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Synthesis, design, and examination of the cytotoxic effects and nuclear condensation properties of novel angrographolide-vanillin-1,2,3-triazoles

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Andrographolide is a bioactive labdane diterpenoid found in the leaves and stems of the *Andrographis paniculata* plant, which is native to South Asian countries such as India and Sri Lanka. Hybrid molecules produced by combining structural elements or pharmacophores from two or more parent compounds retain key features from the parent compound while potentially exhibiting novel or enhanced bioactivities. This paper presents the synthesis of andrographolide-vanillin-1,2,3-triazole hybrid analogues through a copper-catalysed azide-alkyne cycloaddition reaction, commonly known as the "click reaction," which enables the linkage of the three components. A series of 5 novel andrographolide-vanillin-1,2,3-triazole hybrid molecules have been synthesized and evaluated for their cytotoxic efficacy toward HELA cell lines. The results show that compound **7c** potentially induces cytotoxicity at three different concentrations compared with the other compounds in the series. Compared to the other analogues, **7c** induced significant cytomorphological abnormalities, including nuclear condensation in HeLa cells, as observed under both phase-contrast and fluorescence microscopes using Hoechst staining method.

Keywords: *Andrographis paniculata*, Andrographolide, Vanillin, 1,2,3-Triazole, 1,3-Dipolar cycloaddition

Andrographis paniculata Nees belongs to the *Acanthaceae* family, is commonly known as the king of bitters and is a well-known medicinal herb found in India, China and other Asian countries[1,2]. Andrographolide **1** and 14-deoxy-11,12-didehydro andrographolide are the two major bioactive constituents of this plant. The various extracts and principal chemical constituents of the plant have shown multiple pharmacological properties, such as anticancer[3]. Anti-diabetic[4], cardiovascular[5], anti-malarial[6], anti-HIV[7], anti-hyperglycaemic[8], antihepatotoxic[9], immunostimulant[10], anti-inflammatory[11], antibacterial[12] and antimicrobial[13] activities. Structurally, Andrographolide **1** is a bicyclic diterpene with an α-alkylidene-γ-butyrolactone ring, two olefin bonds, and three hydroxyls at C-3, C-14 (secondary) and C-19 (primary), respectively. Structure activity relationship (SAR) studies of **1** revealed that an intact γ-butyrolactone ring, double bonds at C-8 and C-12 and a hydroxyl group at C-14 are important for its cytotoxic activity. Substitution of the acetyl functionality at the C-14 hydroxyl as well as protection of the C-3 and C-19 hydroxyl groups with aromatic aldehydes increased the cytotoxicity[14-17]. Andrographolide **1** analogues were used to design structural diversity with improved bioactivity and water solubility. Thus, there is a need to prepare structurally new andrographolide derivatives by introducing heterocyclic pharmacophores such as 1,2,3- triazoles.

Vanillin is a specialized metabolite and the main ingredient of vanilla extract that occurs in a concertation range of 1.0–2.0% w/w in cured vanilla beans[18]. Vanillin has different functional groups, such as aldehyde, hydroxyl and ether attached to aromatic rings. Vanillin is either isolated from vanilla extract or chemically synthesized from guaiacol. In addition to being known for its flavour and fragrance,

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it has diverse bioactive properties such as anticancer[19], anti-inflammatory[20], neuroprotective[21], sickle cell anaemia[22], antifungal[23], antibacterial[24] properties as well as application in wound healing[25].

During the last two decades, among all heterocycles, substituted 1,2,3-triazoles have received the most attention as pharmacophores in drug discovery[26]. Synthetic 1,2,3-triazoles show diverse biological activities, such as anticancer[27], antibiotic[28], antifungal, antibacterial, antituberculosis, and antiviral activities. Some semisynthetic triazoles have also been reported to have better activities (anticancer, diabetic, lipase, *etc*.) than precursor molecule as well as standards. (*e.g*. podophyllotoxin-triazole-carbamate (**I**)[29], combretastatin A-4 analogues (**II, III**) [30-32], 10β-artemisinin-triazole conjugate (**IV**)[33], *etc*.) (Fig. 1).

Considering the pharmacophore potency of 1,2,3-triazoles and vanillin, we synthesized a series of five novel andrographolide-vanillin-1,2,3-triaole hybrid molecules.

Experimental Section

Chemistry

The melting points were measured employing the Casia-Siamia (VMP-AM) melting point apparatus located in Mumbai, India, and are uncorrected. Infrared (IR) spectra were obtained using a Perkin-Elmer FT-IR 240-C spectrometer from Waltham, MA, USA, with KBr pellets. Nuclear Magnetic Resonance (NMR) spectra were acquired using a Bruker Avance 500 MHz instrument located in Billerica, MA, USA, in both $CDCl_3$ and dimethyl sulfoxide (DMSO-d_6) solvents, with tetramethylsilane (TMS) serving as the internal standard. Reactions were monitored utilizing thin-layer chromatography plates from Merck, Kenilworth, NJ, USA, coated with silica gel and visualized under UV light. The silica gel (100–200 mesh) employed for column chromatography was sourced from Merck.

Cell culture

The HeLa cell line (derived from human cervical cancer) was sourced from the American Type Culture Collection (Manassas, VA, USA). Culture supplies including Dulbecco's modified Eagle's minimal essential medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS), and other necessary materials were procured from Gibco BRL, Life Technologies (Grand Island, NY). Assessment of cell viability was conducted through the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay.

General synthetic procedure and spectral data

Propargylation of vanillin (propargylated vanillin 2 [3-methoxy-4-(prop-2-yn-1-yloxy)benzaldehyde])

Vanillin **1** (1.0 gr, 6.578 m.mole) along with potassium carbonate (1.35 gr, 1.5 eq) was added to dimethyl formamide (DMF) in a clean RB flask and propargyl bromide (1.5 equiv) was added to this solution. The reaction mixture was stirred at RT for 4 hr to afford the crude vanillin propargyl derivative. This crude compound was poured into water (500 mL) and filtered to yield pure propargylated vanillin **2** Yield 95%.

White solid. Yield 95%. m.p. 69-71°C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.88 (s, 1H), 7.55 – 7.36 (m, 2H), 7.27 (s, 1H), 7.15 (d, J = 8.1 Hz, 1H), 4.86 (d, J = 2.4 Hz, 2H), 3.95 (s, 3H), 2.57 (t, J = 2.4 Hz, 1H); ^{13}C NMR (500 MHz, $CDCl_3$): δ 190.90, 152.15, 150.07, 130.97, 126.26, 112.64, 109.52, 77.46, 76.66, 56.63, 56.06; IR (KBr): 2917, 1678, 1587, 1507, 1464, 1423, 1395, 1339, 1265, 1224, 1135, 1011 cm^{-1}.

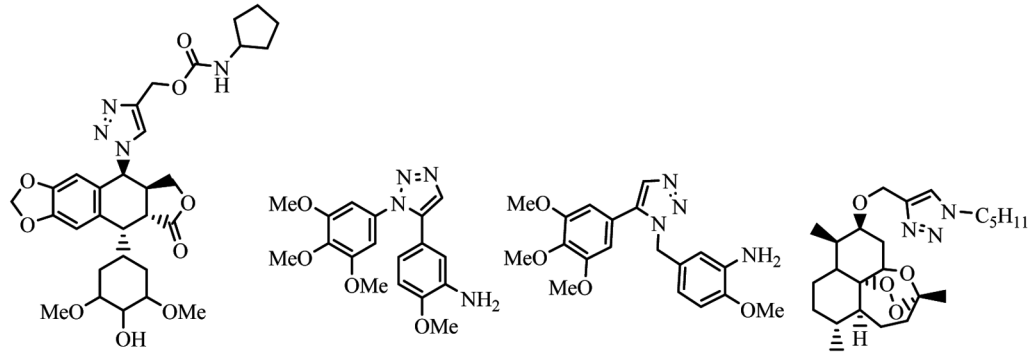


Fig. 1 — Bioactive semi-synthetic triazole derivatives

1,3-diol coupling of andrographolide (3) [(4S,E)-4-hydroxy-3-(2-((4aR,6aS,7R,10bR)-3-(3-methoxy-4-(prop-2-yn-1-yloxy)phenyl)-6a,10b-dimethyl-8-methylenedecahydro-1*H*-naphtho[2,1-d][1,3]dioxin-7-yl)ethylidene)dihydrofuran-2(3*H*)-one])

A mixture of andrographolide (**3**) (1.0 g, 2.857 mmol), propargylated vanillin (**2**) (543 mg, 1.0 eq), and activated amberlyst-15 (100 mg) in dry 1,4-dioxane (10 mL) was refluxed for 5 h under a N_2 atmosphere. The reaction mixture was cooled and filtered to remove the catalyst. The organic layer was evaporated under vacuum, the residue was subjected to column chromatography on silica gel, and elution with petroleum ether/ethyl acetate (80:20) yielded compound **4**.

White solid. Yield 74%. m.p.250-252°C. ^{1}H NMR (500 MHz, $CDCl_3$): δ δ 7.07 (s, 1H), 7.02 (d, *J* = 2.4 Hz, 1H), 6.97 – 6.90 (m, 3H), 5.73 (s, 1H), 4.86 (d, *J* = 2.4 Hz, 1H), 4.64 (s, 2H), 4.45 (dd, *J* = 10.4, 6.2 Hz, 2H), 4.28 (m, 1H), 3.94 (s, 1H), 3.90 (s, 1H), 3.57 (s, 3H), 2.96 (s, 1H), 2.63 (s, 1H), 2.49 (t, *J* = 2.4 Hz, 1H), 2.17 (d, *J* = 3.2 Hz, 1H), 2.06 (s, 3H), 1.90 (m, 1H), 1.48 (s, 2H), 1.30 (t, *J* = 4.1 Hz, 2H), 1.25 (s, 3H), 0.93 (s, 3H), 0.87 (s, 3H); ^{13}C NMR-(500 MHz, $CDCl_3$): δ 170.28, 149.67, 148.62, 147.15, 146.51, 133.10, 118.68, 114.19, 112.65, 109.70, 109.27, 94.98, 78.48, 77.40,75.76, 74.47, 69.48, 66.05, 56.84, 55.87, 54.81, 53.76, 38.87, 37.59, 36.90, 36.06, 31.72, 30.91, 29.20, 26.09, 24.72, 21.77, 15.33; IR (KBR): 2917, 1734, 1639, 1460, 1352, 1294, 1249, 1100, 944, 771, 714, 666, 610 cm^{-1}; HR-MS: *m/z* observed for $C_{31}H_{38}O_7$: 523 (M+ 1).

Synthesis of azides

Aliphatic/benzyl chloride/bromides (**5a-e**) (1 eq.) were added to a mixture of tetrahydrofuran and distilled water (at a 1:3 ratio). Sodium azide (1.5 eq.) was added, and the mixture was refluxed for 8 hr. The aqueous portion was separated and dried over anhydrous Na_2SO_4 until the organic layer become clear. The azide-tetrahydrofuran mixture was transferred to an R.B flask and further processed to afford **6a-e**.

Synthesis of novel andrographolilde-vanillin 1,2,3-triazoles

0.5 grams (0.957 mmole) of compound **4** were dissolved in 10 mL of dry THF, with the addition of a small quantity of copper iodide as a catalyst. Aliphatic/benzyl azides **6a-e** (at 1.0 equivalent) were then introduced into the mixture along with tetrahydrofuran. The reaction was left to proceed at RT under a nitrogen atmosphere for 12 hours. Subsequently, the residue was filtered to separate CuI, followed by concentration under reduced pressure. The resulting crude products were purified *via* column chromatography, utilizing ethyl acetate in hexane mobile phase mixtures, resulting in the isolation of derivatives **7a-e**.

(4S,E)-3-(2-((4aR,6aR,7R,10bR)-3-(4-((1-(3-chlorobenzyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-3-methoxyphenyl)-6a,10b-dimethyl-8-methylenedecahydro-1*H*-naphtho[2,1-d][1,3]dioxin-7-yl)ethylidene)-4-hydroxydihydrofuran-2(3*H*)-one, 7a: White colour. m.p.170-179°C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.30 (s, 1H), 7.44 – 7.32 (m, 2H), 7.36 – 7.26 (m, 1H), 7.14 – 6.91 (m, 3H), 6.66 (s, 1H), 5.84 – 5.67 (m, 2H), 5.64 (s, 2H), 5.11 (s, 2H), 5.04 – 4.85 (m, 3H), 4.68 (s, 2H), 4.42 (dd, *J* = 9.9, 6.1 Hz, 1H), 4.20 (d, *J* = 11.4 Hz, 2H), 4.05 (dd, *J* = 9.9, 1.9 Hz, 2H), 3.73 (d, *J* = 7.1 Hz, 2H), 2.13 – 1.74 (m, 5H), 1.59 (d, *J* = 29.7 Hz, 3H), 1.44 – 1.09 (m, 6H), 0.85 (d, *J* = 26.2 Hz, 4H); IR (KBr): 3419, 2922, 2137, 1650, 1433, 1412, 1318, 1018, 951, 710 cm^{-1}; HR-MS: *m/z* observed for $C_{38}H_{44}ClN_3O_7$: 590 (M+ 1).

(4S,E)-3-(2-((4aR,6aR,7R,10bR)-3-(4-((1-benzyl-1*H*-1,2,3-triazol-4-yl)methoxy)-3-methoxyphenyl)-6a,10b-dimethyl-8-methylenedecahydro-1*H*-naphtho[2,1-d][1,3]dioxin-7-yl)ethylidene)-4-hydroxydihydrofuran-2(3*H*)-one, 7b: White colour. m.p.141-150°C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.26 (s, 1H), 7.43 – 7.22 (m, 3H), 7.08 (d, *J* = 8.3 Hz, 1H), 7.00 – 6.80 (m, 2H), 5.81 – 5.67 (m, 1H), 5.61 (s, 1H), 5.06 (d, *J* = 37.1 Hz, 1H), 4.92 (dd, *J* = 26.0, 20.1 Hz, 2H), 4.68 (s, 2H), 4.41 (dd, *J* = 9.9, 6.1 Hz, 2H), 4.20 (d, *J* = 11.5 Hz, 5H), 4.05 (dd, *J* = 9.9, 1.9 Hz, 1H), 3.72 (d, *J* = 8.2 Hz, 2H), 2.12 – 1.51 (m, 9H), 1.46 – 0.99 (m, 8H), 0.84 (d, *J* = 25.8 Hz, 4H); ^{13}C NMR (500 MHz, DMSO-d_6): δ 170.5, 149.1, 147.8, 147.7, 146.7, 136.4, 134.6, 133.1, 129.5, 128.6, 127.9, 123.3, 119.0, 113.7, 110.5, 109.3, 94.7, 80.3, 70.8, 69.0, 65.1, 62.3, 55.8, 55.5, 54.1, 53.8, 48.8; IR (KBr): 3389, 3006, 2919, 2599, 2153, 1657, 1434, 1411, 1317, 1013, 952, 707 cm^{-1}.

(4S,E)-4-hydroxy-3-(2-((4aR,6aR,7R,10bR)-3-(3-methoxy-4-((1-(4-methoxybenzyl)-1*H*-1,2,3-triazol-4-yl)methoxy)phenyl)-6a,10b-dimethyl-8-methylenedecahydro-1*H*-naphtho[2,1-d][1,3]dioxin-7-yl)ethylidene)dihydrofuran-2(3*H*)-one, 7c: White colour. m.p.180-185°C. ^{1}H NMR: (500 MHz, DMSO-d_6): δ 8.21 (s, 1H), 7.20 (q, *J* = 8.1 Hz, 8H), 7.10 – 6.87 (m, 5H), 5.80 – 5.68 (m, 3H), 5.55 (s, 3H), 5.09

(s, 3H), 5.01 – 4.61 (m, 1H), 4.41 (dd, *J* = 9.9, 6.2 Hz, 1H), 4.20 (d, *J* = 11.6 Hz, 1H), 4.06 (d, *J* = 9.9 Hz, 1H), 3.72 (s, 1H), 2.68 – 2.63 (m, 1H), 2.42 – 2.32 (m, 4H), 2.29 (s, 6H), 1.62 (s, 2H), 1.37 (d, *J* = 12.5 Hz, 1H), 1.15 (d, *J* = 3.3 Hz, 2H), 0.85 (d, *J* = 26.2 Hz, 3H). IR (KBr): 3414, 3010, 2921, 2143, 1652, 1434, 1412, 1318, 1019, 951, 709 cm^{-1}.

(4S,E)-3-(2-((4aR,6aR,7R,10bR)-3-(4-((1-(2,4-dichlorobenzyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-3-methoxyphenyl)-6a,10b-dimethyl-8-methylenedecahydro-1*H*-naphtho[2,1-d][1,3]dioxin-7-yl)ethylidene)-4-hydroxydihydrofuran-2(3*H*)-one, 7d: White colour. m.p.160-164°C. ^{1}H NMR: (500 MHz, DMSO-d_6): δ 8.24 (s, 1H), 7.71 (d, *J* = 2.1 Hz, 2H), 7.61 – 7.38 (m, 2H), 7.19 (d, *J* = 8.4 Hz, 1H), 7.07 (d, *J* = 8.3 Hz, 1H), 7.00 – 6.80 (m, 2H), 6.78 – 6.55 (m, 1H), 5.85 – 5.63 (m, 3H), 5.12 (s, 1H), 4.98 – 4.57 (m, 3H), 4.55 – 4.34 (m, 2H), 4.29 – 4.12 (m, 2H), 4.05 (dd, *J* = 9.9, 1.9 Hz, 2H), 3.85 – 3.59 (m, 3H), 3.58 (dt, *J* = 10.5, 6.9 Hz, 2H), 3.47 (d, *J* = 11.2 Hz, 2H), 2.14 – 1.59 (m, 5H), 1.45 – 1.04 (m, 6H), 0.85 (d, *J* = 26.3 Hz, 4H). IR (KBr): 3414, 3010, 2921, 2143, 1652, 1434, 1412, 1318, 1019, 951, 709 cm^{-1}.

(4S,E)-4-hydroxy-3-(2-((4aR,6aR,7R,10bR)-3-(3-methoxy-4-((1-pentyl-1*H*-1,2,3-triazol-4-yl)methoxy)phenyl)-6a,10b-dimethyl-8-methylenedecahydro-1*H*-naphtho[2,1-d][1,3]dioxin-7-yl)ethylidene)dihydrofuran-2(3*H*)-one, 7e: White colour. m.p.165-171°C. ^{1}H NMR: (500 MHz, DMSO-d_6): δ 8.20 (s, 1H), 7.14 – 6.91 (m, 3H), 6.66 (t, *J* = 6.1 Hz, 2H), 5.74 (dd, *J* = 18.6, 12.5 Hz, 2H), 5.10 (s, 2H), 5.06 – 4.79 (m, 2H), 4.68 (s, 1H), 4.47 – 4.27 (m, 5H), 4.20 (d, *J* = 11.3 Hz, 1H), 4.05 (dd, *J* = 9.9, 1.9 Hz, 1H), 3.80 – 3.63 (m, 7H), 1.85 (dddd, *J* = 111.4, 106.2, 34.4, 23.4 Hz, 8H), 1.42 – 1.12 (m, 8H), 0.85 (dd, *J* = 16.1, 8.8 Hz, 6H). ^{13}C NMR (500 MHz, DMSO-d_6): δ 170,5, 149.1, 147.9, 147.7, 146.7, 133.1, 129.5, 119.1, 113.6, 110.5, 109.3, 94.7, 80.3, 74.8, 69.0, 65.0, 62.2, 55.8, 55.6, 54.1, 49.9, 38.9, 37.5, 36.9, 35.7, 29.9, 28.5, 26.1, 24.4, 22.9, 22.0, 15.4, 14.3; IR (KBr): 33342, 3009, 2920, 2145, 1654, 1434, 1412, 1318, 1017, 951, 708 cm^{-1}.

General procedure for bioactivity of andrographolide hybrid compounds

HeLa, a human cervical cancer cell line, was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin under a 95% air, 5% CO_2 atmosphere. The cells were passaged twice weekly and utilized for experimentation when reaching 70–80% confluency. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay, which measures mitochondrial activity through formazan formation. Briefly, cells were seeded overnight in 96-well plates at a density of 1 × 105 cells per well and subsequently treated with various concentrations (0.25, 0.50, and 0.75 mg/mL) for 24 hours. Following treatment, 100 µl of MTT solution was added and incubated at 37°C for 4 hours. After removing the culture supernatants, the resulting formazan crystals were dissolved in DMSO, and absorbance values were measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Tecan Austria GmbH). Relative cell viability was determined by comparing absorbance values with those of the untreated control group.

Statistical analysis

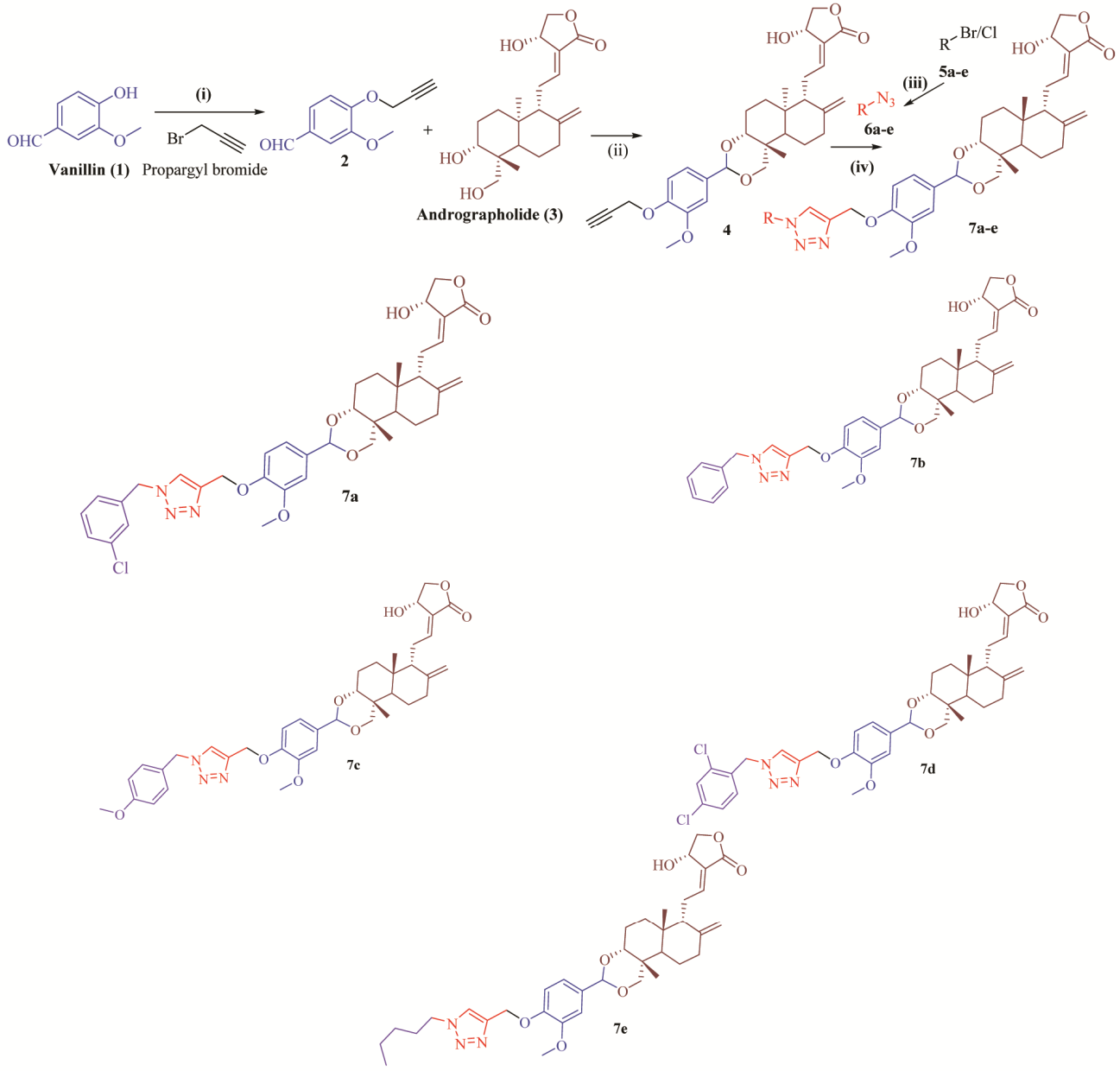
Statistical analysis involved the utilization of one-way analysis of variance (ANOVA) for all assessments, employing data derived from independent experiments. Results are expressed as means ± standard deviation (S.D.). The mean values were determined from data obtained from a minimum of three distinct experiments carried out on different days, utilizing freshly prepared reagents

Results and Discussion

Chemistry

Synthesis of andrographolide-vanillin 1,2,3-triazoles

As a continuation of our earlier work on the Semisynthesis of natural bioactive molecules[34,35], we isolated andrographolide from *A. paniculata* as previously reported[36]. The strategy used for the synthesis of different andrographolide-vanillin-1,2,3 triazoles is outlined in Scheme 1. A series of compounds (**7a–e**) were synthesized in four steps. In the first step, vanillin **1** was reacted with propargyl bromide in the presence of potassium carbonate as a catalyst and N, N-dimethyl formamide was used as a solvent to yield propargylated vanillin **2**. In the second step, andrographolide was condensed with propargylated vanillin **2** in basic medium (activated Amberlyst-15 in dry 1,4-dioxane was refluxed for 5 h under a N_2 atmosphere.) to give 1,3-diol protected andrographolide **4**. In the third step, a series of benzyl/aliphatic azides **6a-e** were synthesized from the respective bromides/chlorides using NaN_3 and THF: water[37]. In the final step, **4** was further reacted with



Reaction Conditions: (i) K_2CO_3, DMF, RT, 4 h; (ii) 1,4-Dioxane, Amberlyst-15, 5 h, Reflux; (iii) NaN_3, THF:H_2O (1:3), reflux 8 h; (iv) Dry THF, CuI, RT, Overnight

Scheme 1 — Semi-synthesis of novel andrographolide-vanillin 1,2,3-triazoles

appropriately substituted aromatic/aliphatic azides by a click reaction to afford compounds **7a–e**.

All the synthesized compounds were characterized by IR, ^{1}H, and ^{13}C NMR spectroscopy and mass spectrometry. In the ^{1}H NMR spectrum of compound **2**, the absence of a hydroxyl peak at δ 6.55 indicates that the hydroxyl group was linked to the propargyl group, which was further confirmed by the presence of an alkyl proton at δ 2.57. Similarly, in the ^{13}C NMR spectrum the resonances at δ 56.63, 76.66 and 77.46 indicate that a propargyl group was present in the molecule. However, in the ^{1}H NMR spectrum of compound **4**, the singlet at δ 5.73 indicated that the 1,3 diol was coupled with propargylated vanillin and the peak at δ 109.70 was further confirmed by ^{13}C NMR. In HR-MS spectrum of compound **4**, a molecular ion peak at *m/z*: 523 (M+ 1) was observed for $C_{31}H_{38}O_7$.

Anti-Cancer Activity

All the synthesized derivatives (**7a-e**) were evaluated for their *in vitro* anti-proliferative activity against the human cervical carcinoma cell line (HeLa). The cytotoxicity of the test compounds was determined by measuring the number of live cells after 24 h of treatment by MTT assay. The treatment concentrations of all compounds are summarized along with their % cell viability in Table 1.

The effects of the synthesized analogues on cell morphology and nuclear condensation were assessed using phase-contrast and fluorescence microscopy techniques. Cells (1×105/well) were exposed to a concentration of 0.75 mg/mL for 24 hours, and their altered morphology was examined using a phase-contrast microscope (DMI6000B, Leica Microsystems, Wetzlar, Germany). Subsequently, the cells were stained with Hoechst dye to visualize nuclear/chromosomal condensation induced by the test compound. Cultured in 96-well plates (1×105 cells/well) in triplicates, the cells were incubated overnight at 37°C, followed by fixation with 4% paraformaldehyde in PBS for one day at -4°C. After two washes with phosphate-buffered saline (PBS), Hoechst 33342 stain (1 µg/mL) was added to the fixed cells and incubated for 20 minutes at RT to visualize nuclear condensation. Fluorescence microscopy (CTR 6000; Leica, Wetzlar, Germany) was then used to capture images of Hoechst-stained cells.

Cytomorphological abnormalities induced by the test compounds (**7a-e**) were observed under both phase contrast and fluorescence microscopes Fig. 2 and figure S1, S2, S3 and S4. The effects of the lead compound **7c** were documented through micrographs obtained from both light and fluorescence microscopy to evaluate their cytotoxicity. In the control group, which did not receive the test compound, cells exhibited normal, healthy, and intact nuclei without cytological abnormalities, as depicted in Fig. 2. However, cells treated with a concentration of 0.75 mg/mL of the highly cytotoxic test compound **7c** for 24 hours displayed significant morphological changes, including blebbing of the cellular membrane, chromatin condensation, fragmentation, and formation of apoptotic bodies. Most treated cells showed signs of apoptosis, while severe damage, including cell membrane rupture and cytoplasmic release, was observed in some cells figure S1, S2, S3 and S4. The enhanced activity of Compound **7c** when compared to other analogues could be due to presence

Table 1 — Cytotoxicity assay of andrographolide hybrids against HeLa cell lines by MTT assay

Compd	Control	Concentration (mg/mL)		
		0.25	0.50	0.75
7a	0.9858	11.436	16.52588	8.223233
7b	1.08348	11.06767	13.28295	22.5784
7c	2.76	66.76473	72.45165	85.72051
7d	1.7922	15.3001	26.19631	37.41033
7e	2.85	9.590826	19.88533	43.20563

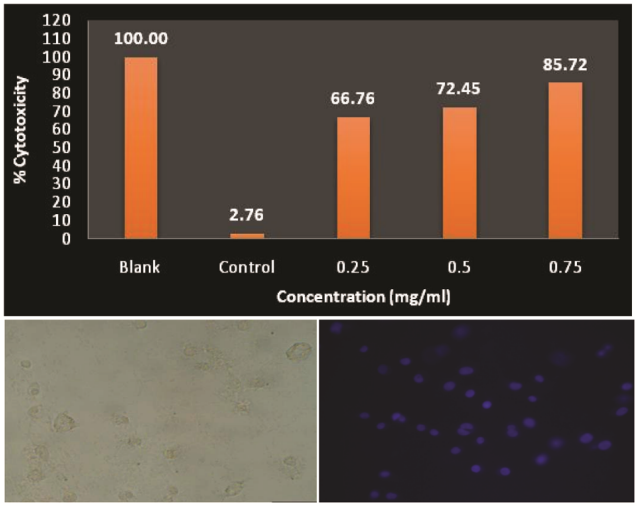


Fig. 2 — Cytotoxicity of **7c** and Hoechst strained cells visualized and photographed under florescence microscopy

of methoxy groups on the aromatic ring, which increase its lipophilicity. This probably facilitates better cell membrane permeability, allowing the compound to enter the cells more effectively and interact with intracellular target. Also, the electron-donating methoxy group and the electron-withdrawing properties of the triazole ring create a dipole moment that can enhance the compound's binding to negatively charged cellular components such as DNA and proteins. This electronic effect could increase the compound's cytotoxic efficacy by promoting stronger interactions at the molecular level.

Supplementary Information

Supplementary information is available in the website http://nopr.niscpr.res.in/handle/123456789/58776.

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