferative efficacy, tumour growth suppression in xenograft animal models and promising results of many of its synthetic variants were well validated [2,3,6-8]. Moreover, unlike clinically used tubulin targeted drugs such as taxanes (polymerizes microtubules and promotes bundling) and vincas (depolymerizes microtubules), noscapine does not significantly change the polymer and monomer ratio of microtubules (referred as "kindler" microtubule poison). Noscapine is extraordinary compared to other microtubule targeted agents based on a clinical perspective because it lacks substantial cytotoxicity to normal cells. Several studies substantiated very little or no toxicity exhibited by noscapine in healthy volunteers [9-11]. Noscapine has an improved ADME profile [12] and has no significant organ toxicity. Many noscapine derivatives have been synthesized to improve its anticancer efficacy. For example, first-generation analogues such as halogenated (fluoro, chloro, bromo, and iodo-noscapine), nitro, amino, and azido noscapine derivatives were developed by modifying the C-9 position of the isoquinolone ring system of the noscapine scaffold [13-16]. By changing the benzofuranone ring system of noscapine, second-generation analogues were synthesized, including O-alkylated and acylated noscapinoids with higher activity than noscapine [17]. Subsequently, third-generation noscapinoids were synthesized by manipulating the isoquinolone ring system by N functionalization, which were reported to exhibit substantial antiproliferative activity against various cancer types [18]. Moreover, steps have been taken to synthesize a panel of hybrid noscapine analogues (biaryl-noscapinoids) by combining a biaryl pharmacophore with higher cytotoxic activity [19].

Keeping this in mind, *in silico* efforts was used to screen out a panel of 9-N-arylmethylamino derivatives of noscapine **6-8** by altering its scaffold structure, followed by chemical synthesis and experimental evaluation of their anticancer activity using two human breast cancer cell lines (MCF-7 and MDAMB-231). Further, the binding affinity **6-8** with tubulin, cell cycle arrest at G2/M phase and efficient induction of apoptosis in cancer cells, were studied.

2. MATERIALS AND METHODS

Protein preparation

The crystal structure of the amino noscapine-tubulin complex (PDB ID: 6Y6D, resolution 2.20 Å) [20] was obtained and used for the structure-based screening of promising derivatives of noscapine from the library. As reported earlier, the protein

structure was prepared using a multistep protein preparation wizard (Schrodinger) with the default parameter set up [21].

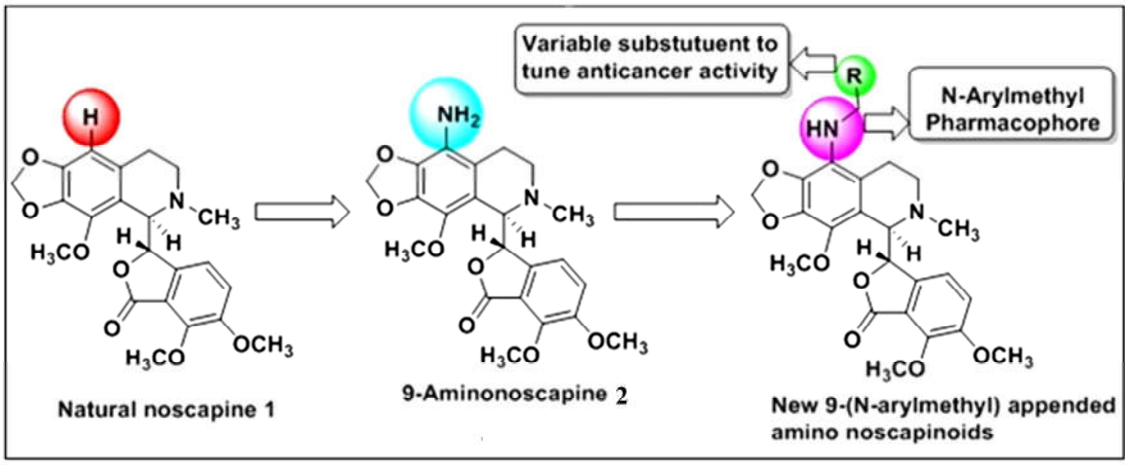


Figure 1: In silico combinatorial derivatization of the scaffold structure of noscapine with alkyl or arylalkyl units to develop a library of 9-(N-arylalkylamino) noscapinoids.

Construction of library of 9-N-arylalkylamino derivatives of noscapine

Several studies suggested the modification at C-9 position of the isoquinoline ring system of noscapine scaffold with halogens (fluoro-, chloro-, bromo- and iodo-noscapine), nitro, amino and azido groups exerted relatively higher anticancer activity compared to the lead molecule, noscapine. Keeping in view, the functionalization of C-9 position of noscapine was envisaged with alkyl or arylalkyl units to construct a library of 9-(N-arylalkylamino) noscapine derivatives (Figure 1). The library was used to screen out a panel of promising derivatives based on molecular docking with tubulin.

Preparation of molecular structure of noscapinoids

The molecular structures of the 9-(N-arylalkylamino) noscapine derivatives (Figure 1) were built using ChemDraw and were imported to a project table using Maestro (Schrödinger). The molecular structures were energy minimized using Macromodel (Schrödinger) and OPLS 2005 force field with PRCG algorithm as reported previously [21]. Further optimization of molecular structures was performed by Jaguar (Schrödinger) based on hybrid density functional theory using Becke's three-parameter exchange potential and the Lee-Yang-Parr correlation function (B3LYP). Ligprep (Schrödinger) was used to generate different conformations of each of the compound.

Molecular docking of noscapinoids

The library of 9-(N-arylalkylamino) noscapine derivatives was used for the structure-based screening with tubulin using Glide docking (Schrödinger) as described

previously [21]. Briefly, all the molecules were docked onto the noscapine binding cavity [20] by creating a grid box of 12 Å x 12 Å x 12 Å dimension and using Glide XP docking algorithm [22,23]. The docked poses of the molecules were evaluated by Glide XPScore [22,23] and the top three molecules (Figure 2) based on docking score were screened out for chemical synthesis and experimental evaluation.

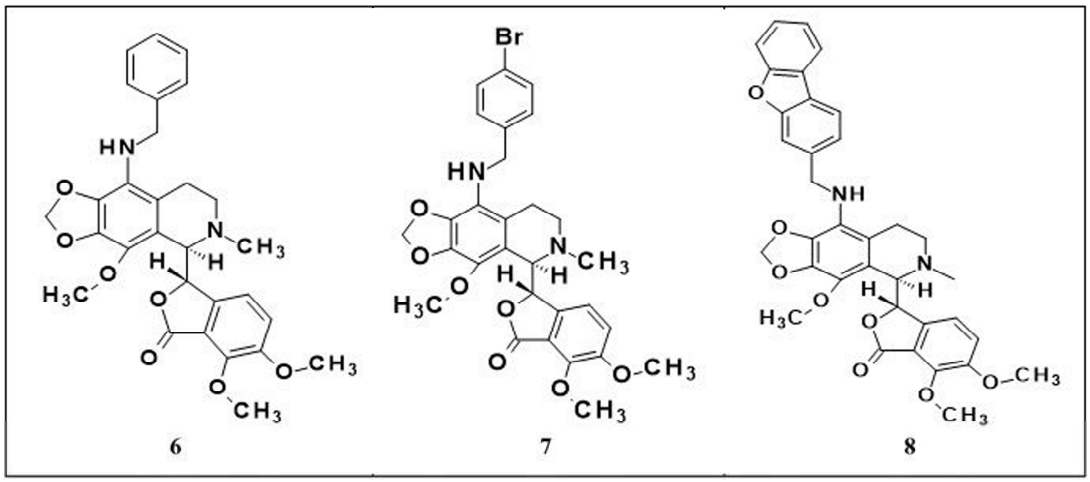


Figure 2: Molecular structure of three top-ranked 9-(N-arylmethylamino) noscapinoids, 6-8, screened out from the library with better tubulin binding affinity.

Chemical synthesis of selected derivatives of noscapine 6-8

The selected derivatives of noscapine, **6-8** from the library were chemically synthesized from the noscapine as starting material based on synthetic protocol (Figure 3). The 9-amino noscapine was synthesized from the noscapine in two steps, (i) bromination of noscapine with aqueous HBr/Br_2-H_2O and (ii) amination with CuI, NaN_3, L-Proline in DMSO as described previously [24]. The 9-amino noscapine **6** (1 mM) solution in ethanol (15 ml) was refluxed for 24 hours with different substituted aromatic aldehydes (1 mM) and the crude residue was extracted using dichloromethane (2x15 ml). Under reduced pressure, the crude residue was chromatographed over triethylamine silica bed with petroleum ether/ethyl acetate (7:3) as eluent to realize 9-(arylimino) noscapinoids, **3-5** as solid products. The 9-(arylimino) noscapinoids, **3-5** (1 mM), were then reduced to 9-(N-arylmethylamino) noscapinoids, **6-8**, by treating them with sodium cyanoborohydride (1.2 mM) in methanol (10 ml) for 4 hours at room temperature. NMR (^{1}H and ^{13}C), IR spectroscopy, and mass (HRMS) spectrometry were used to characterize the intermediates and end products.

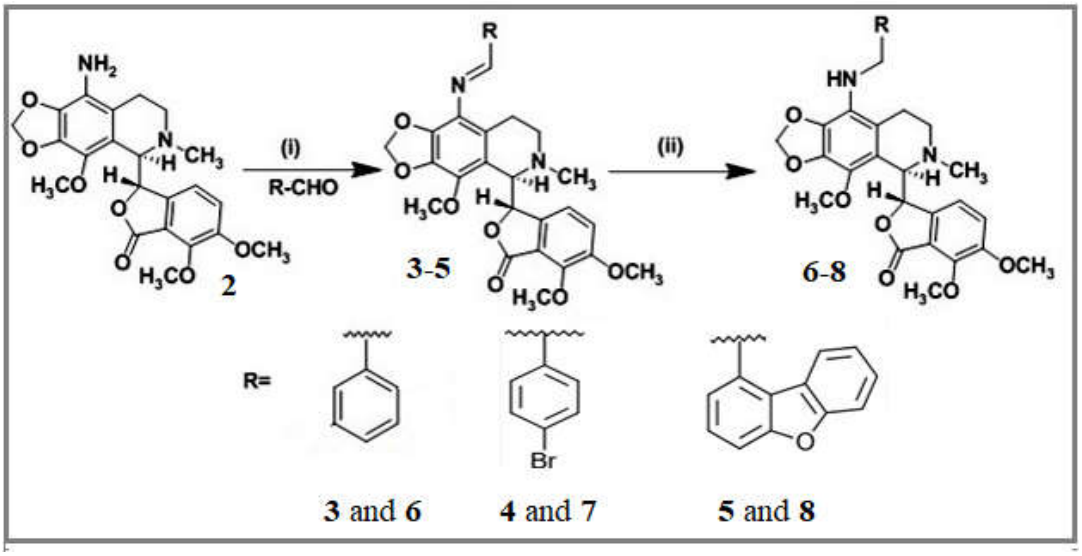


Figure 3: Synthetic scheme of 9-(N-arylmethylamino) noscapinoids, 6-8. Reaction Conditions: i) RCHO, EtOH, Reflux, 24 hours; (ii) NaCNBH3, Methanol, RT, 4 hours. 9-amino-noscapine was transformed to 9-(arylimino) noscapinoids 3-5, which were subsequently reduced to 9-(N-arylmethylamino) noscapinoids, 6-8.

Structural characterization of 9-imine noscapinoids (12-14) (*S*)-3-((*R*)-9-((*E*)-benzylideneamino)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-[1,3] dioxolo [4,5-*g*]isoquinolin-5-yl)-6,7-dimethoxyisobenzofuran-1(3*H*)-one (3):

Nature: White solid. mp: 88-90 °C. IR (KBr): 3426, 2937, 1759, 1627, 1497, 1385, 1266, 1123, 1034, 756, 693 cm^{-1}. ^{1}H NMR (500 MHz, CDCl3) : δ 8.85 (s, 1H, N=C*H*), 7.89 (d, *J* = 3.6 Hz, 2H, Ar-H), 7.50-7.42 (m, 3H, Ar-H), 6.99 (d, *J* = 8.2 Hz, 1H, Ar-H), 6.31 (d, *J* = 8.2 Hz, 1H, Ar-H), 5.99 (d, *J* =20.44Hz, 2H, O-C*H*2-O), 5.58 (d, *J* = 4.2 Hz, 1H, Ar-CH, (C3-phthalide)), 4.40 (d, *J* = 4.2 Hz, 1H, Ar-CH, (C5'-isoquinoline)), 4.10 (s, 3H, -OCH3), 4.04 (s, 3H, -OCH3), 3.85 (s, 3H, -OCH3), 3.02-2.94 (m, 1H, -C*H*H-*N*-CH3 (C7'-isoquinoline)), 2.72-2.65 (m, 1H, -CH*H*-*N*-CH3 (C7'-isoquinoline)), 2.55 (s, 3H, *N*-C*H*3), 2.44-2.36 (m, 1H, Ar-C*H*H (C8'-isoquinoline)), 2.06-1.97 (m, 1H, Ar-CH*H* (C8'-isoquinoline)). ^{13}C NMR (100 MHz, CDCl3): δ 168.1, 161.8, 152.1, 147.6, 141.3, 141.1, 139.0, 138.7, 136.9, 134.5, 131.1, 129.0, 128.6, 128.3, 125.7, 119.8, 118.3, 117.7, 117.6, 100.8, 81.8, 62.2, 60.9, 59.5, 56.7, 49.4, 45.9, 22.7. MS (ESI-MS) *m/z* : 517 [M+H]+ HRMS (ESI) : Calcd for C29H29N2O7 [M+H]+: 517.19693, found: 519.19596.

(*S*)-3-((*R*)-9-((*E*)-(4-bromobenzylidene)amino)-4-methoxy-6-methyl-5,6,7,8-tetrahydro- [1,3] dioxolo[4,5-*g*]isoquinolin-5-yl)-6,7-dimethoxyisobenzofuran-1(3*H*)-one (4):

Nature: White solid. mp: 93-95 °C. IR (KBr) : 3449, 2936, 2795, 1758, 1626, 1494, 1383, 1267, 1124, 1034, 970, 821 cm^{-1}. ^{1}H NMR (500 MHz, CDCl3) : δ 8.84 (s, 1H, N=CH), 7.76 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.58 (d, *J* = 8.5 Hz, 2H, Ar-H), 6.99 (d, *J* = 8.3 Hz, 1H, Ar-H), 6.34 (d, *J* = 8.3 Hz, 1H, Ar-H), 5.99 (dd, *J* = 1.3, 14.9 Hz, 2H, O-C*H*2-O), 5.57 (d, *J* = 4.4 Hz, 1H, Ar-CH, (C3-phthalide)), 4.38 (d, *J* = 4.4 Hz, 1H, Ar-CH, (C5'-isoquinoline)), 4.10 (s, 3H, -OCH3), 4.03 (s, 3H, -OCH3), 3.85 (s, 3H, -OCH3), 3.04-2.93 (m, 1H, -C*H*H-*N*-CH3 (C7'-isoquinoline)), 2.75-2.68 (m, 1H, -CH*H*-*N*-CH3 (C7'-isoquinoline)), 2.54 (s, 3H, *N*C*H*3), 2.46-2.37 (m, 1H, Ar-C*H*H (C8'-isoquinoline)), 2.10-2.00 (m, 1H, Ar-CH*H* (C8'-isoquinoline)). ^{13}C NMR (100 MHz, CDCl3): δ 168.1, 160.1, 152.1, 147.6, 141.4, 139.2, 139.0, 135.9, 134.5, 131.8, 129.6, 129.3, 125.4, 125.2, 119.8, 118.3, 117.8, 117.6, 100.9, 81.7, 62.2, 60.9, 59.5, 56.7, 49.3, 45.8, 22.7. MS (ESI-MS) *m/z*: 595 [M+H]+ HRMS (ESI) : Calcd for $C_{29}H_{28}BrN_2O_7$ [M+H]+: 595.10744, found: 595.10635.

(*S*)-3-((*R*)-9-(((*E*)-dibenzo[b,d]furan-4-ylmethylene)amino)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-[1,3]dioxolo[4,5-*g*]isoquinolin-5-yl)-6,7-dimethoxyisobenzofuran-1(3*H*)-one (5):

Nature: White solid. mp: 114-116 °C. IR (KBr): 3447, 2933, 1758, 1625, 1497, 1447, 1385, 1269, 1185, 1122, 1035, 754cm^{-1}. ^{1}H NMR (400MHz, CDCl3) : δ 9.51 (s, 1H, N=CH), 8.21 (d, *J* = 7.0 Hz, 1H, Ar-H), 8.04 (dd, *J* = 0.9, 7.5 Hz, 1H, Ar-H), 7.9 (d, *J* = 7.5 Hz, 1H, Ar-H), 7.65 (d, *J* = 8.1 Hz, 1H, Ar-H), 7.54-7.48 (m, 1H, Ar-H), 7.46-7.37 (m, 2H, Ar-H), 7.00 (d, *J* = 8.1 Hz, 1H, Ar-H), 6.31 (d, *J* = 8.1 Hz, 1H, Ar-H), 6.07 (dd, *J* = 1.2, 20.6 Hz, 2H, O-C*H*2-O), 5.61 (d, *J* = 4.4 Hz, 1H, Ar-CH, (C3-phthalide)), 4.43 (d, *J* = 4.4 Hz, 1H, Ar-CH, (C5'-isoquinoline)), 4.10 (s, 3H. -OCH3), 4.07 (s, 3H, -OCH3), 3.84 (s, 3H, -OCH3), 3.12-3.03 (m, 1H, -C*H*H-*N*-CH3 (C7'-isoquinoline)), 2.75-2.67 (m, 1H, -CH*H*-*N*-CH3 (C7'-isoquinoline)), 2.57 (s, 3H, *N*-C*H*3), 2.47-2.39 (m, 1H, Ar-C*H*H (C8'-isoquinoline)), 2.11-2.02 (m, 1H,Ar-CH*H* (C8'-isoquinoline)). 13C NMR (125MHz, CDCl3) : δ 168.1, 156.1, 155.9, 155.4, 152.1, 147.6, 141.2, 139.2, 138.9, 134.5, 129.3, 127.4, 125.9, 125.0, 124.4, 123.6, 123.0, 122.9, 121.6, 120.7, 119.9, 118.2, 117.7, 111.7, 101.0, 80.8, 62.2, 60.9, 59.5, 56.6, 49.6, 45.5, 22.9. MS (ESI-MS) *m/z*: 600 [M+H]+ HRMS (ESI) : Calcd for $C_{35}H_{31}N_2O_8$ [M+H]+: 607.20749, found: 607.20712.

Structural characterization of N-alkyl amine analogues of noscapine (6-8)

(*S*)-3-((*R*)-9-(benzylamino)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-[1,3]dioxolo[4,5-*g*]iso quinolin-5-yl)-6,7-dimethoxyisobenzofuran-1(3*H*)-one (6):

Nature : White solid. mp : 135-137 °C. IR (KBr) : 3420, 2928, 2886, 2801, 1765, 1620, 1501, 1458, 1423, 1275, 1064, 1037, 997, 971, 882, 756, 704 cm^{-1}, ^{1}H NMR (500 MHz, CDCl3) : δ 7.38-7.24 (m, 5H, Ar-H), 6.80 (d, *J* = 8.2 Hz, 1H, Ar-H), 5.98-5.91 (m, 3H,

Ar-H, 2 O-C*H*2-O), 5.55 (d, *J* = 4.1 Hz, 1H, Ar-CH, (C3-phthalide)), 4.45-4.38 (m, 2H, *N*-C*H*2), 4.30 (d, *J* = 14.0 Hz, 1H, Ar-H, (C5'- isoquinoline)), 4.07 (s, 3H, -OCH3), 3.95 (s, 3H, - OCH3), 3.82 (s, 3H, -OCH3), 2.57-2.49 (m, 4H, - C*H*H-*N*-CH3 (C7'-isoquinoline), *N*-C*H*3), 2.34-2.25 (m, 2H, -CH*H*-*N*-CH3 (C7'-isoquinoline),Ar-C*H*H (C8'-isoquinoline)), 1.62-1.54 (m, 1H, Ar-CH*H* (C8'-isoquinoline)). 13C NMR (100 MHz,CDCl3) : δ 168.0, 152.0, 147.5, 140.9, 140.3, 137.2, 135.3, 134.6, 128.5, 127.6, 127.1, 124.2, 121.0, 120.0, 117.9, 117.7, 117.6, 100.6, 81.8, 62.2, 60.8, 59.6, 56.7, 51.0, 49.4, 46.0, 22.6. MS (ESI-MS) *m/z* : 519 [M+H]+ HRMS (ESI) : Calcd for $C_{29}H_{31}N_2O_7$ [M+H]+: 519.21258, found: 519.21180.

(*S*)-3-((*R*)-9-((4-bromobenzyl)amino)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-[1,3]dioxolo[4,5-*g*]isoquinolin-5-yl)-6,7-dimethoxyisobenzofuran-1(3H)-one (7):

Nature : White solid. mp : 88-90 °C. IR(KBr) : 3403, 2928, 2852, 1756, 1622, 1496, 1441, 1384, 1268, 1036, 1009, 970, 794 cm^{-1}. ^1H NMR (400 MHz, CDCl3) : δ 7.46 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.20 (d, *J* = 8.1 Hz, 2H, Ar-H), 6.82 (d, *J* = 8.3 Hz, 1H, Ar-H), 5.99 (d, *J* = 8.3 Hz, 1H, Ar-H), 5.93 (dd, *J* = 1.1, 13.6 Hz, 2H, OC*H*2-O), 5.56 (d, *J* = 4.0 Hz, 1H, Ar-CH, (C3-phthalide)), 4.38 (dd, *J* = 4.0, 14.5 Hz, 2H, *N*-C*H*2), 4.27 (d, *J* = 14.5 Hz, 1H, Ar-CH, (C5'-isoquinoline)), 4.07 (s, 3H, -OCH3), 3.93 (s, 3H, -OCH3), 3.85 (s, 3H, -OCH3), 2.61-2.53 (m, 1H, -C*H*H-*N*-CH3 (C7'-isoquinoline)), 2.51 (s, 3H, *N*-C*H*3), 2.36-2.27 (m, 2H, CH*H*-*N*-CH3 (C7'-isoquinoline), Ar-C*H*H (C8'-isoquinoline)), 1.69-1.56 (m, 1H,Ar-CH*H* (C8'-isoquinoline)). ^{13}C NMR (75 MHz, CDCl3) : δ 168.0, 152.1, 147.6, 140.9, 139.4, 137.2, 135.3, 134.7, 131.5, 129.3, 123.8, 120.9, 120.8, 120.0, 117.9, 117.7, 117.6, 100.6, 81.8, 62.2, 60.8, 59.6, 56.7, 50.2, 49.1, 45.8, 22.4. MS (ESI-MS) *m/z* : 597 [M+H]+ HRMS (ESI) : Calcd for $C_{29}H_{30}BrN_2O_7$ [M+H]+: 597.12309, found: 597.12316.

(S)-3-((*R*)-9-((dibenzo[b,d]furan-4-ylmethyl)amino)-4-methoxy-6-methyl-5,6,7,8-tetra hydro-[1,3]dioxolo[4,5-*g*]isoquinolin-5-yl)-6,7-dimethoxyisobenzofuran-1(3*H*)-one (8):

Nature: White solid. mp : 113-115 °C. IR (KBr) : 3404, 2938, 1752, 1619, 1497, 1448, 1267, 1185, 1039, 1010, 971, 757 cm^{-1}. ^1H NMR (500 MHz, CDCl3) : δ 7.95 (d, *J* = 7.6 Hz, 1H, Ar-H), 7.87 (d, *J* = 7.6 Hz, 1H, Ar-H), 7.55 (d, *J* = 8.2 Hz, 1H, Ar-H), 7.49-7.44 (m, 1H, Ar-H), 7.40 (d, *J* = 7.3 Hz, 1H, Ar-H), 7.36 (t, *J* = 7.4 Hz, 1H, Ar-H), 7.31 (t, *J* = 7.4 Hz, 1H, Ar-H), 6.65 (d, *J* = 8.2 Hz, 1H, Ar-H), 5.96 (dd, *J* = 1.2, 16.02 Hz, 2H, O-C*H*2-O), 5.88 (d, *J* = 8.2 Hz, 1H, Ar-H), 5.53 (d, *J* = 4.1 Hz, 1H, Ar-CH, (C3-phthalide)), 4.75 (dd, *J* = 14.4, 30.9 Hz, 2H, *N*-CH2), 4.37 (d, *J* = 4.12 Hz, 1H, Ar-CH, (C5'-isoquinoline)), 4.05 (s, 3H, -OCH3), 3.95 (s, 3H, -OCH3), 3.70 (s, 3H, -OCH3), 2.50-2.44 (m, 4H, -C*H*H-*N*-CH3 (C7'-isoquinoline), *N*-C*H*3), 2.39-2.33 (m, 2H, -CH*H*-*N*-CH3 (C7'-isoquinoline), Ar-C*H*H (C8'-isoquinoline)), 1.63-1.54 (m, 1H, Ar-CH*H* (C8'-

isoquinoline)). ^{13}C NMR (125 MHz, CDCl3) : δ 168.0, 155.9, 154.2, 152.0, 147.4, 140.8, 137.4, 135.3, 134.7, 127.2, 126.6, 124.2, 124.1, 122.9, 122.8, 121.3, 120.6, 120.0, 119.6, 117.8, 117.7, 117.6, 111.5, 100.6, 81.9, 62.2, 60.8, 59.6, 56.6, 49.5, 46.1, 45.8, 22.6. MS (ESI-MS) *m/z* : 609 [M+H]+ HRMS (ESI) : Calcd for $C_{35}H_{33}N_2O_8$ [M+H]+: 609.22314, found: 609.22364.

Cell culture and reagents

The cell culture media and chemical reagents were supplied by Sigma. MCF-7 and MDAMB-231 human breast cancer cell lines were acquired from the National Centre for Cell Science's cell repository in Pune, India. Dimethyl sulfoxide (DMSO) was used to create stock solutions (100 mM) of 9-(N-arylmethylamino) noscapinoids **6-8** and was stored at 4 °C. Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37 °C with 5% CO_2 and 95% humidity. Cells with a 70-80% confluence were sub-cultured for bioassays with trypsin-EDTA (0.25%).

***In vitro* cell proliferation assay**

Two human breast cancer cell lines, MCF-7 and MDAMB-231, were used to determine the antiproliferative activity of selected noscapinoids. In a 96-well plate, cells were plated at a density of $5x10^3$ cells per well and treated with (5-100 M) doses of noscapine and 9-(N-arylmethylamino) noscapinoids **6-8** for 72 hours before being stained with 0.4 percent sulforhodamine B in 1% acetic acid. Washing was done with 1 percent acetic acid to remove the unbound dye. After washing the protein bound dye was recovered with 10 mM Tris base and the absorbance was measured at 564 nm using a SPECTRAmax PLUS 384 microplate spectrophotometer. Quest GraphTM IC_{50} Calculator (AAT Bioquest, Inc., Sunnyvale, CA, USA, https://www.aatbio.com/tools/ic50-calculator) was used to calculate the IC_{50} value that represents the drug concentration necessary to induce 50% cell death.

Flow cytometry analysis of cell cycle progression

The interference in cell cycle progression of MDAMB-231 cells with the treatment of 9-(N-arylmethylamino) noscapinoids **6-8** is studied as per the methods described earlier [21]. Briefly, the MDAMB-231 cancer cells were grown in DMEM medium and treated with noscapine and 9-(N-arylmethylamino) noscapinoids, **6-8**, at IC_{50} concentration for 72 h. The cells were analyzed using flow cytometer (BD FACS Aria-III) to calculate the percentage of cells at different stages of mitotic cell division.

Cellular apoptosis assay

The Apoptosis detection kit (Sigma) was used to detect apoptosis of MDAMB-231 cancer cells utilising the Annexin-V-FITC apoptosis detection technique as described

previously [21]. Briefly, the cancer cells were grown on a 12 well culture plate and were treated with IC_{50} concentrations of noscapine and 9-(N-arylmethylamino) noscapinoids **6-8.** The trysinized cells were labelled with surface marker antibodies such as biotin-conjugated Annexin V, FITC-conjugated streptavidin, and propidium iodide (PI) in 1X binding buffer and were analysed using flow cytometer. The proportion of viable cells (Annexin V/PI), early apoptotic cells (Annexin V+/PI), late apoptotic/necrotic cells (Annexin V+/PI+), and late necrotic cells (Annexin V/PI+) was calculated.

DAPI staining

DAPI labelling was used to visualize apoptotic cells treated with test chemicals using fluorescence microscopy. MDAMB-231 cells were grown on poly-L-lysine coated cover slips in 6-well plates for 72 h before being treated with 25 µM concentration of noscapine and 9-(N-arylmethylamino) noscapinoids **6-8**. After incubation, the cover slips were fixed in cold methanol, rinsed in PBS, and stained with DAPI. The morphology of apoptotic cells (nuclear condensation, membrane bleb development, and apoptotic bodies) was compared to that of untreated cells using a fluorescent microscope (Nikon Eclipse Ts2R-FL).

3. Results and Discussion

Molecular docking and screening of promising derivatives

Several synthetic derivatives of noscapine have been developed previously to increase the anticancer activity of noscapine. Many of these derivatives were experimentally tested to inhibit the proliferation of cancer cells of different tissue origin at a lower concentration, bind to microtubules at high affinity, interfere with the cell cycle progression and induce apoptosis in cancer cells [13,14,18,19,24-27]. In a quest to develop new noscapine derivatives, we have applied *in silico* combinatorial approach to design a library of 9-(N-arylalkylamino) noscapinoids by derivatization at the C-9 position of noscapine scaffold (Figure 1). In addition, the library of design derivatives of noscapine was docked onto the noscapine binding site of tubulin using Glide XP docking and ranked them with respect to their docking score. Finally, we have screened out three derivatives **6-8** that revealed a relatively higher docking score of -4.577 kcal/mol (for **6**), -4.715 kcal/mol (for **7**) and -5.336 kcal/mol (for **8**) compared to noscapine with docking score – 1.927 kcal/mol. All three 9-(N-arylmethylamino) noscapinoids docked well at the interface of α- and β- tubulin (Figure 4). Their mode of interaction with the binding site amino acids was demonstrated by ligplot (Figure 5).

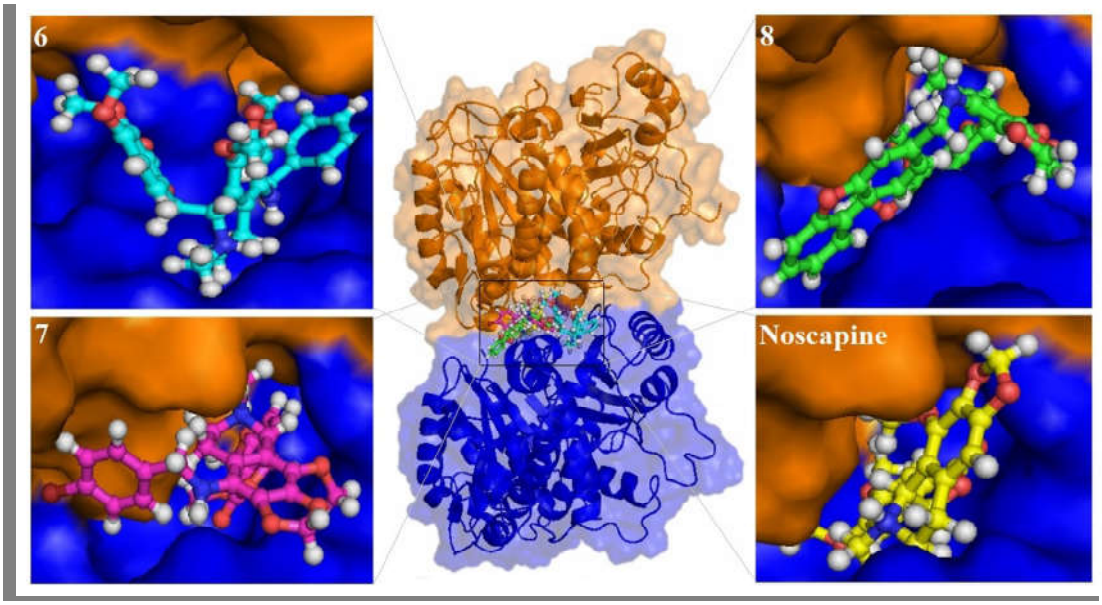
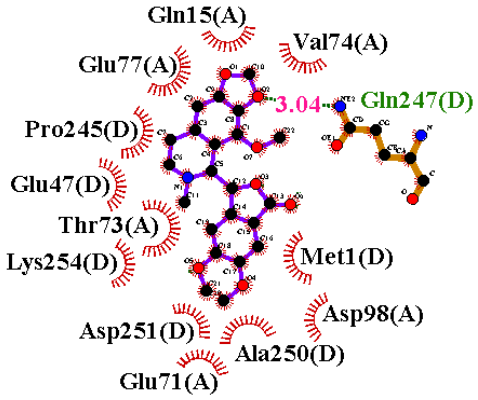
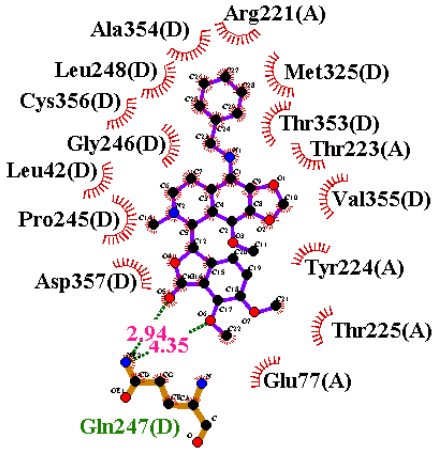


Figure 4: The three 9-(N-arylmethylamino) noscapinoids 6–8 was found to be accomodated well inside the binding pocket of noscapine at the interface of α-and β-tubulin. α-tubulin is represented in blue color and β-tubulin is represented in brown color. The binding domain is represented as a macromodel surface.

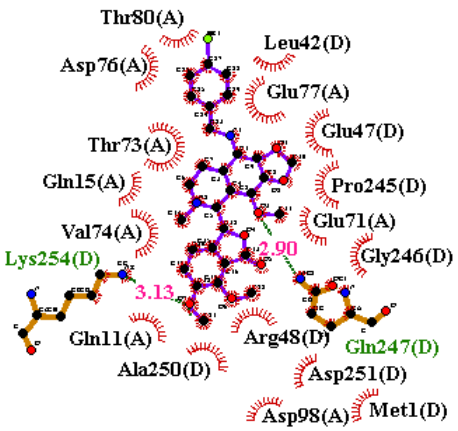
(a) Noscapine_Tubulin complex



(b) Noscapinoid, 6_Tubulin complex



(c) Noscapinoid, 7_Tubulin complex



(d) Noscapinoid, 8_Tubulin complex

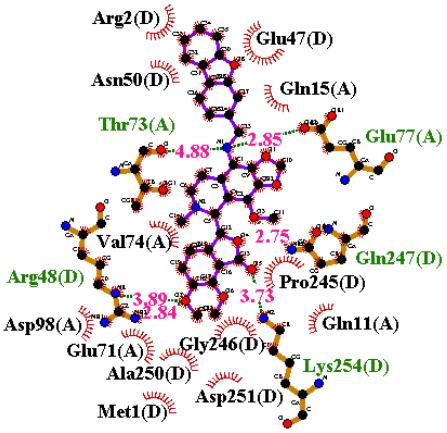
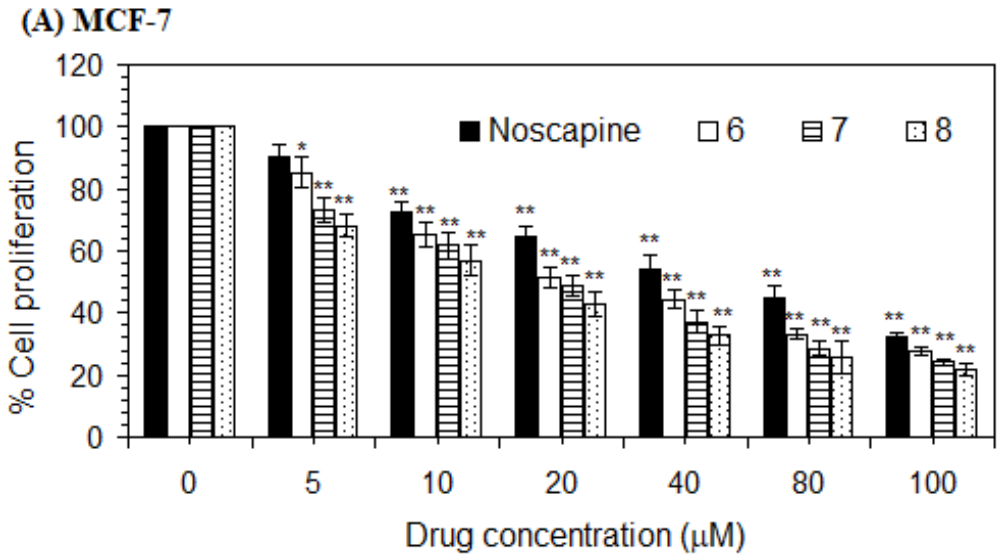


Figure 5. Ligplot analysis demonstrating the interaction between the binding site amino acids of tubulin with 9-(N-arylmethylamino) noscapinoids, (a) Noscapine, (b) 6, (c) 7 and (d) 8. Hydrogen bonds are represented by dashed lines and numbers represent hydrogen bond lengths in Å. Interactions among hydrophobic molecules are shown as arcs with radial spokes. LIGPLOT was used to develop the graphic. Only the residues within 5 Å of the docked ligands were shown in the images.

Inhibition of proliferation of MCF-7 and MDAMB-231

The goal was to see how the newly designed 9-(N-arylmethylamino) noscapinoids, **6-8**, affected cancer cell growth. The antiproliferative effect of these noscapinoids was tested in two human breast cancer cells, MCF-7 (estrogen- and progesterone-receptor positive) and MDAMB-231 (estrogen- and progesterone- receptor negative). In both cell lines, 9-(N-arylmethylamino) noscapinoids, 6-8 showed antiproliferative activity at a lower concentration than noscapine (Figure 6). In MCF-7 cells, the IC_{50} value for noscapine was determined to be 45.9 µM, whereas it was found to be 26.9 µM for **6**, 19.7 µM for **7**, and 14.5 µM for **8**, indicating promising antiproliferative activity. In MDAMB-231 cells, parenthetically similar IC_{50} values of 59.3 µM for noscapine, 41.2 µM for **6**, 22.7 µM for **7**, and 21.4 µM for **8** were calculated. Remarkable differences in IC_{50} values between the cancer cell types suggested that these 9-(N-arylmethylamino) noscapinoids, **6-8**, suppress cancer cell growth regardless of hormone receptor status. (Table 1)



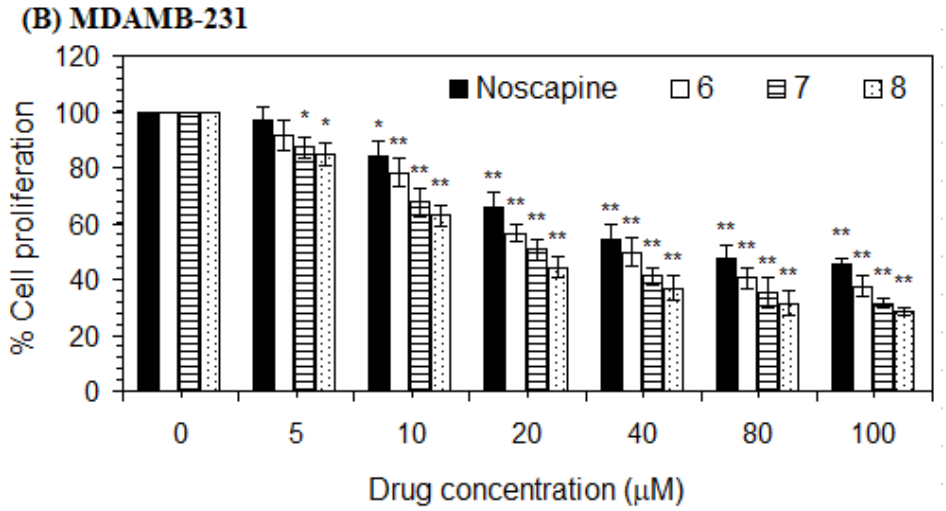


Figure 6: Inhibiting the proliferation of human breast cancer cells, (A) MDAMB-231 and (B) MCF-7 with the treatment of increasing concentration of noscapine and its 9-(N-arylmethylamino) noscapinoids 6-8 for 72 hours. Each value is the average of three independent trials.

Table 1. IC_{50} values of novel 9-(N-arylmethylamino) noscapinoids (6-8) using two human breast adenocarcinoma cell lines: MCF-7 and MDAMB-231. The novel derivatives were found to have improved anti-proliferative activity compared to noscapine.

Breast cancer cell lines	IC_{50} (μM)			
	Noscapine	15	16	17
MCF-7	45.9 ± 4.6	26.9 ± 3.8	19.7 ± 2.6	14.52 ± 2.9
MDAMB-231	59.3 ± 5.3	41.2 ± 4.2	27.7 ± 2.4	21.4 ± 3.6

Induction of apoptosis to cancer cells

The newly designed 9-(N-arylmethylamino) noscapinoids, **6-8**, induced apoptosis to MDAMB-231 cancer cells more effectively than noscapine. A representative figure of flow cytometry analysis of the different populations of cell types is included as Figure 7. After 72 hours of culture, there were only a few early apoptotic (2.5%) and late apoptotic (1.0%) cells in the control untreated cell culture, which were deemed background cell death due to frequent damage throughout cell culture (Table 2). In relation to the control untreated cells, the percentages of early apoptotic cells and late apoptotic cells with treatment of IC_{50} concentration of noscapine and 9-(N-arylmethylamino) noscapinoids **6-8** were found to be relatively higher (Table 2). This corroborates with the previous findings [7,14] using nitro and halogen derivatives of noscapine. We have recently obtained similar results using different derivatives of noscapine [21,28].

Further, fluorescence imaging using DAPI staining revealed apoptosis to MDAMB-231 cancer cells characterized by chromatin condensation and numerous fragmented nuclei (Figure 8).

Table 2. Percentage of early apoptotic (Q1), late apoptotic (Q2), viable (Q3) and necrotic (Q4) cells measured by flow cytometry.

Viability/Apoptotic	Control	Noscapine	15	16	17
Q1	2.5%	15%	45%	56%	61%
Q2	1%	30%	3%	4%	2%
Q3	94%	50%	50%	35%	33%
Q4	0.5%	1%	2%	3%	7%

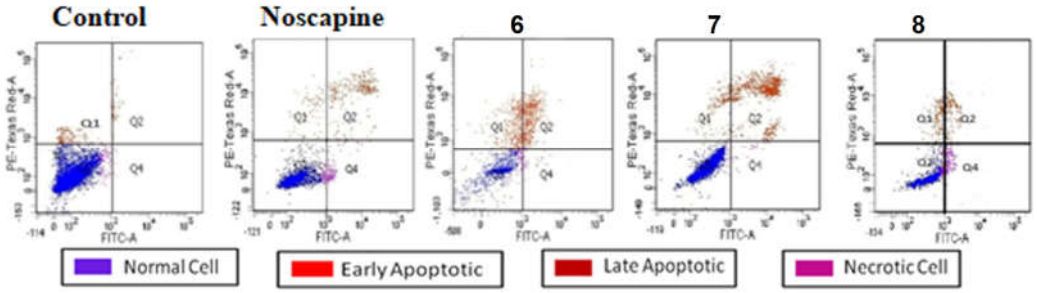


Figure 7: Flow cytometry investigation of MDAMB-231 cells treated with 9-(N-arylmethylamino) noscapinoids 6-8 and noscapine and compared to non treated cells. The fluorescent dye, propidium iodide (PI) was used in combination with the Alexa Fluor 488 conjugate of Annexin-V to discern between three sub-populations: PI-positive and Alexa Fluor 488-positive, indicating late apoptotic cells (PI+, Alexa Fluor 488+), PI-negative and Alexa Fluor 488-negative, indicating viable cells (PI-, Alexa Fluor 488-), PI-negative and Alexa Fluor 488-positive, indicating early apoptotic cells (PI-, Alexa Fluor 488+).

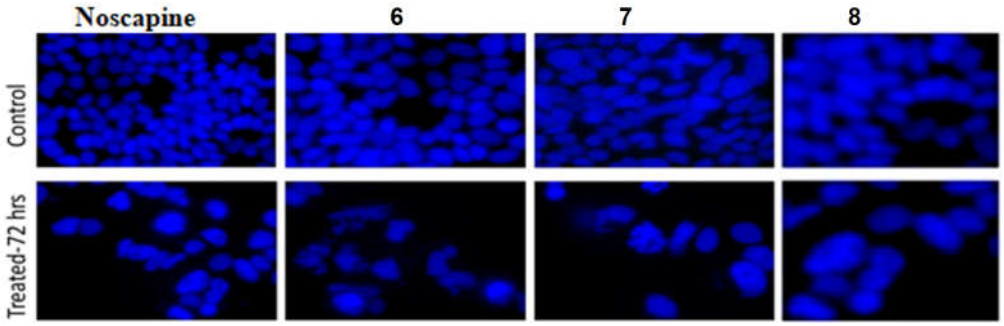


Figure 8: The changes in morphological characters such as chromatin condensation, plasma membrane blebbing and appearance of small, apoptotic bodies indicated the apoptotic cells. Panels show morphological features of cells stained with DAPI from control cells (upper panels) and

cells treated with IC_{50} concentration of noscapine and 9-(N-arylmethylamino) noscapinoids, 6-8 (lower panels) for 72 hours using fluorescence microscopy. The apoptotic cancer cells were evident after 72 hours of drug treatment.

Alteration of cell cycle profile during mitotic cell division

It was demonstrated previously that noscapine and its derivatives interfere with the mitosis cell cycle profile and arrest the cancer cells at G2/M phase [21,28]. Interference with the cell cycle profile, later on, leads to the induction of apoptosis to cancer cells. We have also looked into the alteration of cell cycle profile of MDAMB-231 cancer cells with the treatment of noscapine and 9-(N-arylmethylamino) noscapinoids, **6-8** at IC_{50} concentration using FACS analysis (Figure 9). The increase of fluorescently labelled DNA is thought to indicate cell cycle advancement and cell death. The G1 phase is represented by the unreplicated complement of 2N DNA cells, whereas the G2 and M phases are represented by the duplicated 4N DNA cells. During DNA duplication, the cells between the 2N and 4N peaks indicate the S phase, when DNA is produced. Less than 2N DNA is found in populations of dying cells whose DNA is degraded to varying degrees. In comparison to untreated cells, FACS analysis indicated a significant increase of cells in G2/M phase after 72 h of treatment with noscapine and 9-(N-arylmethylamino) noscapinoids, **6-8** (Table 3). The typical hypodiploid DNA content peak (sub-G1) was seen to rise at 72 h of treatment, in contrast to G2/M block. The emergence of cells with hypodiploid DNA content over time indicates the presence of fragmented DNA, which indicates dying cells.

Table 3. Interference on cell cycle profile of MDAMB-231 cells treated with IC_{50} concentration of noscapine and its 9-(N-arylmethylamino) noscapinoids, 6-8.

	72 hours			
	Sub-G_1	G_0/G_1	S	G_2/M
Control	1.3	19.4	23.3	10.4
Noscapine	5.7	15.5	14.6	19.7
15	10.5	14.8	11.7	29.6
16	14.9	13.5	9.5	85.4
17	18.4	11.9	6.7	89.3

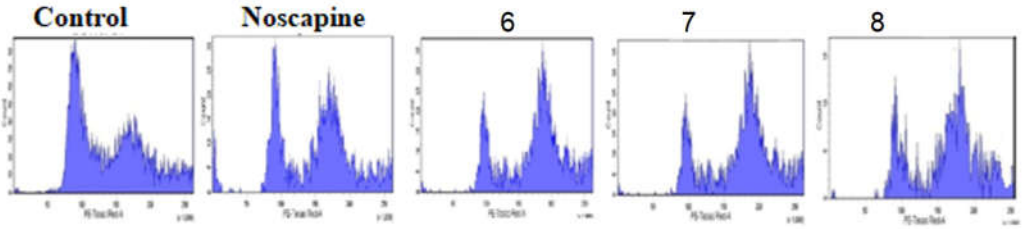


Figure 9: Noscapine and its 9-(N-arylmethylamino) noscapinoids 6-8 disrupt cell cycle progression during the G2/M phase, followed by the emergence of a hypodiploid (sub-G1) DNA peak, suggesting apoptotic cells.

4. Conclusion

In the present study, a panel of 9-(N-arylmethylamino) noscapinoids were strategically designed to accelerate the anticancer activity of the lead molecule, noscapine. The simplest method was provided for the direct and regioselective modification of noscapine scaffold to develop 9-(N-arylmethylamino) noscapinoids with relatively higher yields. The three newly developed 9-(N-arylmethylamino) noscapinoids have shown higher antiproliferative activity in cancer cells based on extensive molecular modeling and cellular evaluation using two human breast cancer cell lines MCF-7 and MDAMB-231. The study suggested that these novel compounds may prove efficacious not only for treating breast carcinoma, but also for other cancer types. Further, the study inferred to examine the therapeutic efficacy of these novel compounds through *in vivo* animal studies with the goal to be used for human clinical trials.

ACKNOWLEDGEMENT

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