Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 2974–2977

Synthesis and preliminary antitumor activity evaluation of a DHA and doxorubicin conjugate

Yuqiang Wang,[a,b,*] Lianfa Li,[b] Wei Jiang,[b] Zhaoqi Yang[a] and Zaijun Zhang[a]

[a]*Institute of New Drug Research, Jinan University College of Pharmacy, Guangzhou 510632, China*
[b]*Panorama Research Inc., 2462 Wyandotte Street, Mountain View, CA 94043, USA*

Received 30 January 2006; revised 21 February 2006; accepted 24 February 2006
Available online 24 March 2006

Abstract—A conjugate of DHA and doxorubicin (DHA–Dox) was synthesized, and its antitumor activity was evaluated in vitro against L1210 leukemia cells and in experimental animal tumor models including L1210 leukemia and B16 melanoma. DHA–Dox showed a greatly improved antitumor efficacy compared to free doxorubicin.

Cancer is the second major cause of death in the majority of the developed countries. Although there has been significant progress in the treatment of cancer during the last several years, there are still no cures for most forms of human cancer. The main reason for the difficulty in treatment of cancer is that most chemotherapeutic drugs lack specificity toward cancer, that is, while cancer cells are killed, normal tissues/cells are damaged at the same time. These side effects often limit dose intensification.[1] One strategy to circumvent this problem is to deliver the chemotherapeutic agents specifically to tumor tissue through the use of drug conjugates.

Essential fatty acids have been found to exhibit anticancer activities in vitro and in experimental animal tumor models as well as in patients with cancer. In addition, certain essential fatty acids have synergistic effects with anticancer drugs. Consumption of diets containing *n*-3 fatty acids such as *cis*-4,7,10,13,16,19-docosahexenoic acid (DHA) was found to inhibit tumorigenesis,[2,3] the growth of rodent tumors,[4] and human breast cancer xenografts.[5,6] Inhibition of tumor growth by *n*-3 fatty acids was mediated through the binding of *n*-3 fatty acids to their receptors on tumor cells, leading to a decrease in intracellular cyclic AMP via a G_i protein-coupled signal transduction pathway.[7] Experiments demonstrate that tumors take up a large proportion of certain kinds of natural fatty acids from blood for use as biochemical precursors and energy sources.[8]

Bradley et al. reported the synthesis of DHA–paclitaxel, a 2′-*O*-acyl conjugate of DHA and paclitaxel (Fig. 1), to target paclitaxel to tumors to decrease toxicity to normal tissues and increase the therapeutic index relative to free paclitaxel.[9] DHA is an ω-3 fatty acid, a constituent of cell membranes in the brain and elsewhere, and is used as a precursor for metabolic and biochemical pathways.[4] Because DHA is found in human milk, and is added to infant formula in Europe and the United States, it should not have any additional toxicity to paclitaxel.

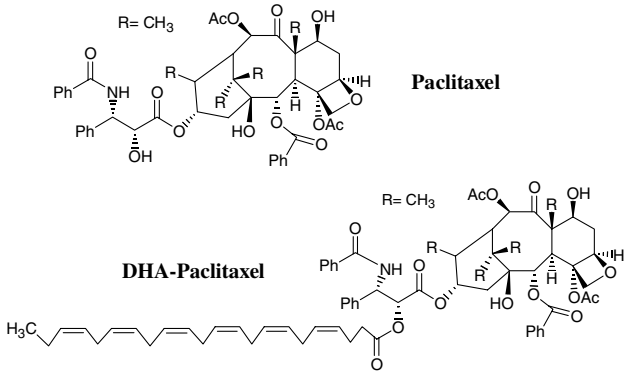


Figure 1. Structures of DHA and DHA–paclitaxel.

Keywords: Anticancer; Doxorubicin; Drug delivery; DHA.
* Corresponding author. Tel.: +1 650 694 4996; fax: +1 650 694 7717; e-mail: yuqiangwang2001@yahoo.com

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.02.066

Pharmacokinetic studies of paclitaxel and DHA–paclitaxel in normal rats suggest that most of the DHA–paclitaxel is confined within the intravascular plasma volume, whereas paclitaxel is rapidly cleared from plasma and distributed into large volumes of peripheral tissue space.[9] When paclitaxel at 20 mg/kg, DHA–paclitaxel at 27.4 mg/kg (a dose equimolar with 20 mg/kg of paclitaxel), and DHA–paclitaxel at 120 mg/kg (a dose equitoxic with 20 mg/kg of paclitaxel) were injected through the tail vein of mice bearing M109 tumors weighing approximately 100 mg, the plasma concentration of paclitaxel remained >2 μM for only 16 h. In contrast, paclitaxel derived from DHA–paclitaxel at an equitoxic dose of 120 mg/kg remained >2 μM for 10 days after injection (tumors grow at concentration below 2 μM).[9] Although less potent than free paclitaxel, DHA–paclitaxel has a significantly higher therapeutic index than free paclitaxel in mice bearing tumors. In addition, DHA–paclitaxel has decreased side effects.

We have recently reported the synthesis and antitumor efficacy evaluations of DHA–HCPT, a conjugate of DHA and 10-hydroxycamptothecin (HCPT) (Fig. 2).[10]

DHA–HCPT showed greatly improved therapeutic efficacies in tumor models tested, compared to the free HCPT.[10] For example, DHA–HCPT (ILS: 154% and two long-term survivors) was at least twice as effective as the free HCPT (ILS: 77% and no long-term survivor) in the L1210 leukemia mouse model at optimal dose. Based on these experimental results, we think that conjugating DHA to existing anticancer drugs might be a valid strategy to further improve the drug's anticancer efficacy. Herein, we report a novel conjugate of DHA and doxorubicin (Dox), DHA–Dox, (Fig. 3), in which DHA was used as a carrier to deliver Dox to tumors to increase its therapeutic index.

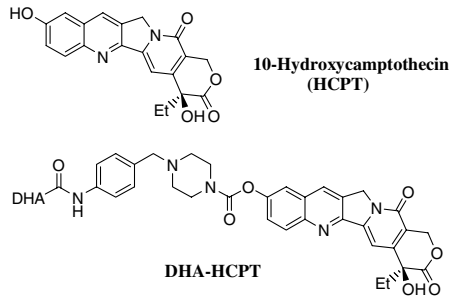


Figure 2. Structures of HCPT and DHA–HCPT.

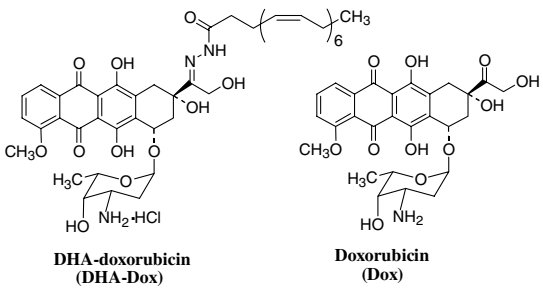
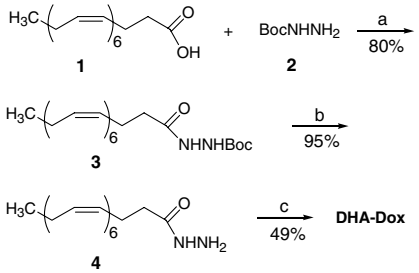


Figure 3. Structures of DHA–Dox and Dox.

Dox is one of the most widely used anticancer agents for the treatment of human cancers including leukemia, lymphoma, breast and ovarian carcinomas, and many other solid tumors.[11,12] Although Dox has been used extensively in clinics over the past three decades, its use is still limited by severe acute and chronic systemic toxicities including myelosuppression, gastrointestinal disorders, stomatitis, cumulative cardiotoxicity, and extravasation.[12] Based on the fact that conjugates of DHA with paclitaxel and HCPT have better therapeutic efficacy than their corresponding free drugs, it is reasonable to believe that a conjugate of DHA and Dox may have superior therapeutic efficacy to free Dox.

DHA–Dox is designed to be double tumor-selective. First, because of the presence of DHA, it should be selectively accumulated in tumors, leading to a selective tumor cell killing. Second, the hydrazone bond used to link DHA and Dox is expected to be selectively cleaved in tumor cells, leading to a selective toxicity to tumor cells. Like other hydrazone-linked Dox derivatives, DHA–Dox is expected to be stable at physiologic pH and is decomposed to release free Dox at a lower pH. For example, Kaneko et al.[13] reported that the hydrazone linker was almost completely broken within 4 h to release free Dox at pH 4.5 at 37 °C. In contrast, there was little decomposition within 5 h at pH 7.4 at 37 °C. The hydrazone bond has been used in Mylotarg, a clinically used drug for treatment of leukemia, and in other drugs such as the Br96–Dox conjugate and other Dox analogues.[14] Because the pH in tumors is lower than in normal tissue,[15–18] more free Dox is expected to be released from DHA–Dox in tumors than in normal tissue, leading to a selective toxicity to tumors. Because of the double tumor-selectivity, DHA–Dox is expected to be superior to free Dox.

DHA–Dox was synthesized as illustrated in Scheme 1.[19] The commercially available DHA (**1**) and Boc-protected hydrazine ($BocNHNH_2$) (**2**) were coupled in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) affording compound **3** with a 80% yield. The Boc-protected group of the latter compound was removed with gaseous hydrogen chloride in ethyl acetate affording compound **4** with a 95% yield. The targeted DHA–Dox was synthesized by treatment of Dox and



Scheme 1. Synthesis of DHA–Dox. Reagents: (a) EDCI, DMF; (b) HCl/EtOAc; (c) Dox, CH_3CN, TFA.

compound **4** in the presence of trifluoroacetic acid (TFA) in acetonitrile with a 49% yield.

The antitumor activity of DHA–Dox was first tested in vitro against L1210 leukemia cells, and the results are shown in Table 1. The IC_{50} values of DHA–Dox and free Dox are 1.4 and 0.15 μM, respectively. These results are expected because prodrugs are generally less potent than their corresponding free drugs.

DHA–Dox was then tested against L1210 leukemia in mice, and the results are shown in Table 2. At an optimal dose of 16 mg/kg, DHA–Dox produced an ILS of 107%, which is twice of that produced by the free Dox (ILS: 53% at 5 mg/kg). Furthermore, DHA–Dox (weight loss: 4%) was less toxic than Dox (weight loss: 7%) to the host animals. On a molar basis, approximately 5-fold more DHA–Dox (32 mg/kg) can be safely administered compared with the free Dox (5 mg/kg). At doses that produced the same therapeutic efficacy (ILS of 53%), DHA–Dox (8 mg/kg, weight loss: 2%) was much less toxic to the animals than the free Dox (5 mg/kg, weight loss: 7%). These results demonstrate that DHX–Dox is more efficacious than the free Dox, suggesting that the former is more tumor-selective than the latter.

The antitumor activity of DHA–Dox was then tested in mice bearing B16 melanoma, and the results are shown in Table 3. DHA–Dox was once again proven to be twice as efficacious as Dox in the B16 melanoma model. At an optimal dose of 30 mg/kg, DHA–Dox produced a 70% tumor growth inhibition (TGI), while the free Dox, at a dose of 5 mg/kg, only had a 35% TGI. Furthermore, DHA–Dox (weight loss: 6%) was much less toxic to the animals than the free Dox (weight loss: 10%). These results demonstrate, once again, that the therapeutic index of DHA–Dox is significantly higher than that of the free Dox.

We have synthesized a conjugate of DHA and Dox. In animal tumor models, DHA–Dox is significantly more efficacious than free Dox. It is not clear, however, if this increased therapeutic efficacy is due to an increased uptake of DHA–Dox by tumors, an increased half-life of DHA–Dox or other reasons. These data and those reported previously suggested that DHA may be used as a vehicle to target anticancer drugs to tumors to increase their therapeutic efficacy.

Table 1. Cytotoxicity against L1210 leukemia cells in vitro[a]

Compound	IC_{50}[b] (μM)
Dox	0.15 ± 0.01
DHA–Dox	1.4 ± 0.35

[a] The assay was set up in triplicate in 96-well flat-bottomed microtiter plates. All cells were seeded at 5000 cells/well in RPMI-1640 plus 10% FCS. Drugs were added, and the total volume was adjusted to 0.2 mL/well. Total incubation time was 48 h with the addition of ^3H thymidine for the last 24 h of incubation. The assay was harvested and radioactivity was counted.

[b] IC_{50} values are defined as the minimal drug concentration necessary to inhibit incorporation of [^3H] thymidine by 50% and are averages of three experiments.

Table 2. Antitumor activity in mice bearing L1210 leukemia[a]

Compound	Dose (μg/kg)	% weight change[b]	% ILS[c]	30-day survivors
DHA–Dox	32	−11	80	0
	16	−4	107	1
	8	−2	53	0
Dox	8	−19	53	0
	5	−7	53	0

[a] Male BDF1 mice (6/group) were injected ip with 10^5 cells on day 0. Drugs were administered ip on days 1 and 5. The median number of days of survival of the vehicle-treated mice was 7.5.

[b] Group bodyweight change between days 0 and the day at which time the group of mice had the lowest weight.

[c] % increase in life span (ILS) = $[(T/C) - 1)] \times 100$, where T and C are the median survival times of the treated groups and the control group, respectively.

Table 3. Antitumor activity in mice bearing B16 melanoma[a]

Compound	Dose (mg/kg)	% weight change[b]	Tumor weight (g)	% TGI[c]	P value[d]
Control	—	—	2.38 ± 1.21	—	—
DHA–Dox	30	−6	0.72 ± 0.34	70	<0.01
	20	−6	1.13 ± 0.46	53	<0.01
Dox	5	−10	1.54 ± 0.48	35	<0.01

[a] Male BDF_1 mice (7/group) were injected sc with 10^6 cells on day 0. Drugs were administered ip on days 1 and 5.

[b] Group bodyweight change was between days 0 and the day at which time the group of mice had the lowest weight.

[c] % tumor growth inhibition (TGI) = $[1 - (T/C)] \times 100$, where T and C are the median tumor weights of the treated groups and the control group, respectively.

[d] Comparing to the control.

References and notes

1. David, E. S.; John, E. E.; Deborah, L. G. *Tetrahedron Lett.* **1995**, *36*, 1413.
2. Yvon, A. M.; Wadsworth, P.; Jordan, M. A. *Mol. Biol. Cell* **1999**, *10*, 947.
3. Jordan, M. A.; Toso, R. J.; Thrower, D.; Wilson, L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9552.
4. Anderson, G. J.; Connor, W. E.; Corliss, J. D. *Pediatr. Res.* **1990**, *27*, 89.
5. Weisenberg, R. C. *Science* **1972**, *177*, 1104.
6. Schiff, P. B.; Horwitz, S. B. *Biochemistry* **1981**, *20*, 3247.
7. Sauer, L. A.; Dauchy, R. T.; Blask, D. E. *Cancer Res.* **2000**, *60*, 5289.
8. Sauer, L. A.; Dauchy, R. T. *Br. J. Cancer* **1992**, *66*, 297.
9. Bradley, M. O.; Webb, N. L.; Anthony, F. H.; Devanesan, P.; Witman, P. A.; Hemamalini, S.; Chander, M. C.; Baker, S. D.; He, L.; Horwitz, S. B.; Swindell, C. S. *Clin. Cancer Res.* **2001**, *7*, 3229.
10. Wang, Y.; Li, L.; Jiang, W.; Larrick, J. W. *Bioorg. Med. Chem.* **2005**, *13*, 5592.
11. Myers, C. E.; Chabner, B. A. In *Cancer Chemotherapy—Principles and Practice*; Chabner, B. A., Collins, J. M., Eds.; Lippincott: Philadephia, 1990; pp 356–381.
12. Dorr, R. T.; Von Hoff, D. D.. *Cancer Chemotherapy Handbook*, 2nd ed.; Appleton and Lange: Norwalk, CT, 1994.

13. Kaneko, T.; Willner, D.; Monkovic, I.; Knipe, J. O.; Braslawsky, G. R.; Greenfield, R. S.; Vyas, D. M. *Bioconjug. Chem.* **1991**, *2*, 133.
14. Kratz, F.; Warnecke, A.; Scheuermann, K.; Stockmar, C.; Schwab, J.; Lazar, P.; Druckes, P.; Esser, N.; Drevs, J.; Rognan, D.; Bissantz, C.; Hinderling, C.; Folkers, G.; Fichtner, I.; Unger, C. *J. Med. Chem.* **2002**, *45*, 5523.
15. Wike-Hooley, J. L.; Haveman, J.; Reinhold, H. S. *Radiother. Oncol.* **1984**, *2*, 343.
16. Negendank, W. *NMR Biomed.* **1992**, *5*, 303.
17. Griffiths, J. R. *Br. J. Cancer* **1991**, *64*, 425.
18. Gillies, R. J.; Liu, Z.; Bhujwalla, Z. *Am. J. Physiol.* **1994**, *267*, C195.
19. Preparation of compound **3**. To DHA (328 mg, 1.0 mmol) in DMF (8 mL) were added $BocNHNH_2$ (158.4 mg, 1.2 mmol) and EDCI (580 mg, 3.0 mmol). The reaction mixture was stirred at room temperature overnight. To the reaction mixture was added water, extracted with EtOAc. The organic phase was then washed with brine, dried with Na_2SO_4, and concentrated in vacuum. The product was purified by column chromatography eluting with hexane/EtOAc (5:1) to afford 354 mg of **3** (80% yield). ^{1}H NMR (DMSO-d_6): 9.48 (s, 1H), 8.66 (s, 1H), 5.39–5.25 (m, 12H), 2.82–2.77 (m, 8H), 2.29–2.24 (m, 2H), 2.12–2.02 (m, 4H), 1.99 (s, 2H), 1.44–1.39 (m, 9H), 0.92 (t, 3H, J = 7.8 Hz).

Preparation of compound **4**. To compound **3** (350 mg) was added HCl/EtOAc (8 mL). The reaction mixture was stirred at room temperature for 2 h. Solvent was evaporated. To the residue was added ether, and the solid was filtered to afford 255 mg of **4** (85%). ^{1}H NMR (DMSO-d_6): 10.94 (s, 1H), 5.34–5.26 (m, 12H), 2.84–2.77 (m, 10H), 2.32–2.26 (m, 4H), 2.07–2.00 (m, 2H), 0.93 (t, 3H, J = 7.2 Hz).

Preparation of DHA–Dox. To Dox (300 mg, 0.52 mmol) and compound **4** (230 mg, 0.67 mmol) in methanol (100 mL) was added trifluoroacetic acid (30 μL). The reaction mixture was stirred in the dark at room temperature overnight. Solvent was removed in vacuo. The product was purified by column chromatography eluting with dichloromethane to afford DHA–Dox as a dark solid (188 mg, 49% yield), mp: 169 °C (dec). ^{1}H NMR (DMSO-d_6): 10.35 (s, 1H), 7.93–7.90 (m, 2H), 7.67–7.65 (m, 2H), 5.76 (br s, 1H), 5.51 (s, 1H), 5.40–5.18 (m, 12H), 5.09–4.89 (m, 2H), 4.41 (br s, 2H), 4.03–3.94 (m, 4H), 3.54 (br s, 1H), 2.75–2.67 (m, 8H), 2.57 (t, 1H, J = 6.40 Hz), 2.33–2.12 (m, 2H), 2.08–1.97 (m, 8H), 1.87 (t, 1H, J = 3.60 Hz), 1.73–1.69 (dd, 1H, J = 3.60, 11.59 Hz), 1.17 (d, 3H, J = 6.40 Hz), 0.89, (t, 3H, J = 7.2 Hz). MS (*m*/*z*), 773.4 ($M+Na^+$).