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Research paper

Structure–activity relationship study of anticancer thymidine–quinoxaline conjugates under the low radiance of long wavelength ultraviolet light for photodynamic therapy

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ABSTRACT

Thymidine quinoxaline conjugate (dT-QX) is a thymidine analog with selective cytotoxicity against different cancer cells. In this study, the structure activity relationship study of dT-QX analogs was carried out under the low radiance of black fluorescent (UVA-1) light. Significantly enhanced cytotoxicity was observed under UVA-1 activation among analogs containing both thymidine and quinoxaline moieties with different length of the linker, stereochemical configuration and halogenated substituents. Among these analogs, the thymidine dichloroquinoxaline conjugate exhibited potent activity under UVA-1 activation as the best candidate with EC_{50} at 0.67 μM and 1.3 μM against liver and pancreatic cancer cells, respectively. In contrast, the replacement of thymidine moiety with a galactosyl residue or the replacement of quinoxaline moiety with a fluorescent pyrenyl residue or a simplified diketone structure resulted in the full loss of activity. Furthermore, it was revealed that the low radiance of UVA-1 at 3 mW/cm^2 for 20 min was sufficient enough to induce the full cytotoxicity of thymidine dichloroquinoxaline conjugate and that the cytotoxic mechanism was achieved through a rapid and steady production of reactive oxygen species.

1. Introduction

Photodynamic therapy (PDT) of cancer is a treatment of tumor cells using external light irradiation and photosensitizers to induce *in situ* production of high levels of cytotoxic reactive oxygen species (ROS) [1,2]. PDT has been used in clinic to treat non-small lung cancer, esophageal cancer, bladder cancer and breast cancer [1–3], control cholangiocarcinoma for liver transplantation [4], and improve the survival time of cholangiocarcinoma patients in combination with anticancer drugs such as gemcitabine and/or cisplatin [5]. Porphyrin-based PDT sensitizers including protoporphyrin IX, prodrug aminolevulinic acid [1–4], chlorins [6] and phthalocyanines [7] can be activated by either blue (405 nm) or red light (625 nm) and cause effective killing of cancer cells at a radiance dose ranging between 2 and 12 J/cm^2. Although the long wavelength red light has deeper penetration into tissues than the blue light, the latter has higher absorbance efficiency and is thus, more effective at the same dose than the former [8,9]. Further functionalized porphyrin hydroxypyridinone or EDTA conjugates [10,11], porphyrin dimers [12] and a mixture of two porphyrin-based sensitizers [13] show improved activity against various cancer cells and tumor models at a low dose. Non-porphyrin based heterocyclic sensitizers including psoralen [14], 2-aryl benzothiazoles [15], furocoumarins [16], polypyridyl ruthenium complex [17], bicyclic triazoles [18], polyheteroaromatic 3B [19] and benzophenothiazinium dyes [20] are mostly activated under ultraviolet A light (UVA, 320–400 nm). On the other hand, neither porphyrin based sensitizers nor the non-porphyrin based ones are anticancer agents themselves in the absence of light radiation [1,2]. Alternatively, sensitizers with built-in selectivity toward cancer cells would have dual cytotoxic potential under PDT conditions for cancer treatment. Recently, porphyrin conjugates with tumor-specific ligands were investigated to increase selective

List of abbreviation: API, atmospheric pressure ionization; dT-QX, thymidine quinoxaline conjugate; CFL, compact fluorescent lamp; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodimimde; OHCT, oxo-2-hydroxy-cis-terpenone; PDT, photodynamic therapy; ROS, reactive oxygen species; RFI, relative fluorescence intensity; UVA, ultraviolet A light (320–400 nm); UVA-1, long wavelength ultraviolet A light (340–400 nm).

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accumulation of sensitizers in cancer cells to improve the efficacy, although further demonstration *in vivo* is needed [22]. Synthetically modifying the cytotoxic UVA fluorophores to increase their selectivity towards cancer cells, is a facile and effective approach compared to the conjugation of porphyrins with tumor-specific ligands. In addition, long wavelength UVA light irradiation (UVA-1, 340–400 nm) has clinically demonstrated high efficacy and low side effects in the treatment of systemic lupus erythematosus patients [21]. However, there are few reports of sensitizers with built-in selective anticancer activity against cancer cells [23].

We have recently developed a selective anticancer thymidine quinoxaline conjugate that showed significantly high cytotoxicity against liver, breast and brain cancer cells with EC_{50} in the range of 6–42 μM, but exhibited a low activity in the normal liver cell line at 200 μM [24]. The selectivity has been attributed to the abnormally high levels of thymidine kinase 1 that phosphorylates thymidine to the 5′-phosphate form as an additional supply to the thymidine triphosphate needed for the elevated DNA synthesis in cancer cells versus that of normal cells [25]. Unfortunately, the selective cytotoxicity was low in a different liver cancer Bel-7402 cell line that also has abnormally high levels of thymidine phosphorylase that deactivated the thymidine analogs as the catabolic pathway, and thus limiting the potential anticancer activity [25]. On the other hand, the cytotoxic chemophore quinoxaline was found to be a fluorophore under the UVA light [25–28], yet the biological potential of thymidine quinoxaline conjugates under UVA irradiation has not been reported. Therefore, the thymidine quinoxaline conjugate served as a good candidate for the investigation of sensitizers with built-in selectivity towards cancer cells. In this study, we reported the structure activity relationship study of thymidine quinoxaline conjugates (dT-QX) under the low radiance black fluorescent (UVA-1) light in two cancer cell lines that dT-QX had low activity in the dark. The low radiance of light activation has been shown to be least immunosuppressive [29] and has fewer side-effects in PDT of breast cancer patients [30]. In addition, the optimal irradiation condition and the cytotoxic mechanism were investigated.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (MO, USA), J&K Scientific Ltd. (Beijing, China), or Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) and used without further purification. NMR spectra were recorded with Bruker Avance-400 NMR spectrometer (Madison, WI, USA). Abbreviations used for the coupling patterns in proton NMR signals are: singlet (s), doublet (d), triplet (t), quartet (q), quintet (qui), multiplet (m) and broad signal (br). Atmospheric pressure ionization (API) mass analysis was carried out with a 1100 LC/MSD Trap System (Agilent Technology, Santa Clara, CA, USA), and high resolution mass analysis (HRMS) was carried out with 4800 MALDI TOF/TOF Analyzer (AB SCIEX, Framingham, MA, USA). Oxo-2-hydroxy-cis-terpenone (OHCT) and compounds **1c**, **2c**, **3c** and **C6** were synthesized and reported previously [24,31,32].

2.1. Synthesis

2.1.1. Bromo-N-(prop-2-yn-yl)alkanamides (1a–d)

To a solution of propargylamine hydrogen chloride (500 mg, 5.46 mmol) and bromoalkanoic acid (1.1 mol equiv.) in dry CH_2Cl_2 (20 mL) were added triethylamine (555 mg, 5.5 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (2.1 g, 10 mmol). The resulting suspension was stirred under N_2 at room temperature for 18 h. The reaction solution was worked up with brine (200 mL) and CH_2Cl_2 (200 mL × 3). The organic layers were collected, dried with $MgSO_4$ and concentrated. A flash chromatographic separation (0–40% EtOAc in hexanes) afforded the desired products. Because the yield was low, three to four batches of **1a**–**d** were carried out to produce enough materials that were stored at −20 °C. Only ^{1}H NMR analysis was carried out on the bromo-intermediates due to the low stability.

***1a**: colorless oil (270 mg, 23% yield).* ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 6.78 (br s, 1H; NH), 4.10 (dd, ^3J(H,H) = 3.6 Hz, ^4J(H,H) = 1.6 Hz, 2H; CH_2), 4.07 (s, 2H; CH_2), 2.28 (t, ^4J(H,H) = 1.6 Hz, 2H; CH_2) ppm as reported [33].

***1b**: colorless oil (320 mg, 28% yield).* ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.76 (br s, 1H; NH), 4.06 (dd, ^3J(H,H) = 5.2 Hz, ^4J(H,H) = 1.6 Hz, 2H; CH_2), 3.61 (t, ^3J(H,H) = 6.0 Hz, 2H; CH_2), 2.39 (t, ^3J(H,H) = 7.2 Hz, 2H; CH_2), 2.24 (t, ^3J(H,H) = 2.4 Hz, 1H; CH), 2.10–2.14 (m, 2H; CH_2) ppm; API-MS: calcd for $C_7H_{10}BrNO$ 203.0 [M^+], 205.0 [$(M + 2)^+$], found 202.9, 204.9.

***1d**: colorless oil (157 mg, 11% yield, 1:1 cis/trans amide mixture based on ^{1}H NMR analysis).* ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.66 (br s, 1H; NH), 4.07 (dd, ^3J(H,H) = 5.1 Hz, ^4J(H,H) = 1.7 Hz, 2H; CH_2), 3.55 (t, ^3J(H,H) = 5.8 Hz, 1H; 0.5CH_2), 3.42 (t, ^3J(H,H) = 5.8 Hz, 1H; 0.5CH_2), 2.26 (t, ^3J(H,H) = 2.1 Hz, 1H; CH), 2.22 (t, ^3J(H,H) = 7.1 Hz, 2H; CH_2), 1.87 (t, ^3J(H,H) = 7.3 Hz, 1H; 0.5CH_2), 1.78 (t, ^3J(H,H) = 7.5 Hz, 1H; 0.5CH_2), 1.66–1.68 (m, 2H; CH_2) 1.44–1.47 (m, 2H; CH_2), 1.34–1.37 (m, 4H; 2CH_2) ppm; API-MS: calcd for $C_{11}H_{19}BrNO$ 260.1 [$M + H^+$], 262.1 [$(M + 2)+H^+$], found 260.8, 261.8.

2.1.2. N-(Prop-2-yn-1-yl)-{[(4bS,8aR)-4b,8,8-trimethyl-9,10-dioxo-4b,5,6,7,8,8a,9,10-octahydrophenanthren-2-yl]oxy}alkanamides (2a–d)

To a solution of compound **1** (2 mmol) and OHCT (600 mg, 1.1 mol equiv.) in dry DMF (20 mL) were added anhydrous potassium carbonate (1.4 g) and silver carbonate (0.3 g). The resulting suspension was stirred under N_2 at room temperature for 18 h. The reaction solution was worked up with brine (200 mL) and CH_2Cl_2 (200 mL × 3). The organic layers were collected, dried with $MgSO_4$ and concentrated. A flash chromatographic separation (0–40% EtOAc in hexanes) afforded the desired products.

***2a**: colorless oil (378 mg, 51% yield).* ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.64 (d, ^4J(H,H) = 2.4 Hz, 1H; CH), 7.46 (d, ^3J(H,H) = 8.8 Hz, 1H; CH), 7.30 (dd, ^3J(H,H) = 8.8 Hz, ^4J(H,H) = 2.4 Hz, 1H; CH), 6.95 (br, 1H; NH), 4.60 (s, 2H, CH_2), 4.19 (t, ^3J(H,H) = 2.8 Hz, 2H; CH_2), 2.69 (s, 1H, CH), 2.57 (d, ^3J(H,H) = 14.4 Hz, 1H; CH), 2.29 (d, ^4J(H,H) = 1.6 Hz, 1H; CH), 1.35–1.63 (m, 5H; 5CH), 1.21 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.36 (s, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): 198.2, 180.9, 167.1, 156.1, 144.0, 135.0, 126.6, 122.8, 114.5, 78.9, 72.0, 68.8, 67.5, 41.9, 39.2, 35.4, 31.3, 28.8, 24.1, 18.8 ppm; API-MS: calcd for $C_{22}H_{26}NO_4$ 368.2 [$M + H^+$], found 368.1.

***2b**: colorless oil (285 mg, 36% yield).* ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.60 (d, ^4J(H,H) = 2.6 Hz, 1H; CH), 7.41 (d, ^3J(H,H) = 8.8 Hz, 1H; CH), 7.25 (dd, ^3J(H,H) = 8.8 Hz, ^4J(H,H) = 2.5 Hz, 1H; CH), 5.76 (br, 1H; NH), 4.12 (m, 4H; 2CH_2), 2.70 (s, 1H, CH), 2.58 (d, ^3J(H,H) = 14.1 Hz, 1H; CH), 2.44–2.46 (m, 2H; CH_2), 2.16–2.22 (m, 3H; CH and CH_2), 1.35–1.63 (m, 5H; 5CH), 1.22 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.43 (s, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): 198.9, 181.2, 171.7, 157.8, 142.8, 134.6, 126.1, 123.9, 112.8, 79.5, 71.7, 68.9, 67.2, 41.9, 39.2, 35.4, 32.6, 31.1, 29.3, 24.9, 18.8 ppm; API-MS: calcd for $C_{24}H_{30}NO_4$ 396.2 [$M + H^+$], found 396.1.

***2d**: colorless oil (358 mg, 40% yield).* ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.55 (d, ^4J(H,H) = 2.8 Hz, 1H; CH), 7.36 (d, ^3J(H,H) = 8.8 Hz, 1H;