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Fatty Acyl Amide Dderivatives of Doxorubicin: Synthesis and In Vitro Anticancer Activities

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Fatty-acyl amide derivatives of doxorubicin: Synthesis and *in vitro* anticancer activities

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TITLE RUNNING HEAD. Synthesis and evaluation of doxorubicin-fatty acyl conjugates as anticancer agents

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Abstract

Doxorubicin is ~~an anticancer drug~~ extensively used in anticancer therapy. Doxorubicin is highly hydrophilic, has short half-life, and its use is associated with severe side effects at high doses. Fatty acyl amide derivatives of doxorubicin were synthesized with the expectation to improve the lipophilicity and anticancer activity of the drug. The lipophilicity was enhanced with the increase in chain length of fatty acyl moiety. Conjugation of 4′-amino group with fatty acids through an amide bond reduced the anticancer activity in leukemia, breast, ovarian, and colon cancer cell lines, suggesting that the presence of free amino group is required for anticancer activity of doxorubicin. Dodecanoyl-doxorubicin derivative was consistently the most effective among the synthesized derivatives and inhibited the proliferation of colon (HT-29) and ovarian (SK-OV-3) cancer cells by 64% and 58%, respectively, at a concentration of 1 μM after 96 h incubation.

KEYWORDS: Doxorubicin; Prodrug; Fatty acids, Anticancer; Lipophilicity; Sustained delivery.

1. Introduction

Doxorubicin is an anthracycline antibiotic produced by the fungus *Streptomyces peucetius* [1]. The drug is widely used as an anticancer agent in the treatment of leukemia, breast carcinoma, and other solid tumors [2]. Doxorubicin (Fig. 1) has high hydrophilic nature and low bioavailability. To circumvent problems associated with low bioavailability, higher dose is used clinically that may lead to dose-dependent side effects, such as cumulative cardiotoxicity, myelosuppression, nephrotoxicity and extravasation. Doxorubicin has been called as “red devil” or “red death” because of its high antibiotic activity and red color [3].

Please insert Figure 1 here.

Development of compounds with higher therapeutic index is a subject of major interest for the treatment of different cancers. It is widely accepted concept that biological activity and toxicity of low molecular weight antitumor drugs are dependent on their physicochemical and pharmacokinetic properties, such as biodistribution [4-6]. The toxicity and side effects associated with an anticancer drug can be counteracted by altering the pharmacokinetic behavior of the compound. The pharmacological modulations of an anticancer drug create change in its circulation in blood and improve the target accumulation by enhanced permeation rate (EPR).

Various approaches have been applied to improve the drug delivery by chemical modification in a parent drug (prodrug strategy) [7], by attaching the drug molecules to longer circulating particles [8] like polymeric nanoparticles [9], metal nanoparticles [10] and other nanoparticles [11], or by encapsulation in protective sheaths like liposomes, niosomes [12,13], nanomicelle [14], dendrimers [15] or hydrogels/organogels [16].

Doxorubicin has a short half-life in the blood stream and high volume of distribution [17]. In case of doxorubicin, the liposomal formulations have been successfully used in improving its therapeutic efficacy [18-22]. Furthermore, the conjugation of docosahexanoic acid (DHA) to doxorubicin using a hydrazone linker at position 13 formed a lipophilic prodrug, which was able to release doxorubicin at a lower pH. DHA–doxorubicin displayed lower cytotoxicity *in vitro* as compared to that of doxorubicin against a lymphocytic leukemia cell line L1210. Following intraperitoneal injection in L1210 leukemia ascites model, DHA–doxorubicin showed considerably higher anticancer activity than free doxorubicin *in vivo* [23].

In continuation of our efforts to modulate and improve the therapeutic potential of anticancer drugs through chemical modifications [24], herein we report the synthesis of fatty acyl amide derivatives of doxorubicin and evaluation of their *in vitro* anticancer activity in different cancer cell lines. The conjugates were synthesized with the expectation that lipophilic nature of fatty acyl chains will enhance the sustained effect and biological activity of the parent drug.

2. Result and Discussion

2.1. Chemistry

Monosubstituted fatty acyl-doxorubicin conjugates were synthesized by reaction of doxorubicin with fatty acids (i.e., $CH_3(CH_2)_nCOOH$ (n = 0-18), $CH_3CH_2S(CH_2)_{11}COOH$) (one equivalent) in the presence of 1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIPEA) (Scheme 1). The 4′-primary amino group of doxorubicin was selectively reacted with the carboxylic acid moiety of fatty acid under these conditions due to its higher nucleophilic nature compared to the hydroxyl groups. The structures of the synthesized derivatives were confirmed by using infrared spectroscopy, one-dimensional nuclear magnetic resonance (NMR) spectroscopy (^{1}H, ^{13}C or DEPT), 2-dimensional correlative NMR (HSQC and COSY), and high-resolution time-of-flight electrospray mass spectrometry. The IR spectra showed the peak at 1642-1645 cm^{-1} confirming the presence of an amide bond in the final products. Furthermore, C4′ peak in ^{13}C NMR was significantly deshielded after conjugation, suggesting the involvement of 4′-amino group in amide formation. While the doxorubicin was highly soluble in aqueous medium, the fatty acyl amide doxorubicin derivatives were soluble in organic solvents. All the derivatives had dark red brown color. The palmityl and stearyl derivatives of doxorubicin have been reported in Refs [25,26] and were resynthesized here for evaluation of their anticancer activity and showed similar spectral properties to those reported in the literature.

Please insert Scheme 1 here.

Partition coefficients (Log *P*) of **1-7** were determined using biphasic n-octanol/water shake flask system. The concentration of the compounds was measured in both phases using UV spectroscopy. Lipophilicity of many compounds is usually correlated with the membrane permeability and/or biological activity in quantitative structure–activity relationship studies.

Lipophilicity of doxorubicin Log P = 0.6 was consistent with the reported value [27]. The Log P values of the doxorubicin derivatives showed a gradual increase in the lipophilicity of the derivatives with the increase in chain length of the attached fatty acyl groups. For example, the partition coefficient increased in compound **2** with the introduction of acetyl group (Log P = 1.18) (Fig. 2).

Please insert Figure 2 here.

2.2. Biological Activity

The effect of compounds on the cell proliferation of cancer cells was evaluated *in vitro* in human leukemia cell line CCRF-CEM, breast adenocarcinoma MDA-MB-468, ovarian adenocarcinoma SK-OV-3, and colon adenocarcinoma HT-29 cell lines up to 120 h at 1 µM.

At 24 h of incubation, the inhibition of cell proliferation by the derivatives was negligible when incubated with breast cancer cell line (MDA-MB-468) while at 96 h

dodecanoyl (**5**) and tetradecanoyl (**6**) doxorubicin derivatives inhibited the cell proliferation up to 38% and 31%, respectively, when compared to the control (Fig. 3a). These data suggest that even though the compounds **2-10** have higher lipophilicity than that of doxorubicin, they were probably stable intracellularly and did not release substantial amount of doxorubicin even after 96 h in breast cancer cell line.

Please insert Figure 3 here.

Furthermore, the effect of compounds on cell proliferation of other cell lines was evaluated to determine whether fatty acyl-doxorubicin conjugates demonstrate any cell-specific activity. In case of leukemia cell line (CCRF-CEM), the anti-proliferation activity was negligible when compared to doxorubicin (**1**). The tetradecanoyl derivative (**6**) showed only 27% growth inhibition at 96 h (Fig. 3b). Because of high lipophilicity and the prodrug nature, these compounds were expected to have enhanced cellular uptake and/or sustained effect. Thus, the lack of inhibitory activity is postulated to be because of limited intracellular hydrolysis of amide conjugates.

The anti-proliferation profile of compounds **1-10** in SK-OV-3 cell line is shown in Fig. 3c. A number of fatty acyl amide doxorubicin conjugates show efficient inhibition of cell proliferation at 96 h incubation in ovarian cancer cell line. The derivatives showed peculiar parabolic correlation on inhibition of cell proliferation of SK-OV-3 cells. Anti-proliferation activity enhanced with increase in methylene numbers in the fatty acyl chain in compounds **2-4**, reached a maxima in compounds **5** and **6** and then decreased

with increase in carbon number (**8-10**). 12-Thiododecanoyl derivative **7** that has different side chain containing heteroatom sulfur than the other derivatives did not follow the pattern. The maximum activity was shown by dodecanoyl derivative **5**. Dodecanoyl **5** and tetradecanoyl **6** derivatives inhibited the cell proliferation by 58% and 50%, respectively, when compared to the control after 96 h of incubation.

In a time dependent study to evaluate the effect of incubation time (more than 96 h) on cell proliferation inhibitory activity of the derivatives, colon cancer cells HT-29 were incubated for 120 h with the compounds. The compounds showed similar activity pattern in HT-29 cells as seen in SK-OV-3 cells. Dodecanoyl doxorubicin derivative **5** showed inhibited proliferative activity of the cells by 64% while tetradecanoyl derivative **6** exhibited approximately 41% inhibition after 120 h of incubation (Fig. 3d).

Doxorubicin acts as a DNA intercalator, inhibiting the ability of the enzyme topoisomerase II to reseal the DNA double helix strands during the replication and thereby stops the reproduction of cells. Several crystal structures suggest that replication inhibition is directly due to the structure of the doxorubicin; the planar aromatic portion of the molecule intercalates between two base pairs of the DNA helix, while the carbohydrate portion of the drug is positioned to intercalate with the flanking base pairs [28–30]. Thus, the carbohydrate part of doxorubicin is involved in the binding with bases of replicating DNA during its mechanism of action and amine group should be freely available for interaction. Thus, this systematic study performed by fatty acylation of doxorubicin at 4′-amino group of the carbohydrate moiety and the anti-proliferative profile further confirm the requirement for availability of the sugar moiety in doxorubicin. Stable amide was only partially hydrolyzed even after long incubation time

providing anti-proliferative activity in specific cell lines. The inhibitory activity for **5** at 24 h and 96 h suggests that the intracellular hydrolysis of the prodrug to doxorubicin leads to improved anti-proliferative activity after longer incubation period, suggesting the potential benefit of this compound for sustained release effect.

3. Conclusions

Fatty acyl amide derivatives of doxorubicin were synthesized by coupling of 4′-amino group with the fatty acids and were found to be more lipophilic when compared to doxorubicin. In general, the fatty acyl amide derivatives exhibited more anti-proliferative activity in ovarian and colon cell lines when compared to leukemia and breast cancer cells. The overall pattern for the synthesized derivatives for anti-proliferation activity in different cell lines showed that dodecanoyl derivative was the most effective in *in vitro* studies. This compound may have potential application for slow delivery of doxorubicin. Further optimization will be required to generate the lead compounds with optimal anticancer activity. Future studies on designing fatty acyl conjugates of doxorubicin should be also directed on conjugation of other functional groups.

4. Experimental Protocols

4.1. Materials and methods

Doxorubicin was purchased from EuroAsian Chemical Ltd (Mumbai, India). HBTU, anhydrous dichloromethane (DCM), and other chemicals and reagents were

purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI). 12-Thioethyldodecanoic acid was synthesized from 12-bromododecanoic acid and thioethanol as described previouslyin Ref. [31]. HBTU in DCM was used as a coupling reagent. The chemical structures of final products were characterized by nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR) determined on a Varian NMR spectrometer (500 MHz). ^{13}C NMR spectra are fully decoupled. Chemical shifts were reported in parts per millions (ppm) using deuterated solvent peak or tetramethylsilane (internal) as the internal standards. The chemical structures of final products were confirmed by a high-resolution Biosystems QStar Elite time-of-flight electrospray mass spectrometer. Details of procedures and spectroscopic data of the respective compounds are presented below. Final compounds were purified on a Phenomenex Prodigy 10 µm ODS reversed-phase column (2.1 cm × 25 cm) with a Hitachi HPLC system using a gradient system of acetonitrile or methanol and water (CH_3CN or CH_3OH/H_2O, 0–100%, pH 7.0, 60 min). The purity of final products (>99%) was confirmed by analytical HPLC. The analytical HPLC was performed on a Hitachi analytical HPLC system using a C18 Shimadzu Premier 3 µm column (150 cm × 4.6 mm) and a gradient system, and a flow rate of 1 mL/min with detection at 490 nm.

4.2. Chemistry

4.2.1. General synthesis

Fatty acyl amide derivatives of doxorubicin (**2-10**) were synthesized by coupling reaction of doxorubicin and the fatty acids in presence of HBTU. All derivatives were synthesized using a similar procedure. As a representative example the synthesis of tetradecanoyl amide derivative is described here. Doxorubicin (50 mg, 0.09 mmol) was suspended in dry DCM (10 mL). Myristic acid (20.9 mg, 0.09 mmol) and HBTU (115 mg, 0.30 mmol) in dry DCM (20 mL) were added slowly to the reaction mixture. Next, DIPEA (100 mg, 0.77 mmol) was added to the reaction mixture at room temperature. The mixture was stirred for 3 h under nitrogen atmosphere. After completion of the reaction (TLC product R_f = 0.8, doxorubicin R_f = 0.1 in DCM/Methanol (8:2 v/v)), water (50 mL) was added to the mixture and the crude product was extracted with DCM (3 x 40 mL). After removal of DCM under reduced pressure, the crude product was purified by column chromatography over silica gel using DCM/methanol (0-20%) as the eluents to afford product (63 mg, 90.8%)**.** The product was further purified on a reverse phase HPLC using C18 column and gradient methanol/water as mobile phase as described above.

4.2.1.1. (8S,10S)-10-(4-(N-acetylamido)-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (2). Red brown solid (50 mg, 92.8% yield). IR (cm^{-1}, ATR): 2969, 1718 (C=O), 1642 (C=O str, amide), 1611, 1573, 1404, 1373, 1279, 1231, 1200, 1110, 1072, 1013; UV/vis λ (nm): 496, 479, 289, 249, 229; ^{1}H NMR (500 MHz, $CDCl_3$ + CD_3OD, δ ppm): 7.65 (s, 1H, ArC_2H), 7.64 (s, 1H, ArC_3H), 7.38 (s, 1H, ArC_4H), 5.48 (d, 1H, *J* = 5.6 Hz, H-2′), 5.35 (s, 1H, H-5′), 4.78 (s, 2H, $O{=}C_{13}$-CH_2), 4.16-4.25 (m,

2H, H-10 and H-C4′), 4.09 (s, 3H, OC*H*$_3$), 3.60 (s, 1H, H-6′), 3.32 (s, 1H, H-9eq), 2.95 (d, 1H, *J* = 14.5 Hz, H-9ax), 2.22-2.35 (m, 1H, H-3′eq), 1.85-2.18 (m, 5H, H-3′ax, H-7ax, O=C-C*H*$_3$), 1.69-1.73 (m, 1H, H-7eq), 1.26 (d, 3H, *J* = 4.8 Hz, 6′-C*H*$_3$); ^{13}CNMR (125 MHz, $CDCl_3$ + CD_3OD, δ ppm): 214.20 (C_{13}=O), 187.67 (C_{12}=O), 187.21 (C_5=O), 171.32 (NH-C=O), 161.45 (C1), 156.42 (C6), 155.72 (C11), 136.37 (C3), 135.84 (C10a), 134.45 (C4a), 134.19 (C6a), 121.18 (C12a), 120.20 (C2), 119.07 (C4), 111.96 (C11a), 111.70 (C5a), 101.13 (C2′), 76.69 (C8), 69.88 (C6′), 69.06 (C5′), 67.78 (C10), 65.51 (O=*C*-*C*H_2OH), 56.94 (O*C*H_3), 45.83 (NH-C4′), 36.34 (C3′), 33.94 (C9), 29.94 (C7), 22.87 (O=C-*C*H_3), 16.99 (6′-*C*H_3); HR-MS (ESI-TOF): calcd. for $C_{29}H_{31}NO_{12}$ 585.1846; found, 624.0463 $[M + K]^+$.

4.2.1.2. (8S,10S)-10-(4-(N-Octoylamido)-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (3). Red brown solid (57 mg, 92.5% yield). IR (cm^{-1}, ATR): 3346. 2923, 2848, 1716 (C=O), 1642 (C=O str, amide), 1618, 1575, 1445, 1407, 1373, 1279, 1233, 1203, 1109, 1068, 1015, 844, 764; UV/vis λ (nm): 478, 290, 254, 234; ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 13.98 (s, 1H, PhO*H*), 13.25 (s, 1H, PhO*H*), 8.04 (d, *J* = 7.7 Hz, 1H, ArC_2H), 7.79 (t, 1H, *J* = 7.8 Hz, ArC_3H), 7.39 (d, 1H, *J* = 8.5 Hz, ArC_4H), 7.26 (s, 1H, NH), 5.80 (d, 1H, *J* = 8.2 Hz, O*H*), 5.50 (d, 1H, *J* = 3.3 Hz, H-2′), 5.28 (s, 1H, H-5′), 4.76 (s, 2H, O=C_{13}-C*H*$_2$), 4.14-4.19 (m, 2H, H-10 and H-4′), 4.08 (s, 3H, OC*H*$_3$), 3.63 (s, 1H, H-6′), 3.28 (d, 1H, *J* =12.8 Hz, H-9eq), 3.02 (d, 1H, *J* =18.8 Hz, H-9ax), 2.33 (d, 1H, *J* =14.5 Hz, H-3′eq), 2.17 (dd, *J* = 14.6 Hz, *J* = 3.8 Hz, 1H, H-3′ax),

2.10-2.14 (m, 2H, O=C-C*H*$_2$), 1.87 (dd, 1H, *J* = 13.5 Hz, *J* = 5.1 Hz, H-7ax), 1.72-1.77 (m, 1H, H-7eq), 1.56 (t, 2H, *J* = 6.5 Hz, NHCOCH$_2$C*H*$_2$), 1.29 (d, 3H, *J* = 6.5 Hz, 6′-C*H*$_3$), 1.25 (br s, 8H, 4 x C*H*$_2$, methylene envelope), 0.85 (t, 3H, *J* = 5.9 Hz, C*H*$_3$); ^{13}CNMR (125 MHz, $CDCl_3$, δ ppm): 213.94 (C_{13}=O), 187.10 (C_{12}=O), 186.65 (C_5=O), 172.61 (NH-*C*=O), 161.01 (C1), 156.01 (C6), 155.64 (C11), 135.77 (C3) 135.48 (C10a), 133.61 (C4a), 133.55 (C6a), 120.83 (C12a), 119.86 (C2), 118.41 (C4), 111.57 (C11a), 111.37 (C5a), 100.70 (C2′), 76.53 (C8), 69.66 (C6′), 69.63 (C5′), 67.21 (C10), 65.56 (O=*C*-*C*H$_2$OH), 56.66 (O*C*H$_3$), 44.99 (NH-C4′), 36.76 (NHCO*C*H$_2$), 35.71 (C3′), 33.95 (C9), 29.98 (C7), 31.63, 29.16, 28.98 (methylene carbons), 25.68 (NHCOCH$_2$*C*H$_2$), 22.58 (*C*H$_2$CH$_3$), 16.83 (6′-*C*H$_3$) 14.04 (*C*H$_3$); HR-MS (ESI-TOF): calcd. for $C_{35}H_{43}NO_{12}$ 669.2785; found, 692.1190 $[M + Na]^+$.

4.2.1.3. (8S,10S)-10-(4-(N-Decylamido)-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (4). Red brown solid (60 mg, 93.6% yield). IR (cm^{-1}, ATR): 3346, 2923, 2848, 1719 (C=O), 1644 (C=O str, amide), 1614, 1575, 1445, 1407, 1280, 1205, 1017, 980; UV/vis λ (nm): 494, 477, 288, 250, 229; ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 13.92 (s, 1H, PhO*H*), 13.18 (s, 1H, PhO*H*), 7.99 (d, *J* = 7.7 Hz, 1H, ArC$_2$H), 7.74 (t, 1H, *J* = 8.2 Hz, ArC$_3$H), 7.35 (d, 1H, *J* = 8.5 Hz, ArC$_4$H), 7.24 (s, 1H, N*H*), 5.85 (d, 1H, *J* = 4.2 Hz, O*H*), 5.46 (d, 1H, *J* = 3.7 Hz, H-2′), 5.22 (s, 1H, H-5′), 4.73 (s, 2H, O=C$_{13}$-C*H*$_2$), 4.12-4.16 (m, 2H, H-10 and H-4′), 4.04 (s, 3H, OC*H*$_3$), 3.61 (s, 1H, H-6′), 3.23 (d, 1H, *J* =18.9 Hz, H-9eq), 2.96 (d, 1H, *J* = 18.9 Hz, H-9ax), 2.30 (d, 1H, *J* =

14.4 Hz, H-3′eq), 2.15 (d, *J* = 3.9 Hz, 1H, H-3′ax), 2.07-2.12 (m, 2H, O=C-C*H*$_2$), 1.87 (dd, 1H, *J* = 13.5 Hz, *J* = 5.0 Hz, H-7ax), 1.70-1.76 (m, 1H, H-7eq), 1.52 (t, 2H, *J* = 7.0 Hz, NHCOCH$_2$C*H*$_2$), 1.25 (d, 3H, *J* = 6.5 Hz, 6′-CH$_3$), 1.22 (br s, 12H, 6 x CH$_2$, methylene envelope), 0.80-0.86 (m, 3H, C*H*$_3$); ^{13}CNMR (125 MHz, $CDCl_3$, δ ppm): 213.54 (C_{13}=O), 186.65 (C_{12}=O), 186.21 (C_5=O), 172.32 (NH-*C*=O), 160.61 (C1), 155.80 (C6), 155.23 (C11), 135.35 (C3), 135.08 (C10a), 133.23 (C4a), 133.17 (C6a), 120.44 (C12a), 119.45 (C2), 118.02 (C4), 111.15 (C11a), 110.96 (C5a), 100.29 (C2′), 76.14 (C8), 69.24 (C6′), 69.22 (C5′), 66.84 (C10), 65.16 (O=*C*-*C*H$_2$OH), 56.26 (O*C*H$_3$), 44.67 (NH-C4′), 36.35 (NHCO*C*H$_2$), 35.32 (C3′), 33.55 (C9), 29.32 (C7), 31.43, 29.01, 28.97, 28.92, 28.85, 28.82, (methylene carbons), 25.30 (NHCOCH$_2$*C*H$_2$), 22.24 (*C*H$_2$CH$_3$), 16.43 (6′-*C*H$_3$) 13.69 (*C*H$_3$); HR-MS (ESI-TOF): calcd. for $C_{37}H_{47}NO_{12}$ 697.3098; found, 720.1878 $[M + Na]^+$.

4.2.1.4. (8S,10S)-10-(4-(N-Dodecanoylamido)-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (5). Red brown solid (61 mg, 91.4% yield). IR (cm^{-1}, ATR): 3346, 2923, 2848, 1719 (C=O), 1644 (C=O str, amide), 1614, 1575, 1445, 1407, 1280, 1205, 1017, 980; UV/vis λ (nm): 494, 477, 289, 249, 229; ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 13.94 (s, 1H, PhO*H*), 13.19 (s, 1H, PhO*H*), 8.01 (d, *J* = 7.6 Hz, 1H, ArC$_2$H), 7.77 (t, 1H, *J* = 8.3 Hz, ArC$_3$H), 7.37 (d, 1H, *J* = 8.4 Hz, ArC$_4$H), 7.26 (s, 1H, N*H*), 5.86 (d, 1H, *J* = 8.0 Hz, O*H*), 5.44-5.50 (m, 1H, H-2′), 5.23 (s, 1H, H-5′), 4.75 (s, 2H, O=C$_{13}$-C*H*$_2$), 4.14-4.16 (m, 2H, H-10 and H-4′), 4.06 (s, 3H, OC*H*$_3$), 3.62 (s, 1H, H-

6′), 3.23 (d, 1H, *J* = 18.9 Hz, H-9eq), 2.96 (d, 1H, *J* =18.7 Hz, H-9ax), 2.32 (d, 1H, *J* =14.0 Hz, H-3′eq), 2.17 (d, *J* = 3.1 Hz, 1H, H-3′ax), 2.09-2.12 (m, 2H, O=C-C*H*$_2$), 1.84 (dd, 1H, *J* = 13.3 Hz, *J* = 5.1 Hz, H-7ax), 1.72-1.78 (m, 1H, H-7eq), 1.54 (d, 2H, *J* = 6.5 Hz, NHCOCH$_2$C*H*$_2$), 1.25 (d, 3H, *J* = 6.5 Hz, 6′-C*H*$_3$), 1.25 (br s, 16H, 8 x CH$_2$, methylene envelope), 0.84-0.87 (m, 3H, C*H*$_3$); ^{13}CNMR (125 MHz, $CDCl_3$, δ ppm): 213.90 (C$_{13}$=O), 186.99 (C$_{12}$=O), 186.54 (C$_5$=O), 172.61 (NH-*C*=O), 160.98 (C1), 156.17 (C6), 155.59 (C11), 135.72 (C3) 135.44 (C10a), 133.59 (C4a), 133.58 (C6a), 120.80 (C12a), 119.82 (C2), 118.40 (C4), 111.52 (C11a), 111.33 (C5a), 100.69 (C2′), 76.51 (C8), 69.64 (C6′), 69.60 (C5′), 67.22 (C10), 65.53 (O=*C*-*C*H$_2$OH), 56.64 (O*C*H$_3$), 45.01 (NH-C4′), 36.74 (NHCO*C*H$_2$), 35.68 (C3′), 33.91 (C9), 29.95 (C7), 31.87, 29.68, 29.59, 29.55, 29.44, 29.32, 29.29, 29.22 (methylene carbons), 25.67 (NHCOCH$_2$*C*H$_2$), 22.64 (*C*H$_2$CH$_3$), 16.81 (6′-*C*H$_3$), 14.08 (*C*H$_3$); HR-MS (ESI-TOF): calcd. for $C_{39}H_{51}NO_{12}$ 725.3411; found, 748.2137 $[M + Na]^+$ and 764.1691 $[M + K]^+$.

4.2.1.5. (8S,10S)-10-(4-(N-Tetradecanoylamido)-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (6). Red brown solid (63 mg, 90.8% yield)**.** IR (cm^{-1}, ATR): 3346, 2923, 2848, 1719 (C=O), 1644 (C=O str, amide), 1614, 1575, 1445, 1407, 1280, 1205, 1017, 980; UV/vis λ (nm): 494, 477, 289, 249, 229; ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 13.92 (s, 1H, PhO*H*), 13.18 (s, 1H, PhO*H*), 7.99 (d, *J* = 7.6 Hz, 1H, ArC$_2$H), 7.73 (t, 1H, *J* = 8.3 Hz, ArC$_3$H), 7.34 (d, 1H, *J* = 8.4 Hz, ArC$_4$H), 7.24 (s, 1H, N*H*), 5.85 (d, 1H, *J* = 8.4 Hz, O*H*), 5.46 (d, 1H, *J* = 3.7 Hz, H-2′), 5.22 (s, 1H, H-5′), 4.73

(s, 2H, O=C_{13}-C*H_2*), 4.12-4.16 (m, 2H, H-10 and H-4′), 4.04 (s, 3H, OC*H_3*), 3.61 (s, 1H, H-6′), 3.22 (d, 1H, *J* = 18.9 Hz, H-9eq), 2.95 (d, 1H, *J* =18.7 Hz, H-9ax), 2.30 (d, 1H, *J* = 14.4 Hz, H-3′eq), 2.15 (d, *J* = 3.1 Hz, 1H, H-3′ax), 2.06-2.13 (m, 2H, O=C-C*H_2*), 1.82 (dd, 1H, *J* = 13.3 Hz, *J* = 5.1 Hz, H-7ax), 1.68-1.77 (m, 1H, H-7eq), 1.49-1.55 (m, 2H, $NHCOCH_2CH_2$), 1.25 (d, 3H, *J* = 6.5 Hz, 6′-CH_3), 1.23 (br s, 20H, 10 x CH_2, methylene envelope), 0.80-0.88 (m, 3H, Hz, C*H_3*); ^{13}CNMR (125 MHz, $CDCl_3$, δ ppm): 213.56 (C_{13}=O), 186.68 (C_{12}=O), 186.22 (C_5=O), 172.20 (NH-*C*=O), 160.61 (C1), 155.81 (C6), 155.25 (C11), 135.37 (C3) 135.07 (C10a), 133.25 (C4a), 133.17 (C6a), 120.46 (C12a), 119.46 (C2), 118.00 (C4), 111.16 (C11a), 110.97 (C5a), 100.29 (C2′), 76.17 (C8), 69.26 (C6′), 69.24 (C5′), 66.83 (C10), 65.18 (O=*C*-*C*H_2OH), 56.26 (O*C*H_3), 44.60 (NH-C4′), 36.40 (NHCO*C*H_2), 35.34 (C3′), 33.57 (C9), 29.61 (C7), 31.53, 29.29, 29.25, 29.29, 29.44, 29.08, 28.85, 28.87 (methylene carbons), 25.33 ($NHCOCH_2CH_2$), 22.31 (*C*H_2CH_3), 16.44 (6′-*C*H_3), 13.74 (*C*H_3); HR-MS (ESI-TOF): calcd. for $C_{41}H_{55}NO_{12}$ 753.3724; found, 776.2407 $[M + Na]^{+\cdot}$.

4.2.1.6. (8S,10S)-10-(4-(N-(12-Thioethyldodecanoylamido))-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (7). Red brown solid (66 mg, 91.4% yield)**.** IR (cm^{-1}, ATR): 3346, 2923, 2848, 1716 (C=O), 1642 (C=O str, amide), 1618, 1575, 1445, 1407, 1373, 1279, 1233, 1203, 1109, 1068, 1015, 844, 764; UV/vis λ (nm): 478, 290, 254, 234; ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 13.94 (s, 1H, PhO*H*), 13.20 (s, 1H, PhO*H*), 8.00 (d, *J* = 7.6 Hz, 1H, ArC_2H), 779 (t, 1H, *J* = 7.8 Hz, ArC_3H), 7.36 (d, 1H, *J* =

8.1 Hz, ArC_4H), 7.24 (s, 1H, N*H*), 5.84 (d, 1H, *J* = 7.4 Hz, O*H*), 5.50 (s, 1H, H-2′), 5.23 (s, 1H, H-5′), 4.74 (s, 2H, O=C_{13}-C*H_2*), 4.12-4.15 (m, 2H, H-10 and H-4′), 4.05 (s, 3H, OC*H_3*), 3.60 (s, 1H, H-6′), 3.24 (d, 1H, *J* = 18.1 Hz, H-9eq), 2.97 (d, 1H, *J* = 17.7 Hz, H-9ax), 2.47-2.48 (m, 4H, SC*H_2*), 2.30 (d, 1H, *J* = 14.7 Hz, H-3′eq), 2.14-2.19 (m, 1H, H-3′a), 2.07-2.12 (m, 2H, O=C-C*H_2*), 1.87 (s, 1H, H-7ax), 1.69-1.75 (m, 1H, H-7eq), 1.56 (br s, 4H, $NHCOCH_2CH_2$, SCH_2CH_2), 1.29 (s merged, 3H, 6′-C*H_3*), 1.23 (br s, 14H, 7 x CH_2, methylene envelope), 0.85 (t, 3H, *J* = 5.9 Hz, C*H_3*); ^{13}CNMR (125 MHz, $CDCl_3$, δ ppm): 213.94 (C_{13}=O), 187.10 (C_{12}=O), 186.65 (C_5=O), 171.91 (NH-*C*=O), 161.01 (C1), 156.01 (C6), 155.64 (C11), 135.77 (C3) 135.48 (C10a), 133.61 (C4a), 133.55 (C6a), 120.83 (C12a), 119.86 (C2), 118.41 (C4), 111.57 (C11a), 111.37 (C5a), 100.70 (C2′), 76.37 (C8), 69.37 (C6′), 69.21 (C5′), 66.83 (C10), 65.17 (O=*C*-*C*H_2OH), 56.27 (O*C*H_3), 43.37 (NH-C4′), 36.36 ($NHCOCH_2$), 35.33 (C3′), 33.07 (C9), 29.32 (C7), 31.53, 31.27, 29.57, 29.27, 29.23, 29.12, 29.04, 28.98, 28.91, 28.83, 28.54, 27.63 (methylene carbons), 25.52 ($NHCOCH_2CH_2$), 22.90 (CH_2CH_3), 22.31 (SCH_2), 18.02 (6′-*C*H_3) 13.74 (*C*H_3); HR-MS (ESI-TOF): calcd. for $C_{41}H_{55}NO_{12}S$ 785.3445; found, 824.1986 $[M + K]^+$.

4.2.1.7. (8S,10S)-10-(4-(N-Hexadecanoylamido)-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (8). Red brown solid (66 mg, 91.8% yield). IR (cm^{-1}, ATR): 3346. 2923, 2848, 1716 (C=O), 1642 (C=O str, amide), 1618, 1575, 1445, 1407, 1373, 1279, 1233, 1203, 1109, 1068, 1015, 844, 764; UV/vis λ (nm): 478, 290, 254, 234; ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 13.98 (s, 1H, PhO*H*), 13.28 (s, 1H, PhO*H*),

8.06 (d, *J* = 7.8 Hz, 1H, ArC_2H), 7.80 (t, 1H, *J* = 8.1 Hz, ArC_3H), 7.40 (d, 1H, *J* = 8.6 Hz, ArC_4H), 7.26 (s, 1H, N*H*), 5.76 (d, 1H, *J* = 8.5 Hz, O*H*), 5.50 (d, 1H, *J* = 3.0 Hz, H-2′), 5.30 (s, 1H, H-5′), 4.76 (s, 2H, $O=C_{13}$-C*H*$_2$), 4.19-4.13 (m, 2H, H-10 and H-4′), 4.09 (s, 3H, OC*H*$_3$), 3.64 (s, 1H, H-6′), 3.30 (d, 1H, *J* = 19.2 Hz, H-9eq), 3.05 (d, 1H, *J* = 20.1 Hz, H-9ax), 2.34 (d, 1H, *J* = 13.4 Hz, H-3′eq), 2.18 (dd, *J* =14.8 Hz, *J* = 3.7 Hz, 1H, H-3′ax), 2.10-2.13 (m, 2H, O=C-C*H*$_2$), 1.87 (dd, 1H, *J* = 13.7 Hz, *J* = 4.5 Hz, H-7ax), 1.71-1.77 (m, 1H, H-7eq), 1.54-1.62 (m, 2H, $NHCOCH_2CH_2$), 1.29 (d, 3H, *J* = 5.3 Hz, 6′-C*H*$_3$), 1.23-1.25 (br s, 24H, 12 x CH_2, methylene envelope), 0.86-0.89 m, 3H, C*H*$_3$); ^{13}CNMR (125 MHz, $CDCl_3$, δ ppm): 213.94 (C_{13}=O), 187.10 (C_{12}=O), 186.65 (C_5=O), 172.61 (NH-*C*=O), 161.01 (C1), 156.01 (C6), 155.64 (C11), 135.77 (C3) 135.48 (C10a), 133.61 (C4a), 133.55 (C6a), 120.83 (C12a), 119.46 (C2), 118.00 (C4), 111.16 (C11a), 110.97 (C5a), 104.60 (C2′), 76.37 (C8), 69.26 (C6′), 69.24 (C5′), 66.83 (C10), 65.18 (O=*C*-*C*H_2OH), 56.66 (O*C*H_3), 44.60 (NH-C4′), 36.38 (NHCO*C*H_2), 33.56 (C3′), 33.09 (C9), 29.58 (C7), 33.07, 33.04, 31.54, 31.53, 29.32, 29.28, 29.26, 29.24, 29.08, 29.04, 28.98, 28.96, 28.85 (methylene carbons), 25.31 ($NHCOCH_2CH_2$), 22.90 (*C*H_2CH_3), 16.44 (6′-*C*H_3) 13.74 (*C*H_3); HR-MS (ESI-TOF): calcd. for $C_{43}H_{59}NO_{12}$ 781.4037; found, 804.2106 $[M + Na]^+$.

4.2.1.8. (8S,10S)-10-(4-(N-Octadecanoylamido)-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (9). Red brown solid (65 mg, 87.3% yield)**.** IR (cm^{-1}, ATR): 3346. 2923, 2848, 1716 (C=O), 1642 (C=O str, amide), 1618, 1575, 1445, 1407,

1373, 1279, 1233, 1203, 1109, 1068, 1015, 844, 764; UV/vis λ (nm): 478, 290, 254, 234; ^{1}H NMR (500 MHz, $CDCl_3$, δppm): 13.93 (s, 1H, PhO*H*), 13.18 (s, 1H, PhO*H*), 8.00 (d, *J* = 7.7 Hz, 1H, ArC_2H), 7.76 (t, 1H, *J* = 8.1 Hz, ArC_3H), 7.37 (d, 1H, *J* = 8.5 Hz, ArC_4H), 7.26 (s, 1H, N*H*), 5.81 (s, 1H, O*H*), 5.48 (br s, 1H, H-2′), 5.26 (s, 1H, H-5′), 4.76 (s, 2H, $O{=}C_{13}$-C*H*$_2$), 4.14-4.19 (m, 2H, H-10 and H-4′), 4.06 (s, 3H, OC*H*$_3$), 3.67 (s, 1H, H-6′), 3.24 (d, 1H, *J* = 18.6 Hz, H-9eq), 2.94-3.02 (m, 1H, H-9ax), 2.32 (d, 1H, *J* = 13.8 Hz, H-3′eq), 2.17 (d, 1H, *J* = 3.8 Hz, H-3′ax), 2.10-2.13 (m, 2H, O=C-C*H*$_2$), 1.87 (dd, 1H, *J* = 14.2 Hz, *J* = 6.2 Hz, H-7ax), 1.73-1.79 (m, 1H, H-7eq), 1.55 (t, 2H, *J* = 6.5 Hz, $NHCOCH_2CH_2$), 1.28 (d, 3H, *J* = 6.6 Hz, 6′-C*H*$_3$), 1.25 (br s, 28H, 14 x CH_2, methylene envelope), 0.86-0.89 (m, 3H, C*H*$_3$); ^{13}CNMR (125 MHz, $CDCl_3$, δ ppm): 213.94 (C_{13}=O), 186.99 (C_{12}=O), 186.55 (C_5=O), 172.88 (NH-*C*=O), 160.97 (C1), 156.21 (C6), 155.58 (C11), 135.74 (C3), 135.43 (C10a), 133.64 (C4a), 133.61 (C6a), 120.78 (C12a), 119.83 (C2), 118.41 (C4), 111.50 (C11a), 111.37 (C5a), 100.64 (C2'), 76.49 (C8), 69.60 (C6′), 69.56 (C5′), 67.25 (C10), 65.55 (O=*C*-*C*H_2OH), 56.63 (O*C*H_3), 45.12 (NH-C4′), 36.75 (NHCO*C*H_2), 35.72 (C3′), 33.46 (C9), 29.93 (C7), 31.92, 29.74, 29.70, 29.65, 29.64, 29.63, 29.60, 29.59, 29.48, 29.46, 29.43, 29.36, 29.26, 29.24, 29.06 (methylene carbons), 25.71 ($NHCOCH_2CH_2$), 22.69 (*C*H_2CH_3), 16.82 (6′-*C*H_3), 14.12 (*C*H_3); HR-MS (ESI-TOF): calcd. for $C_{45}H_{63}NO_{12}$ 809.4350; found, 832.2217 $[M + Na]^+$.

4.2.1.9. (8S,10S)-10-(4-(N-Icosanoylamido)-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (10). Red brown solid (68 mg, 88.2% yield)**.** IR (cm^{-1},

ATR): 3346, 2923, 2848, 1716 (C=O), 1642 (C=O str, amide), 1618, 1575, 1445, 1407, 1373, 1279, 1233, 1203, 1109, 1068, 1015, 844, 764; UV/vis λ (nm): 478, 290, 254, 234; ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 13.98 (s, 1H, PhO*H*), 13.24 (s, 1H, PhO*H*), 8.04 (d, *J* = 5.9 Hz, 1H, ArC_2H), 7.79 (t, 1H, *J* = 6.2 Hz, ArC_3H), 7.39 (d, 1H, *J* = 6.7 Hz, ArC_4H), 7.27 (s, 1H, N*H*), 5.82 (d, 1H, *J* = 6.4 Hz, O*H*), 5.50 (br s, 1H, H-2′), 5.28 (s, 1H, H-5′), 4.76 (s, 2H, $O{=}C_{13}$-C*H*$_2$), 4.17-4.18 (m, 2H, H-10 and H-4′), 4.07 (s, 3H, O*C*H_3), 3.63 (s, 1H, H-6′), 3.28 (d, 1H, *J* = 18.5 Hz, H-9eq), 3.01 (d, 1H, *J* = 19.5 Hz, H-9ax), 2.33 (d, 1H, *J* = 14.9 Hz, H-3′eq), 2.17 (m, 1H, H-3′ax), 2.10-2.13 (m, 2H, O=C-C*H*$_2$), 1.84-1.87 (m, 1H, H-7ax), 1.72-1.77 (m, 1H, H-7eq), 1.54 (t, 2H, *J* = 6.0 Hz, $NHCOCH_2C$*H*$_2$), 1.29 (d, 3H, *J* = 6.2 Hz, 6′-C*H*$_3$), 1.25 (br s, 32H, 16 x CH_2, methylene envelope), 0.89 (t, 3H, *J* = 5.1 Hz, C*H*$_3$); ^{13}CNMR (125 MHz, $CDCl_3$, δ ppm): 213.94 (C=O), 187.10 (C_{12}=O), 186.65 (C_5=O), 172.61 (NH-*C*=O), 160.61 (C1), 155.80 (C6), 155.26 (C11), 135.37 (C3) 135.11 (C10a), 133.22 (C4a), 133.14 (C6a), 120.83 (C12a), 119.47 (C2), 118.00 (C4), 111.19 (C11a), 111.00 (C5a), 100.30 (C2'), 76.14 (C8), 69.28 (C6′), 69.24 (C5′), 66.81 (C10), 65.17 (O=*C*-*C*H_2OH), 56.33 (O*C*H_3), 44.58 (NH-C4′), 36.38 (NHCO*C*H_2), 35.32 (C3′), 33.59 (C9), 29.59 (C7), 33.07, 31.53, 29.30, 98.08, 29.03, 28.97, 28.85 (methylene carbons), 25.30 ($NHCOCH_2$*C*H_2), 22.90 (*C*H_2CH_3), 16.44 (6′-*C*H_3) 13.74 (*C*H_3); HR-MS (ESI-TOF): calcd. for $C_{47}H_{67}NO_{12}$ 837.4663; found 860.2712 $[M + Na]^+$.

4.2.2. Partition coefficient

Partition coefficients of doxorubicin derivatives were determined using n-octanol/water distribution shake method. In a typical method 100 µg of the doxorubicin derivative was partitioned between 300 µL of 1-octanol and 300 µL of distilled water. The mixture was shaken for 30 min and the organic and aqueous phases were allowed to separate. The concentration of the doxorubicin derivatives was measured in both the phases using UV spectroscopy by comparing with a standard solution UV spectrum. The experiment was repeated three times and average of the reading was taken. The partition coefficient of the sample was then determined by the following equation:

$$\frac{\text{Concentration of sample in n-octanol}}{\text{Concentration of sample in water}} = P$$

and represented as Log *P* where *P* is partition coefficient.

4.3. Cell culture

Human leukemia cell line CCRF-CEM (ATCC no. CCL-119), breast adenocarcinoma MDA-MB-468 (ATCC no. HTB-132), ovarian adenocarcinoma SK-OV-3 (ATCC no. HTB-77), and colon adenocarcinoma HT-29 (ATCC no. HTB-38) were obtained from American Type Culture Collection. Cells were grown on 75 cm^2 cell culture flasks with RPMI-16 medium for leukemia and EMEM medium for other cell lines and supplemented with 10% Fetal bovine serum (FBS), and 1% penicillin-streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 0.9 % NaCl) in a humidified atmosphere of 5% CO_2, 95% air at 37 ºC.

4.4. Cell proliferation assay

Cell proliferation assay of doxorubicin derivatives synthesized was evaluated in MDA-MB-468, CCRF-CEM, SK-OV-3, and HT-29 cells, and was compared with that of doxorubicin. Cell proliferation assay was carried out using CellTiter 96 aqueous one solution cell proliferation assay kit (Promega, USA). As a representative example, CCRF-CEM cells were suspended at 5 × 10^5/mL and 100 µL of the cell suspension was placed in each well of the 96 well culture plate. Cells were incubated with doxorubicin and its derivatives (1 µM) in 4% DMSO and tested in triplicate. Incubation was carried out at 37 ºC in an incubator supplied with 5% CO_2 for 24-120 h. At the end of the sample exposure period (24-120 h), 20 µL CellTiter 96 aqueous solution was added. The plate was returned to the incubator for 1 h in a humidified atmosphere at 37 ºC. The absorbance of the formazan product was measured at 490 nm using microplate reader. The percentage of cell survival was calculated as OD value of cells treated with test compound – OD value of culture medium/(OD value of control cells – OD value of culture medium) × 100%.

Supplementary Information

Supplementary data include ^{1}H NMR, ^{13}C NMR, DEPT, HSQC, COSY and analytical HPLC for representative compounds.

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