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Research article

Synthesis and Cytotoxicity Evaluation of Novel C-3 Aminocarbamate Pregnenolone Derivatives

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Abstract

Cancer is one of the leading causes of death worldwide. There are many ongoing studies in the search for new treatments or drugs to combat cancer. Similarly, in this research, twelve 3-aminocarbamate pregnenolones (**2a**–**2l**) were designed, synthesized, and evaluated for their cytotoxicity against five cancer cell lines: Human hepatocellular carcinoma (HepG2), Human colon adenocarcinoma (HT-29), Human oral cavity carcinoma (KB), Human breast adenocarcinoma (MCF-7), Murine leukemia (P388), and one normal cell line, African green monkey kidney fibroblast (Vero), using the MTT assay. Notably, 3-aminobenzylcarbamate pregnenolone (**2b**), 3-diaminoheptylcarbamate pregnenolone (**2f**), and 3-diaminopropanolcarbamate derivative (**2i**) were the most potent against these cancer cell lines. Specifically, for P388 cell lines, these compounds were more potent than the positive control drug, vinblastine sulfate salt. Results from the SAR study demonstrated that the length of the alkyl chain of diaminocarbamate derivatives was crucial for their anticancer properties. These findings will be useful in the future research and development of anticancer drugs.

Keywords: aminocarbamate; pregnenolone; cytotoxicity; SAR; steroid

1. Introduction

Steroids are a group of natural products found in plants and animals and are essential to living organisms (Lednicer, 2011). All steroids have a characteristic core structure composed of 17 carbon atoms arranged into four fused rings (A, B, C, and D), as shown in Figure 1. Steroids are crystalline compounds with chemical properties similar to other analogs, but their reactions are highly stereo- and regio-selective (Erkılıç, 2008). The reactivities of steroidal functional groups depend on their positions, and many steroids are amphipathic molecules with both polar and non-polar regions (Yang et al., 2017; Ehsan et al., 2018). Various steroids have been found to have pharmacological effects and they are

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commonly used in medicine to treat various symptoms and diseases (Bohl, 2018). Furthermore, steroids include animal and plant hormones, such as progesterone (a female sex hormone) (Baulieu & Schumacher, 2000) and castasterone (a plant hormone) (Bishop & Koncz, 2002). Due to the diverse and interesting chemical and biological properties of steroids, chemists have functionalized and modified steroid structures, evaluated their bioavailability, and developed them into new drugs (Graf et al., 2009; Maltais & Poirier, 2011; Midzak et al., 2012; Gupta et al., 2013).

Pregnenolone (Figure 1) is a major precursor of steroid hormones, including estrogen, progesterone, and testosterone (Saudan et al., 2005). It is biologically active and is used both as a medicine and a dietary supplement. Several pregnenolone derivatives have been synthesized and evaluated for their potential antifungal (Lone & Bhat, 2013), antimicrobial (Banday et al., 2011), and anticancer activities (Banday et al., 2010, 2014; Choudhary et al., 2011; Szalóki et al., 2014; Iqbal & Siddiqui, 2021; Tufail et al., 2021; Yadav et al., 2021). Certain positions on the pregnenolone backbone are commonly modified, such as C-3, C-20, and C-21. Of these, C-20 and C-21 are derivatized more often than other positions. For example, C-21 on the D-ring of pregnenolone was modified to produce chalcone derivatives, which were found to exhibit antimicrobial properties (Banday et al., 2011). Moreover, pregnenolone-derived chalcones and oximes were further functionalized into pyrazoles and other related compounds (Figure 1) (Banday et al., 2010, 2014; Choudhary et al., 2011; Tufail et al., 2021). These compounds exhibited promising anticancer properties. C-21 was also derivatized to alkyne derivatives in addition to chalcones, and the cytotoxicity of these steroids was recently studied against specific cell models (Figure 1) (Szalóki et al., 2014).

According to the provided information, while the C-20 and C-21 positions of pregnenolone are commonly subjected to functionalization, reserch studies on C-3 modification are scarce. Yadav et al. (2021) recently utilized palladium-catalyzed cross-coupling reactions to convert the C-3 group of pregnenolone into ester derivatives. These newly synthesized compounds exhibited effectiveness against lung cancer cells (Figure 1). The results from this research caught our attention in exploring modifications to the C-3 functional group of pregnenolone and then evaluating their cytotoxicity.

Herein, we aimed to synthesize novel C-3 amino pregnenolones (Figure 2) and conduct preliminary cytotoxicity tests against five cancer cell lines: HepG2 (Human hepatocellular carcinoma), HT-29 (Human colon adenocarcinoma), KB (Human oral epidermal carcinoma), MCF-7 (Human breast carcinoma), and P388 (Murine leukemia). Vinblastine sulfate salt was used as a positive control experiment, and Vero (African green monkey kidney fibroblast) cell lines were employed to represent normal cell lines. Carbamate functional groups piqued our interest due to the numerous studies demonstrating the anticancer activity of organic carbamates (Chaturvedi, 2013; Ghosh & Brindisi, 2015; Matošević & Bosak, 2020; Song et al., 2022). Previously, anticancer properties of some steroidal carbamates such as C-17 phenylcarbamate steroid (Bratoeff et al., 2007), piperazine-carbamate steroid (Pathak et al., 2019), diamine-carbamate ergosterol peroxide (Bu et al., 2017), and diosgenin carbamate (Pacheco et al., 2012) were reported (Figure 3).

Furthermore, most carbamates being studied here were derived from diamine and morpholine moieties because compounds bearing these amines were found to have excellent biological properties (Lee et al., 1995; Merlani et al., 2006; Antinarelli et al., 2012; Taha et al., 2017; Arshad et al., 2019; Fiorot et al., 2019; Goud et al., 2019; Ajduković et al., 2021; Lenci et al., 2021), particularly anticancer properties. Our findings can contribute to the development of novel anticancer drugs.

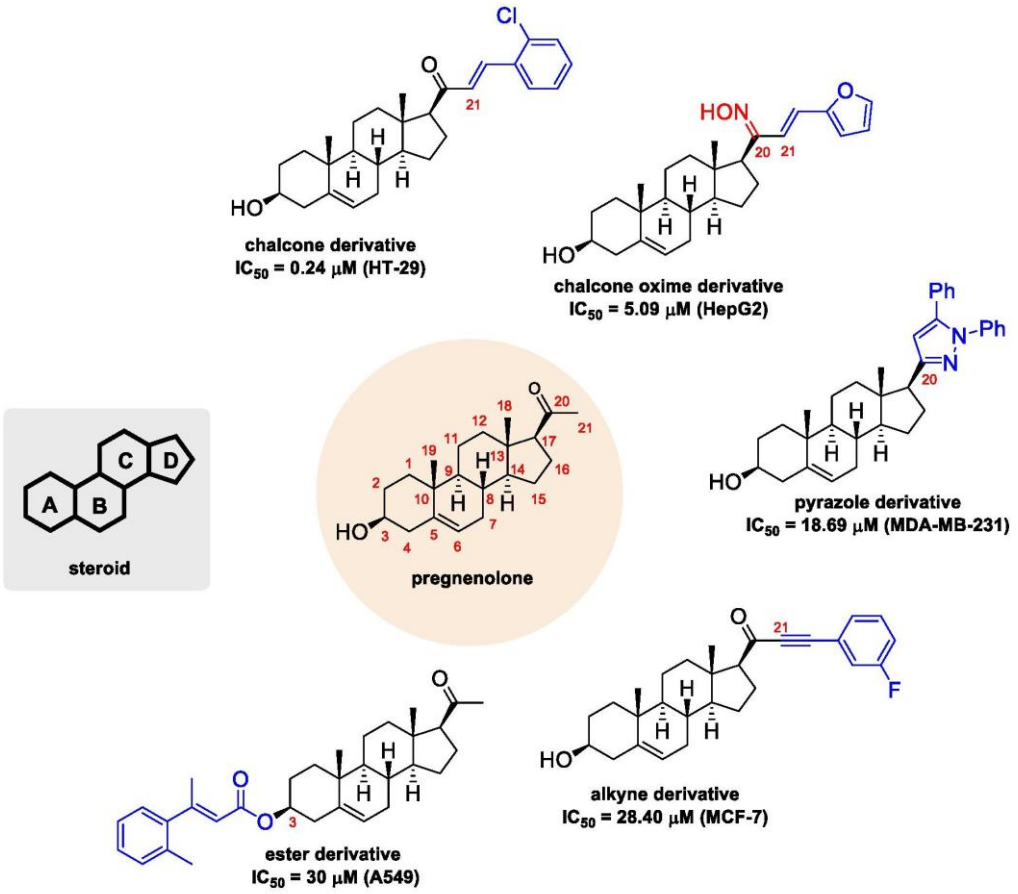

Figure 1. Steroids core structure, pregnenolone, and its anticancer derivatives

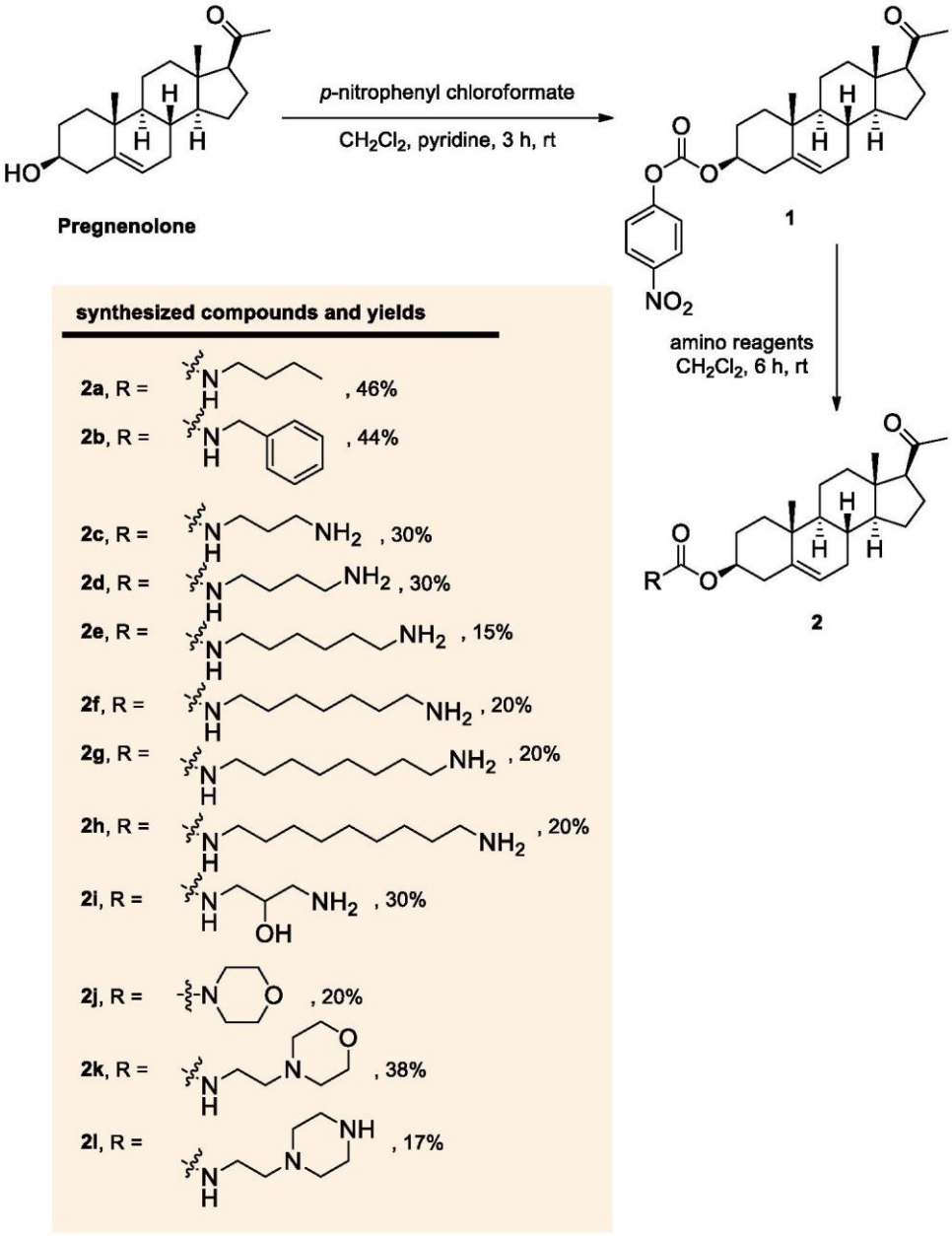

Figure 2. Synthesis of C-3 aminocarbamate pregnenolones (**2a**–**2l**)

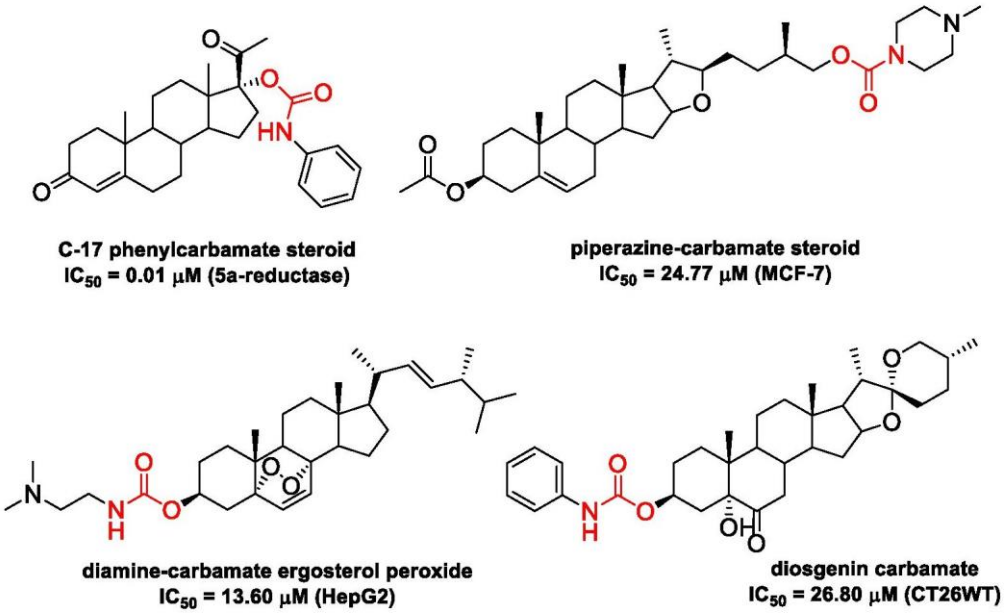

Figure 3. Some steroidal carbamates with anticancer properties

2. Materials and Methods

2.1 General experimental procedure

^{1}H and ^{13}C NMR spectra were recorded on a 300 MHz Bruker Avance device (Switzerland) at the Department of Chemistry, School of Science, KMITL. Spectra were acquired in $CDCl_3$ with TMS as an internal standard for both proton and carbon spectra. Chemical shift values were reported in δ (ppm), and coupling constants were given in Hz. Mass spectra were recorded on an Agilent 1260 Infinity Series at the Faculty of Science, Naresuan University, while IR spectra were recorded on a Perkin Elmer (Spectrum GX 60237) (Connecticut, USA), at the Department of Chemistry, School of Science, KMITL. Melting points were determined using a Gallenkamp Sanyo apparatus (Osaka, Japan). All chemicals and solvents were of analytical grade. Dichloromethane (DCM) was distilled over calcium hydride. Pregnenolone was purchased from Sigma (Massachusetts, USA). All reactions were monitored by thin-layer chromatography (TLC) using Merck aluminum plates coated with silica gel (Merck Kieselgel 60 F254). Spots were visualized after applying anisaldehyde-sulfuric acid spray and heat. Compounds were purified by column chromatography (Scharlau silica gel).

2.2 Synthesis of 4-nitrophenylcarbonate pregnenolone (1)

Following previously reported procedures (Yamada et al., 2012, Vergallo et al., 2020), pregnenolone (300.0 mg, 0.95 mmol) was dissolved in DCM (15 mL). Then pyridine (1.5 mL, 18.5 mmol) and 4-nitrophenyl chloroformate (248.9 mg, 1.23 mmol) were added to the solution. The mixture was stirred for 3 h, or until complete conversion, as monitored by

TLC. After the reaction was complete, the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography (hexane: ethyl acetate, 97:3) to yield a white solid (420.9 mg, 0.87 mmol, 92% yield) with the following properties: R_f 0.62 (hexane:ethyl acetate, 6:4); m.p. 165-168°C; IR (KBr): 2945, 1762, 1702, 1524, 1491, 1254, 1216 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.27 (d, *J* = 9.2 Hz, 2H), 7.38 (d, *J* = 9.2 Hz, 2H), 5.43 (d, *J* = 4.9 Hz, 1H), 4.70-4.53 (m, 1H), 2.58-2.44 (m, 2H), 2.24-1.89 (m, 5H), 2.12 (s, 3H), 1.82-1.40 (m, 9H), 1.34-1.09 (m, 4H), 1.05 (s, 3H), 0.64 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.4, 155.6, 151.8, 145.3, 138.8, 125.2 (2 × CH), 123.2, 121.8 (2 × CH), 79.6, 63.6, 56.8, 49.8, 43.9, 38.7, 37.8, 36.8, 36.5, 31.8, 31.7, 31.5, 27.5, 24.5, 22.8, 21.0, 19.2, 13.2. HRMS (ESI) Exact mass calcd for $C_{28}H_{35}NNaO_6$ $[M+Na]^+$: 504.2362, found 504.2362.

2.3 General procedure for the synthesis of C-3 aminocarbamate pregnenolone derivatives (2a–2l)

As mentioned earlier, most C-3 aminocarbamates studied here were derived from diamine and morpholine moieties because compounds bearing these functional groups were found to have excellent biological properties (Lee et al., 1995; Merlani et al., 2006; Antinarelli et al., 2012; Taha et al., 2017; Arshad et al., 2019; Fiorot et al., 2019; Goud et al., 2019; Ajduković et al., 2021; Lenci et al., 2021).

Amino reagents (1.2 mmol) were added to a solution of 4-nitrophenylpregnenolone carbonate (**1**) (1.0 mmol) in DCM (20 mL) at room temperature. The mixture was stirred for 6 h, and the reaction progress was monitored by TLC. Upon completion, water was added, and the mixture was extracted with DCM (3 × 20 mL). The combined organic phase was washed with water and brine, then dried over anhydrous Na_2SO_4. After solvent removal under reduced pressure, the residue was purified by column chromatography on silica gel to yield the desired compounds **2a**–**2l**.

*Butylcarbamate pregnenolone (**2a**)*. Compound **2a** was obtained at 191.2 mg (0.46 mmol, 46% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 9:1); R_f 0.45 (hexane: ethyl acetate, 7:3); m.p. 186-188°C; IR (KBr): 3363, 2936, 2851, 1702, 1453, 1357 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.39-5.29 (m, 1H), 4.68-4.59 (m, 1H), 4.52-4.41 (m, 1H), 3.17-3.09 (m, 2H), 2.53 (t, *J* = 8.9 Hz, 1H), 2.10 (s, 3H), 2.04-0.83 (m, 23H), 0.98 (s, 3H), 0.90 (s, 3H), 0.60 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.6, 156.1, 139.8, 122.0, 74.2, 63.6, 56.8, 49.8, 44.0, 40.6, 38.7, 38.5, 36.9, 36.5, 32.0, 31.8, 31.7, 31.5, 28.1, 24.4, 22.8, 21.0, 19.8, 19.2, 13.6, 13.2; HRMS (ESI) Exact mass calcd for $C_{26}H_{41}NNaO_3$ $[M+Na]^+$: 438.2984, found 438.2960.

*Benzylcarbamate pregnenolone (**2b**)*. Compound **2b** was obtained at 197.8 mg (0.44 mmol, 44% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 7:3); R_f 0.50 (hexane: ethyl acetate, 8.5:1.5); m.p. 166-167°C; IR (KBr): 3363, 2936, 2851, 1702, 1453, 1357 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.35-7.25 (m, 5H), 5.39-5.34 (m, 1H), 5.03-4.92 (m, 1H), 4.59-4.47 (m, 1H), 4.35 (s, 2H), 2.53 (t, *J* = 9.0 Hz, 1H), 2.41-0.85 (m, 19H), 2.12 (s, 3H), 1.00 (s, 3H), 0.62 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.7, 156.1, 139.8, 138.6, 128.6 (2 × CH), 127.5, 127.4 (2 × CH), 122.2, 74.4, 63.7, 56.8, 49.8, 45.0, 44.0, 38.8, 38.5, 37.0, 36.5, 31.8, 31.7, 31.5, 28.1, 24.4, 22.8, 21.0, 19.3, 13.2; HRMS (ESI) Exact mass calcd for $C_{29}H_{39}NNaO_3$ $[M+Na]^+$: 472.2828, found 472.2826.

*1,3-Diaminopropylcarbamate pregnenolone (**2c**)*. Compound **2c** was obtained at 125.0 mg (0.30 mmol, 30% yield), as a pale yellow solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 7.6:2.4); R_f 0.26 (hexane: ethyl acetate, 6:4); m.p. 109-111°C; IR (KBr): 3355, 2940, 2851, 1702, 1438, 1357 cm^{-1}; ^{1}H-NMR ($CDCl_3$,

300 MHz): δ 5.42-5.31 (d, *J* = 4.6 Hz, 1H), 5.15-5.01 (m, 1H), 4.56-4.38 (m, 1H), 3.30-3.13 (m, 2H), 2.53 (t, *J* = 8.7 Hz, 1H), 2.40-0.79 (m, 25H), 2.11 (s, 3H), 1.00 (s, 3H), 0.62 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.6, 156.6, 139.8, 122.1, 74.2, 63.7, 56.8, 49.9, 44.0, 38.8, 38.5, 37.4, 37.0, 36.6, 31.8, 31.7, 31.5, 30.6, 29.7, 28.1, 24.5, 22.8, 21.0, 19.3, 13.2; HRMS (ESI) Exact mass calcd for $C_{25}H_{41}N_2O_3$ $[M+H]^+$: 417.3117, found 417.3119.

*1,4-Diaminobutylcarbamate pregnenolone (**2d**)*. Compound **2d** was obtained 129.2 mg (0.30 mmol, 30% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 7.5:2.5); R_f 0.26 (hexane: ethyl acetate, 6:4); m.p. 129-130°C; IR (KBr): 3363, 2936, 2851, 1702, 1453, 1357 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.35 (s, 1H), 4.65 (s, 1H), 4.56-4.39 (m 1H), 3.20-3.02 (m, 2H), 2.51 (t, *J* = 8.7 Hz, 1H), 2.40-1.78 (m, 8H), 2.10 (s, 3H), 1.73-0.80 (m, 20H), 0.98 (s, 3H), 0.60 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.5, 156.1, 139.8, 122.0, 73.9, 63.6, 56.8, 49.8, 43.9, 40.8, 38.7, 38.5, 36.9, 36.5, 31.8, 31.7, 31.5, 29.9, 29.1, 28.1, 26.6, 24.4, 22.8, 21.0, 19.3, 13.1; HRMS (ESI) Exact mass calcd for $C_{26}H_{43}N_2O_3$ $[M+H]^+$: 431.3274, found 431.3254.

*1,6-Diaminohexylcarbamate pregnenolone (**2e**)*. Compound **2e** was obtained at 68.8 mg (0.15 mmol, 15% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 8.4:1.6); R_f 0.38 (hexane: ethyl acetate, 6:4); m.p. 193-194°C; IR (KBr): 3362, 2936, 2851, 1702, 1453, 1357 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.36 (s, 1H), 4.72 (s, 1H), 4.55-4.40 (m, 1H), 3.16 (d, *J* = 4.2 Hz, 2H), 2.52 (t, *J* = 8.6 Hz, 1H), 2.40-0.78 (m, 31H), 2.11 (s, 3H), 1.00 (s, 3H), 0.61 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.5, 156.2, 139.8, 122.1, 74.1, 63.6, 56.8, 49.8, 43.9, 40.5, 38.8, 38.5, 37.0, 36.5, 31.9, 31.8, 31.7, 31.5, 29.6, 28.1, 27.3, 24.4, 22.8, 21.0. 19.3, 13.2; HRMS (ESI) Exact mass calcd for $C_{28}H_{47}N_2O_3$ $[M+H]^+$: 459.3587, found 459.3578.

*1,7-Diaminoheptylcarbamate pregnenolone (**2f**)*. Compound **2f** was obtained at 94.5 mg (0.20 mmol, 20% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 8.7:1.3); R_f 0.36 (hexane: ethyl acetate, 6:4); m.p. 175-178°C; IR (KBr): 3359, 2927, 2852, 1698, 1455, 1357 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.35 (d, *J* = 4.2 Hz, 1H), 4.67 (s, 1H), 4.55-4.39 (m, 1H), 3.20-3.05 (m, 2H), 2.52 (t, *J* = 8.7 Hz, 1H), 2.41-0.78 (m, 33H), 2.11 (s, 3H), 0.99 (s, 3H), 0.61 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.6, 156.2, 139.8, 122.1, 74.0, 63.7, 56.8, 49.8, 44.0, 40.7, 38.8, 38.5, 37.0, 36.5, 32.0, 31.8, 31.7, 31.5, 29.9, 29.7, 28.1, 26.2, 24.4, 22.8, 21.0, 19.3, 13.2; HRMS (ESI) Exact mass calcd for $C_{29}H_{49}N_2O_3$ $[M+H]^+$: 473.3743, found 473.3742.

*1,8-Diaminooctylcarbamate pregnenolone (**2g**)*. Compound **2g** was obtained at 97.3 mg (0.20 mmol, 20% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 8.9:1.1); R_f 0.38 (hexane: ethyl acetate, 6:4); m.p. 104-106°C; IR (KBr): 3353, 2933, 2852, 1703, 1455, 1381 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.37 (s, 1H), 4.65 (s, 1H), 4.58-4.40 (m 1H), 3.23-3.04 (m, 2H), 2.54 (t, *J* = 8.6 Hz, 1H), 2.42-0.79 (m, 35H), 2.12 (s, 3H), 1.01 (s, 3H), 0.63 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.6, 156.1, 139.8, 122.0, 73.9, 63.6, 56.8, 49.8, 43.9, 40.8, 38.7, 38.5, 36.9, 36.5, 31.8, 31.7, 31.5, 29.9, 29.1, 28.1, 26.6, 24.4, 22.8, 21.0, 19.3, 13.1; HRMS (ESI) Exact mass calcd for $C_{30}H_{51}N_2O_3$ $[M+H]^+$: 487.3900, found 487.3892.

*1,9-Diaminononylcarbamate pregnenolone (**2h**)*. Compound **2h** was obtained at 100.2 mg (0.20 mmol, 20% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 8.8:1.2); R_f 0.38 (hexane: ethyl acetate, 6:4); m.p. 82-85°C; IR (KBr): 3357, 2926, 2852, 1702, 1466, 1380 cm^{-1}; ^{1}H-NMR (CD_3OD, 300 MHz): δ 5.39 (s, 1H), 4.47–4.29 (m, 1H), 3.13-3.21 (m, 2H), 2.69-2.59 (m, 1H), 2.38-2.25 (m, 2H), 2.21-0.81 (m, 36H), 2.12 (s, 3H), 1.03 (s, 3H), 0.62 (s, 3H); ^{13}C-NMR (CD_3OD, 75.5 MHz): δ 212.3, 158.7, 141.3, 123.2, 75.3, 64.7, 68.1, 51.5, 45.1, 41.6, 39.9, 39.7, 38.3, 37.8, 37.6, 33.3, 33.2, 32.9, 31.7, 30.9, 30.7, 30.5, 30.2, 29.5, 29.3, 27.7, 25.6, 25.5, 23.8, 22.2, 19.8, 13.6; HRMS (ESI) Exact mass calcd for $C_{31}H_{53}N_2O_3$ $[M+H]^+$: 501.4056, found 501.4056.

*(1,3-Diaminopropyl-2-ol)carbamate pregnenolone (**2i**)*. Compound **2i** was obtained at 129.8 mg (0.30 mmol, 30% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 6:4); R_f 0.07 (hexane: ethyl acetate, 6:4); m.p. 104-106°C; IR (KBr): 3379, 2845, 2918, 1698, 1455, 1384 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.47-5.28 (m, 2H), 4.53-4.37 (m, 1H), 3.81-3.69 (m, 1H), 3.24 (s, 2H), 2.52 (t, *J* = 8.5 Hz, 1H), 2.39-0.80 (m, 24H), 2.10 (s, 3H), 0.99 (s, 3H), 0.61 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.6, 157.3, 139.7, 122.2, 74.7, 70.5, 63.6, 56.8, 49.8, 43.9, 43.6, 38.7, 38.4, 36.9, 36.5, 32.0, 31.8, 31.7, 31.5, 28.0, 24.4, 22.8, 21.0, 19.3, 13.2; HRMS (ESI) Exact mass calcd for $C_{25}H_{41}N_2O_4$ $[M+H]^+$: 433.3066, found 433.3055.

*Morpholinocarbamate pregnenolone (**2j**)*. Compound **2j** was obtained at 85.9 mg (0.20 mmol, 20% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 9.5:0.5); R_f 0.50 (hexane: ethyl acetate, 6:4); m.p. 157-159°C; IR (KBr): 3357, 2926, 2852, 1702, 1466, 1380 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.34 (s, 1H), 4.57-4.40 (m, 1H), 3.68-3.56 (m, 4H), 3.48-3.36 (m, 4H), 2.50 (t, *J* = 8.6 Hz, 1H), 2.39-0.93 (m, 19H), 2.08 (s, 3H), 0.98 (s, 3H), 0.58 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.4, 154.9, 139.7, 122.1, 74.8, 66.5 (2C), 63.5, 56.7, 49.7, 43.8 (3C), 38.7, 38.4, 36.9, 36.5, 31.7, 31.6, 31.4, 28.0, 24.4, 22.7, 20.9, 19.0, 13.1; HRMS (ESI) Exact mass calcd for $C_{26}H_{39}NNaO_4$ $[M+Na]^+$: 452.2777, found 452.2778.

*(4-(2-Aminoethyl) morpholino)carbamate pregnenolone (**2k**)*. Compound **2k** was obtained at 179.6 mg (0.38 mmol, 38% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 65:35); R_f 0.05 (hexane: ethyl acetate, 6:4); m.p. 177-179°C; IR (KBr): 3357, 2943,1702,1455, 1357 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.36 (s, 1H), 5.12 (s, 1H), 4.58-4.41 (m, 1H), 3.73-3.65 (m, 4H), 3.33-3.20 (m, 2H), 2.58-0.91 (m, 26H), 2.11 (s, 3H), 1.00 (s, 3H), 0.62 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.6, 156.1, 139.8, 122.1, 74.2, 66.9 (2C), 63.7, 57.5, 56.8, 53.3 (2C), 49.8, 44.0, 38.8, 38.5, 37.1, 37.0, 36.5, 31.8, 31.7, 31.5, 28.1, 24.4, 22.8, 21.0, 19.3, 13.2; HRMS (ESI) Exact mass calcd for $C_{28}H_{45}N_2O_4$ $[M+H]^+$: 473.3379, found 473.3382.

*(2-Piperazin-1-yl-ethyl)carbamate pregnenolone (**2l**)*. Compound **2l** was obtained at 80.2 mg (0.17 mmol, 17% yield), as a white solid, after purification by silica gel column chromatography (hexane: ethyl acetate, 60:40); R_f 0.16 (hexane: ethyl acetate, 6:4); m.p. 142-144°C; IR (KBr): 3378, 2925, 2851, 1704, 1433 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.35 (s, 1H), 4.49 (s, 1H), 3.46 (s, 2H), 3.46 (s, 1H), 2.57-0.78 (m, 31H), 2.10 (s, 3H), 1.00 (s, 3H), 0.61 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): δ 209.5, 156.1, 139.8, 122.1, 74.8, 63.6 (2C), 57.1, 56.8 (2C), 52.6, 49.8, 43.9, 43.4, 38.7, 38.5, 37.0, 36.5, 31.8, 31.7, 31.5, 29.6, 28.1, 24.4, 22.8, 21.0, 19.3, 13.1; HRMS (ESI) Exact mass calcd for $C_{28}H_{46}N_3O_3$ $[M+H]^+$: 472.3539, found 472.3512.

2.4 Determination of cytotoxic activity

All cell lines (Human hepatocellular carcinoma (HepG2), Human colon adenocarcinoma (HT-29: ATCC HTB-38), Human oral cavity carcinoma (KB: CLS300446), Human breast adenocarcinoma (MCF-7: ATCC HTB-22), Murine leukemia (P388), and African green monkey kidney fibroblast (Vero: CLS605372)) were obtained from the Animal Cell Culture Laboratory, the Scientific Instruments Center, School of Science, KMITL. The *in vitro* cytotoxicity was also determined at this laboratory.

In vitro cytotoxicity was determined using the MTT colorimetric assay (Poeaim et al., 2016), originally described by Mosmann (1983). The cells were trypsinized and diluted with RPMI 1640 medium containing 5% v/v fetal bovine serum (FBS). Subsequently, the cell lines were seeded into 96-well plates at densities of 1.0-1.8 x 10^5 cells/mL in a 100 µL cell culture and incubated at 37°C for 24 h. After incubation, the cells were treated with

each of the 3-aminocarbamate pregnenolones and incubated for 20 h. For preliminary screening to evaluate the percent cytotoxicity, the final concentration of 3-aminocarbamate pregnenolones in each well was 1,000 µg/mL. However, for calculating the IC_{50}, the concentrations of 3-aminocarbamate pregnenolones were studied in the range of 125-1,000 µg/mL. A 0.2% (v/v) dimethyl sulfoxide (DMSO) solution and vinblastine sulfate salt were used as negative and positive controls, respectively. Subsequently, 50 µL of MTT solution was added to each of the 96-well plates, and cultures were incubated for 4 h. The medium was then removed, and 100 µL of a DMSO: absolute ethanol (1:1) solution was added to each well to stabilize formazan crystals. The absorbance of the converted dye was measured at 570 nm using a microplate reading spectrophotometer (Anthos MultiRead 400, Biochrom, UK). The percentage of inhibition of cell growth was calculated using the following formula:

$$\%\ cytotoxicity = \left(\frac{\text{control} - \text{sample}}{\text{control}}\right) \times\ 100$$

The 50% inhibitory concentration (IC50) was estimated using GraphPad Prism 5 software. Each experimental measurement was replicated three times and expressed as the mean±standard deviation (SD).

3. Results and Discussion

3.1 Chemical synthesis

Our synthetic design involved varying the aminocarbamate substituents at C-3 of pregnenolone. We utilized a variety of amino reagents, including alkylamines (aminobutyl and aminobenzyl), diamines (diaminopropyl, diaminobutyl, diaminohexyl, diaminoheptyl, diaminooctyl, diaminononyl), morpholines (morpholino and aminoethylmorpholino), and piperazine (piperazinylethyl), to explore the structure-activity relationship (SAR) of this class of compounds (Figure 2). Initially, pregnenolone was converted to precursor **1** by reaction with *p*-nitrophenyl chloroformate in dichloromethane and pyridine. Subsequently, the reaction of compound **1** with various amino reagents provided the corresponding 3-aminocarbamate pregnenolones (**2a**–**2l**) in low to moderate yields (Figure 2).

The structures of compounds **1** and **2a**–**2l** were confirmed by spectroscopic data. The nitro precursor (**1**) was identified by comparing its ^{1}H NMR spectrum to that of 4-iodophenylpregnenolone carbonate (Van Dort et al., 1989), a previously reported analog, which exhibited a similar pattern in the ^{1}H NMR spectrum. Additionally, a distinct pattern was observed when comparing the ^{1}H NMR spectrum of compound **1** with that of the pregnenolone starting material (Figure 4). While most protons in both compounds showed similar chemical shifts, the C-3 proton of pregnenolone appeared at around 3.55-3.43 ppm (Figure 4 (A)), whereas the C-3 proton of precursor **1**, bearing an electron-withdrawing carbamate, exhibited signals at around 4.70-4.53 ppm (Figure 4 (B)).

Regarding the aminocarbamate pregnenolone derivatives (**2a**–**2l**), all compounds exhibited a common pattern in their ^{1}H NMR spectra similar to precursor **1**. The main difference was observed in the proton signal of the alkylaminocarbamate and alkyldiaminocarbamate groups attached to the steroid ring. For example, compound **2b** showed a ^{1}H NMR signal of the aromatic ring at around 7.35-7.25 ppm, the methylene proton ($H^{2\prime}$) at 4.35 ppm, and the NH proton at 5.03-4.92 ppm (Figure 4 (C)). In the case of diaminoalkylcarbamates, such as compound **2d**, although the ^{1}H NMR signals of some methylene groups overlapped with those of the steroid ring in the high field region, this compound exhibited a distinct signal of a free amino group (NH_2) at around 3.20-3.02 ppm (Figure 4 (D)).

In terms of the isolated yield of aminocarbamate pregnenolones, it is evident that all compounds were obtained in low to moderate yields. The exact reason behind this remains unclear; however, the TLC results of the reaction mixtures revealed some minor spots likely representing side products formed from the interaction of both amine groups. Moreover, the type of amino reagent appeared to influence the reaction outcome. Monoprimary amines, such as *n*-butylamine and benzylamine, were transformed into products **2a** and **2b** in moderate yields (46% and 44%, respectively). However, for morpholine, a monosecondary amine, a lower yield (20%) of **2j** was obtained, possibly due to steric hindrance. For all diamino carbamates (**2c**–**2i**), only small amounts of products were obtained (15%-30%). Since both amino groups of diamino reagents have similar reactivity, it is presumed that the desired products could further react with other species, such as the carbamate precursor **1**, resulting in a lower yield of the desired compounds. Regarding the use of 2-morpholinoethanamine reagent, product **2k** was obtained in moderate yield (38%). However, when the 2-(piperazin-1-yl)ethanamine reagent was used, the yield of compound **2l** was only 17%. Despite these variations, the reaction conditions were not further optimized as significant amounts of all products were obtained for further studies.

3.2 Evaluation of cytotoxic activity

To investigate the structure-activity relationship (SAR) of pregnenolone and its aminocarbamate derivatives (**2a**–**2l**), these compounds were preliminarily tested *in vitro* against five cancer cell lines: HepG2 (Human hepatocellular carcinoma), HT-29 (Human colon adenocarcinoma), KB (Human oral cavity carcinoma), MCF-7 (Human breast adenocarcinoma), and P388 (Murine leukemia), as well as one normal cell line: Vero (African green monkey kidney fibroblast), using an MTT assay (Poeaim et al., 2016; Mosmann, 1983). The applied concentration was 1,000 µg/mL, and the exposure time was 24 h. The cytotoxicity was reported in terms of % cytotoxicity.

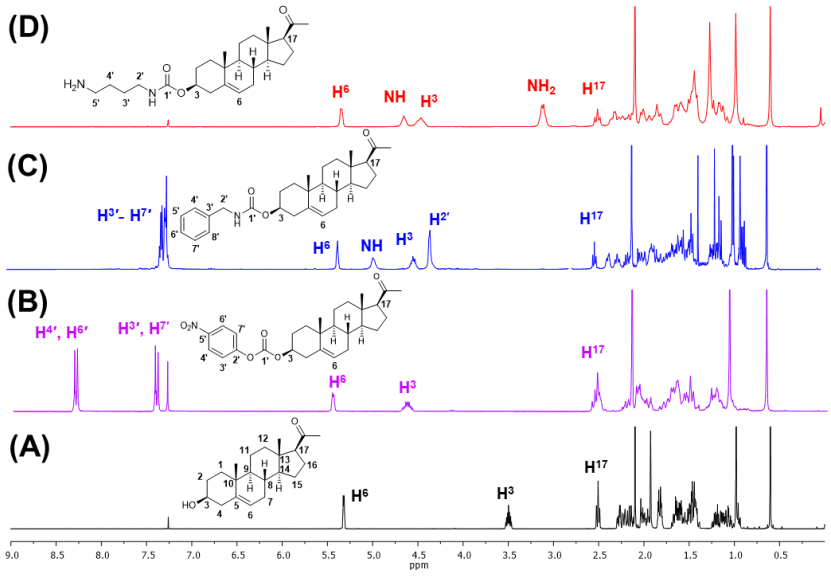

Figure 4. ^{1}H NMR spectra of (A) pregnenolone, (B) nitro precursor **1**, (C) benzylcarbamate (**2b**), and (D) 1,4-diaminobutylcarbamate (**2d**)

As shown in Table 1, all tested compounds inhibited cancer cell growth. The pregnenolone starting material reduced MCF-7 cell growth by 60.45%. Among the aminocarbamate pregnenolones, compound **2k** completely inhibited P388. Compounds **2b** and **2i** were highly active against all tested cell lines, while derivatives **2c**, **2e**, and **2f** strongly inhibited the growth of KB, MCF-7, and P388. Other derivatives exhibited weaker activity against cell line growth, particularly compounds **2a** and **2l**, which showed very low percent cytotoxicity against most tested cell lines. To gain more information about the cytotoxicity of the tested aminocarbamate pregnenolones, all compounds that exhibited a percent cytotoxicity higher than 50% were further evaluated against all types of tested cell lines. The half-maximal inhibitory concentration (IC_{50}) value, indicating the amount of substance needed to inhibit a biological function by half, was determined and is shown in Table 2.

As illustrated in Table 2, pregnenolone and its aminocarbamate derivatives (**2b**–**2l**) exhibited a wide range of IC_{50} values. Some derivatives showed IC_{50} values lower than that of the positive control, vinblastine sulfate salt. For the HepG2 and HT-29 cell lines, 3-aminobenzylcarbamate (**2b**) was the most active compound against both cell lines, with IC_{50} values of 0.79±0.02 µM and 0.58±0.02 µM, respectively. Regarding the KB cell line, the diaminopropanolcarbamate derivative (**2i**) was the most potent, with an IC_{50} value of 0.30±0.02 µM, followed by compounds **2c** and **2e**, which were the second and third most active derivatives, respectively. For the MCF-7 cell line, diaminoheptylcarbamate pregnenolone (**2f**) displayed the lowest IC_{50} of 0.64±0.03 µM, slightly stronger than compounds **2b**, **2c**, and **2i**. However, other tested compounds exhibited weak cytotoxicity against the MCF-7 cell line. In the case of P388 cell line, diaminopropanolcarbamate (**2i**) was the most active compound, with an IC_{50} as low as 0.30±0.00 µM. Diaminohexylcarbamate (**2e**) was the second most active, with an IC_{50} of 0.33±0.02 µM. Compounds **2b**, **2f**, and **2k** exhibited comparable cytotoxicities, whereas other derivatives showed weaker effects. For the normal cell line, Vero, most compounds had weak cytotoxicity, except for 3-aminobenzylcarbamate (**2b**), which showed an IC_{50} of 0.59±0.03 µM.

Table 1. % Cytotoxicity of pregnenolone and 3-aminocarbamate pregnenolones (**2a**–**2l**)

Compounds	% Cytotoxicity*					
	HepG2	HT-29	KB	MCF-7	P388	Vero
Pregnenolone	43.26	21.04	32.34	60.45	22.18	40.65
2a	30.63	47.73	27.21	43.61	32.16	34.07
2b	91.52	95.33	65.85	93.61	95.77	98.32
2c	44.27	38.97	84.65	71.74	97.56	56.88
2d	40.07	42.30	66.74	57.42	37.94	57.12
2e	55.19	25.64	95.30	68.74	96.86	62.38
2f	40.25	19.34	97.80	89.85	99.65	42.40
2g	27.44	22.10	53.88	54.02	57.80	44.40
2h	43.41	23.95	31.99	74.51	28.15	62.71
2i	62.36	64.92	98.69	74.75	92.96	61.97
2j	30.54	13.27	52.95	55.84	13.36	36.52
2k	31.14	10.57	56.75	53.17	100.00	32.04
2l	36.98	18.68	35.88	51.70	26.56	31.44

* All compounds were tested at 1,000 µg/ mL

Table 2. *In vitro* cytotoxic activity (IC_{50} in μM) of pregnenolone and 3-aminocarbamate pregnenolones (**2a**–**2l**)

Compounds	***In vitro* cytotoxicity (IC_{50} in μM)***					
	HepG2	HT-29	KB	MCF-7	P388	Vero
Pregnenolone	-	-	-	2.29±0.18	-	-
2a	-	-	-	-	-	-
2b	0.79±0.02	0.58±0.02	1.47±0.06	0.88±0.01	0.52±0.01	0.59±0.03
2c	-	-	0.49±0.03	0.90±0.02	1.26 ± 0.03	1.46±0.16
2d	-	-	0.95±0.04	1.99±0.14	-	2.02±0.06
2e	2.02±0.08	-	0.72±0.01	1.58±0.03	0.33±0.02	1.40±0.14
2f	-	-	0.97±0.03	0.64±0.03	0.66±0.02	-
2g	-	-	1.94±0.08	1.85±0.10	1.32±0.02	-
2h	-	-	-	1.39±0.18	-	1.61±0.07
2i	1.88±0.07	0.86±0.04	0.30±0.02	0.84±0.07	0.30±0.00	1.33±0.04
2j	-	-	1.53±0.03	1.93±0.02	-	-
2k	-	-	1.44±0.02	1.97±0.07	0.70±0.03	-
2l	-	-	-	1.97±0.09	-	-
Vinblastine sulfate salt	0.71±0.02	1.60±0.26	1.47±0.29	0.19±0.00	8.32±0.39	1.33±0.39

* Values expressed are mean±SD of triplicate measurements
- IC_{50} is not evaluated

The results from Table 1 and Table 2 reveal that, for monoamino compounds (**2a** and **2b**), the derivative with an aromatic ring (**2b**) is more potent than the compound bearing an aliphatic substituent (**2a**). Although the effects of aliphatic and aromatic substituents at C-3 of steroids have rarely been compared, numerous studies have shown that benzyl and its derivatives are important pharmacophores in many anticancer compounds (Subtel'na et al., 2010; Fu et al., 2021). Hussein et al.(2019) investigated the cytotoxicity of thiosemicarbazone derivatives and found that the cytotoxic activity increased with the addition of a terminal phenyl group. They suggested that this group may enhance the interactive properties, allowing the compound to cross cell wall barriers and facilitating interaction with DNA.

For diaminocarbamate pregnenolones (**2c**-**2h**), it can be observed that the chain lengths and polarity of the diamino substituents affect cytotoxicity. Among these diaminos, the compound bearing a short alkyl chain (3 carbons, **2c**) is more potent against the KB cell line than other compounds. For the MCF-7 cell line, a different trend is observed: the compound containing a medium chain length (7 carbons, **2f**) is more active than other derivatives. A similar trend is found for P388 cell line growth, where compounds bearing medium alkyl chains (6-7 carbons, **2e** and **2f**) are more potent than compounds with short (3-4 carbons, **2c** and **2d**) and long (8-9 carbons, **2g** and **2h**) alkyl chains.

However, when a more polar diaminopropanolcarbamate pregnenolone (**2i**) was tested, it became the most active among these diaminocarbamate pregnenolones (**2c**–**2i**). This result highlights the significant effect of the polarity of the tested compounds on their cytotoxicity. Previous studies have also indicated that the polarity of substituents at C-3 of steroids influences their cytotoxicity (Phi et al., 2011; Bu et al., 2017; Huang et al., 2021). Additionally, the effects of the carbon chain length of diamine substituents on the biological activity of organic compounds have been disclosed. Loncle et al. (2007) synthesized 7-

aminosterol squalamine analogs and evaluated them for antimicrobial activity. The results revealed that the nature of the amino group attached to the sterol moiety plays an important role in the antimicrobial properties of the synthesized compounds. Specifically, the presence of an amino group with a well-defined chain length, typically 3-5 carbons between the two amino groups, showed great activity, while other chain lengths (1-2 and 6-11) were less active.

Moreover, our current results highlight that diaminocarbamate pregnenolones (**2c**–**2i**) are more potent than the monoaminocarbamate pregnenolone with an aliphatic substituent (**2a**). A similar finding was previously reported for 17-amino steroids against the growth of some tumor cell lines (Merlani et al., 2006). Although 17β-methylamine, a monoamino steroid, was inactive against the tumor cell lines tested, the 17β-(N-methyl-N-amino) steroid, a diamino derivative, demonstrated distinct antitumor activity against various cancer cell lines. Similarly, many monoamino and diamino pyrimidines were evaluated against cancer cell lines (Madia et al., 2021), revealing that diamino derivatives were more potent than their monoamino counterparts.

Concerning pregnenolones bearing morpholinocarbamate and piperazinylcarbamate groups (**2j**–**2l**), both morpholine-containing compounds (**2j** and **2k**) exhibited similar cytotoxicity against the tested cell lines, with slightly stronger effects compared to piperazinylcarbamate pregnenolone (**2l**). The underlying reason for this discrepancy remains unclear. Nonetheless, numerous reports have demonstrated the effectiveness of morpholine-containing compounds in anticancer activity (Arshad et al., 2019; Fiorot et al., 2019; Goud et al., 2019; Lenci et al., 2021; Taha et l., 2017). These scaffolds hold promise for integration into various core structures in the development of future anticancer drugs.

Overall, the cytotoxicity of our synthesized compounds varied depending on the type of substituent. Additionally, the response of all tested cell lines differed to the synthesized amino steroids. While this study does not address the specific mechanism of action of 3-aminocarbamate pregnenolones on cancer cell lines, it is evident that such mechanisms would be complex and necessitate further investigation.

4. Conclusions

In summary, 3-aminocarbamate pregnenolones (**2a**–**2l**) were successfully prepared at low to moderate yields from pregnenolone by a two-step synthesis, involving carbonation with 4-nitrophenyl chloroformate followed by nucleophilic substitution with amino reagents. Structural confirmation *via* spectroscopic techniques was followed by *in vitro* evaluation of cytotoxicity against five cancer cell lines (HepG2, HT-29, KB, MCF-7, and P388) and one normal cell line (Vero). Notably, all synthesized compounds exhibited toxicity towards cancer cell lines, generally surpassing the cytotoxicity levels of pregnenolone. The results from the structure-activity relationship (SAR) study revealed that a compound bearing an aromatic group (**2b**) had greater activity than one bearing an aliphatic group (**2a**). Additionally, the cytotoxicity of diaminocarbamate derivatives was influenced by the lengths and polarity of the carbon chains of the diamino substituents. Specifically, a compound with a short alkyl chain (**2c**) exhibited higher potency against KB cell line, while those with medium chain lengths (6-7 carbons, **2e** and **2f**) were more effective against the MCF-7 and P388 cell lines compared to short and long chain derivatives. Moreover, diamino compounds showed higher activity than the monoaminobutylcarbamate derivative (**2a**). Notably, 3-aminobenzyl carbamate pregnenolone (**2b**) demonstrated the highest potency against the HepG2 and HT-29 cell lines, while polar diaminopropanolcarbamate

pregnenolone (**2i**) and diaminoheptylcarbamate pregnenolone (**2f**) were most effective against the KB and P388 cell lines, and MCF-7 cell lines, respectively. These findings provide valuable insights for the design of novel anticancer drugs derived from 3-aminocarbamate pregnenolones.

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6. Conflicts of Interest

The authors declare no conflict interest.

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