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Conjugation with α-linolenic acid improves cancer cell uptake and cytotoxicity of doxorubicin

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ABSTRACT

The synthetic DOX–LNA conjugate was characterized by proton nuclear magnetic resonance and mass spectrometry. In addition, the purity of the conjugate was analyzed by reverse-phase high-performance liquid chromatography. The cellular uptake, intracellular distribution, and cytotoxicity of DOX–LNA were assessed by flow cytometry, fluorescence microscopy, liquid chromatography/electrospray ionization tandem mass spectrometry, and the tetrazolium dye assay using the in vitro cell models. The DOX–LNA conjugate showed substantially higher tumor-specific cytotoxicity compared with DOX.

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The major limitation of conventional cancer chemotherapy drugs is their severe adverse side effects on normal tissues, leading to high systemic toxicity and preventing the use of high drug doses that are required for effective killing of cancer cells, thus limiting anti-tumor efficacy.[1] Doxorubicin (DOX) is a highly potent and widely used chemotherapeutic agent. DOX is effective against various types of cancers including breast cancer, urothelial cancer, hematopoietic malignancies and solid tumors. However, its susceptibility to multidrug resistance mechanisms and serious cytotoxicity in normal tissues, such as the induction of myelosuppression and cardiotoxicity, limit the maximum tolerated dose.[2,3] Therefore, DOX is a good candidate for drug targeting research to enhance effectiveness and limit nonspecific toxicity.

In recent years, important advances have been made in the development of new drugs and drug formulations based on specialized carriers that can be conjugated with drugs to reduce their toxicity, improve their solubility, bioavailability and stability (enzymatic, thermal, etc.), eliminate undesirable physiological interactions, and prolong circulation time. The tissue selectivity of chemotherapy can be substantially increased by conjugating (either directly or by using a complex drug delivery system) an anticancer drug with a targeting moiety. Site-specific drug delivery vehicles could potentially make chemotherapy more effective and less toxic by increasing the amount of drug that reaches the intended target.[4,5]

Drug delivery systems using $n-3$ fatty acids have much potential for improving the therapeutic efficacy of anti-cancer drugs. Several studies have demonstrated that $n-3$ fatty acids sensitize tumor cells to effects of anti-cancer drugs either in culture or in tumor-bearing animals and can play an important role in cancer prevention/progression. These nutritionally induced changes in tumor fatty acid composition result in increased sensitivity to chemotherapy, especially in tumor lines that are resistant to chemotherapy, and cause specific enhancement of cytotoxicity to tumor cells and protection of normal cells.[6–8]

Drug delivery systems based on the mainly dietary $n-3$ fatty acids, such as docosahexaenoic acid (DHA), eicosapentaenoic acid or α-linolenic acid (LNA), have received much attention due to their unique structures and characteristics. Incubation of DOX-sensitive and DOX-resistant small-cell lung carcinoma cells with DHA resulted in a significant increase in phospholipid DHA concentration without the loss of cell viability; only the DOX-resistant cell line pre-incubated with DHA showed increased sensitivity to DOX (Fig. 1).[9,10]

The extent of cellular DOX uptake was analyzed by intrinsic DOX fluorescence (red) by flow cytometry. MCF-7, MDA-MB-231 and HepG2 cells were used to assess endocytosis. For drug efflux studies, cells harvested during logarithmic growth were seeded in 96-well plates at a cell density of 5×10^4 cells/ml. After 24 h, cells were loaded with drug by exposure to 5, 10 and 20 μM free

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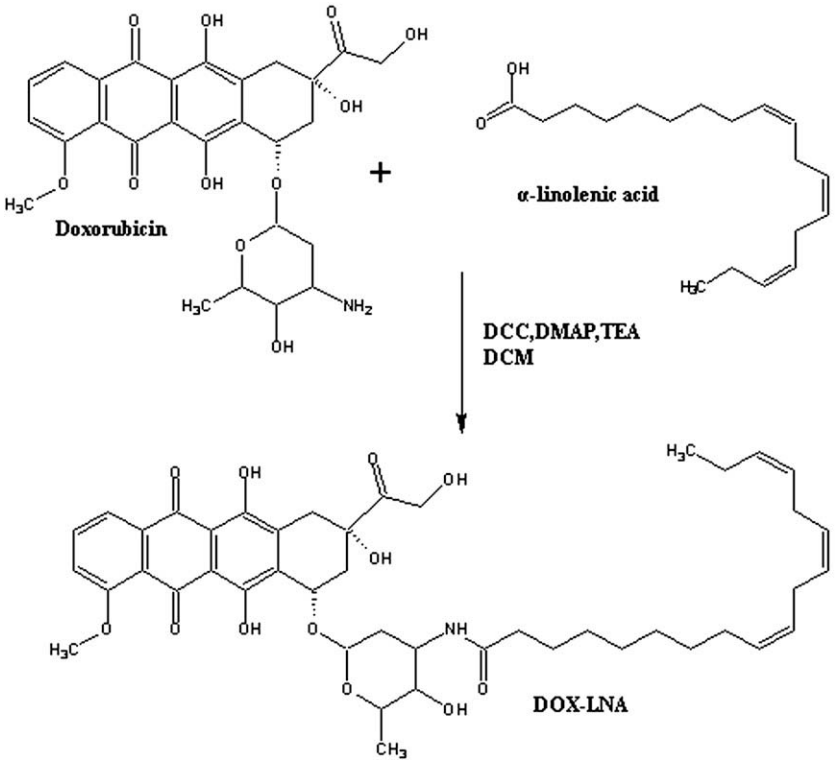

Figure 1. Synthetic pathway of DOX–LNA conjugate.

DOX or DOX–LNA at 37 °C for the indicated incubation times (5–120 min) that were used. Cells were then washed three times with PBS and were removed from plates by trypsinization, centrifuged for 2 min, resuspended in 0.5 ml PBS, and then analyzed by flow cytometry. Fluorescent and light images were recorded to observe the influence of LNA conjugation on intracellular distribution of DOX.

MCF-7, MDA-MB-231 and HepG2 cells were plated in 25-cm^2 flasks as described above, and loaded with drug by exposure to 0.5, 1 and 2 μM DOX or DOX–LNA for 48 h at 37 °C in cell culture media. The drug was removed, and fresh medium was added. The cells were harvested and collected by centrifugation, counted using a Coulter counter, and cell lysate was obtained by subjecting the drug-containing cells to three freeze-thaw cycles in liquid nitrogen. The cell lysate samples (0.2 mL) were extracted with 1 mL ethyl acetate by vigorous vortex mixing for 1 min and centrifuged at 3000*g* at room temperature for 10 min. The solution was spiked with 10 ng resveratrol for use as an internal standard for extraction efficiency. The supernatants were evaporated to dryness under N_2 gas, reconstituted in 100 μl of methanol, and analyzed by LC/ESI-MS/MS. The final DOX level was divided by the number of cells lysed for each sample and reported as nanogram of DOX per cell.

The endocytosis and extent of cellular uptake of DOX and DOX–LNA by MCF-7, MDA-MB-231 and HepG2 cells with higher doses and shorter incubation times were analyzed by fluorescence microscopy and flow cytometry compared to what was used in the LC/MS/MS assay. First, flow cytometry showed that the uptake of LNA–DOX was greater than that of free DOX (Fig. 2A). The intracellular uptake of free and conjugated DOX slowly increased in MCF-7 and MDA-MB-231 cells from 5 min to 120 min of incubation. The maximum uptake of DOX and DOX–LNA was detected in HepG2 cells at 5 min, which was higher than in the other two cell types (Fig. 2B). Furthermore, uptake of DOX and DOX–LNA was dose dependent in MCF-7 and MDA-MB-231 cells.

As shown in Figure 3, DOX–LNA incubated with MCF-7, MDA-MB-231 or HepG2 cells emitted higher fluorescence intensity compared with free DOX, suggesting that LNA–DOX was taken up more rapidly and to a much greater extent than free DOX. After internalization by cancer cells, DOX was localized primarily in the cytoplasm with some accumulation in the nucleus.

We developed a sensitive high-throughput bioanalytical method using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) equipped with an electrospray ionization interface used to generate negative ions $[M-H]^-$ for the estimation of the DOX and DOX–LNA levels in cell lysates (Fig. 4A and B).[11] Intracellular levels of DOX and DOX–LNA were quantified by multiple reaction monitoring (MRM) of the deprotonated precursor ion and the related product ion for DOX using the internal standard method with peak area ratio.[12,13] The intracellular DOX levels were measured by LC/MS/MS after free DOX and DOX–LNA were incubated with MCF-7, MDA-MB-231 or HepG2 cells at lower doses over longer incubation times than were used to evaluate the drug uptake (Fig. 4C). These data showed that DOX–LNA could be more effectively taken up by MCF-7, MDA-MB-231 and HepG2 cells compared to free DOX. The conjugate showed higher cellular uptake efficiency within the cells. The enhanced uptake of DOX–LNA was attributed to facilitated endocytotic transport relative to free DOX, which was transported into cells by passive diffusion.

The tetrazolium dye (MTT) assay was performed to determine the cytotoxicity of free DOX and DOX–LNA in MCF-7, MDA-MB-231 and HepG2 cells as described with minor modifications. Based on these measurements, the IC_{50} (the concentration of active ingredients necessary to inhibit cell growth by 50%) of free DOX and DOX–LNA was calculated. All assays were performed at least three times in quadruplicate to determine the IC_{50}. A decrease in the IC_{50} indicates an increase in drug toxicity. The cytotoxicity of DOX and DOX–LNA against MCF-7, MDA-MB-231 and HepG2 cells using the MTT assay are summarized in Table 1. The results show that DOX–

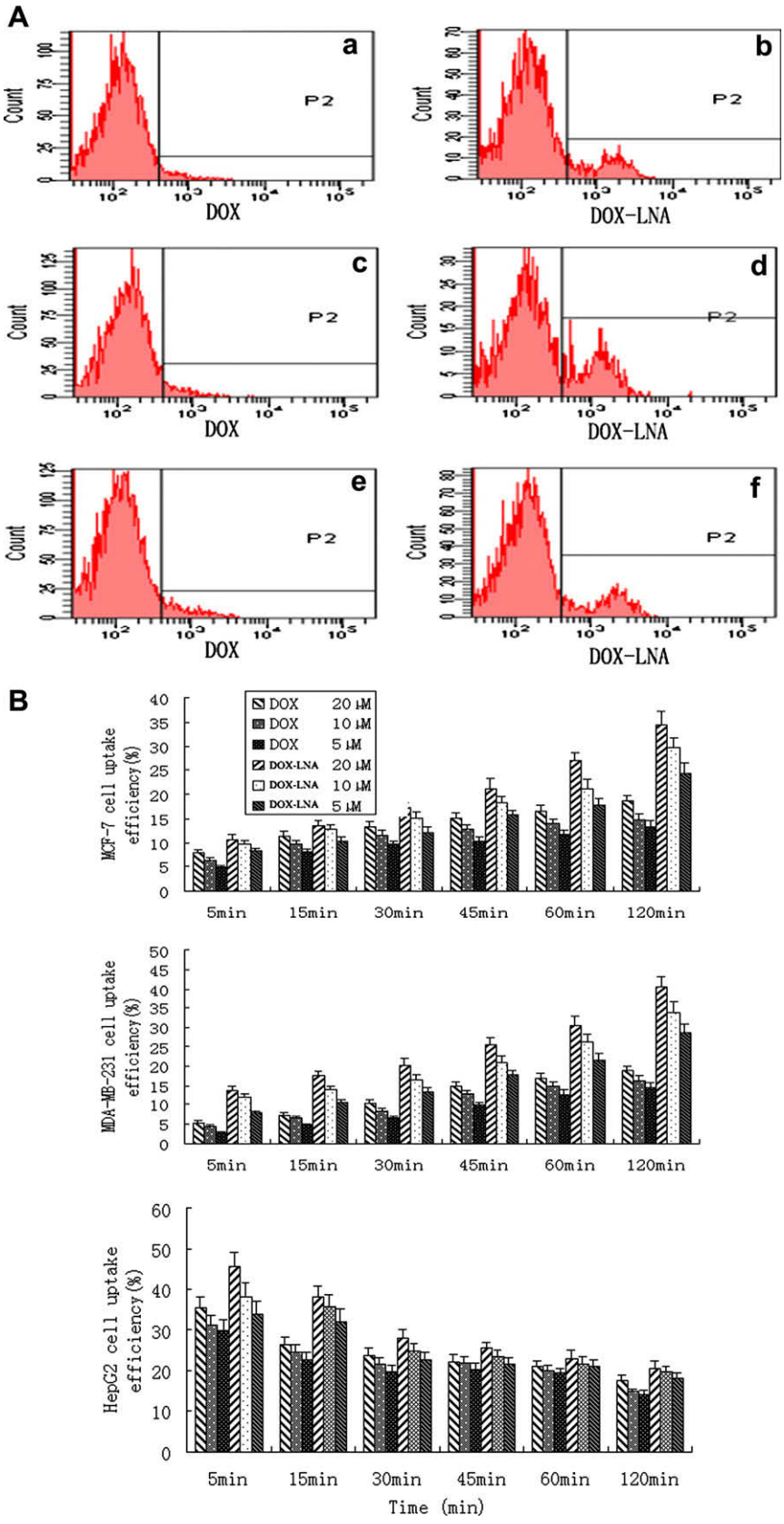

Figure 2. Intracellular uptake of DOX and DOX–LNA. Flow cytometry results was shown in panel A, MCF-7 (a, DOX; b, DOX–LNA), MDA-MB-231 (c, DOX; d, DOX–LNA) and HepG2 (e, DOX; f, DOX–LNA) cells. Cellular uptake of free DOX and its conjugates in MCF-7 (upper graph), MDA-MB-231 (middle graph) and HepG2 cells (lower graph) (B).

LNA had significantly higher specific cytotoxicity and lower IC_{50} compared with DOX. The IC_{50} indicated that DOX–LNA was three-fold more cytotoxic in MDA-MB-231 cells than free DOX, whereas DOX–LNA showed twofold higher cytotoxicity in HepG2 or MCF-7 cells compared to free DOX. These IC_{50} values suggested that DOX–LNA was more cytotoxic against MDA-MB-231 cells than against

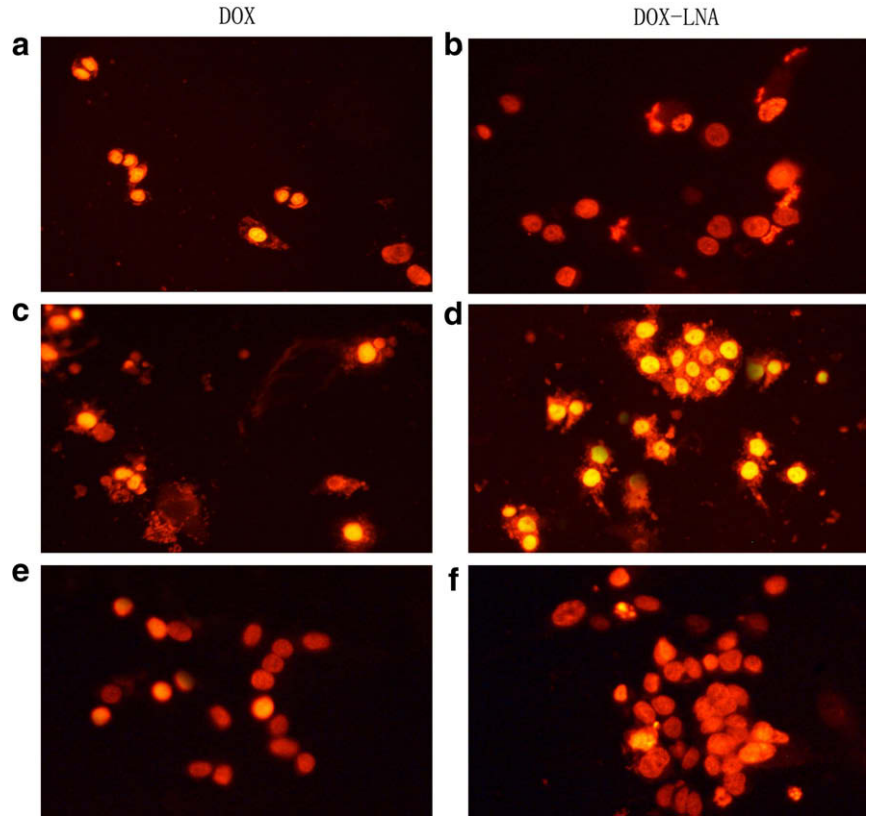

Figure 3. Intracellular localization of DOX and DOX–LNA. Typical fluorescence and composite images of MCF-7 (a, DOX; b, DOX–LNA), MDA-MB-231 (c, DOX; d, DOX–LNA) and HepG2 (e, DOX; f, DOX–LNA) cells incubated for 10 min with free DOX and DOX–LNA conjugate.

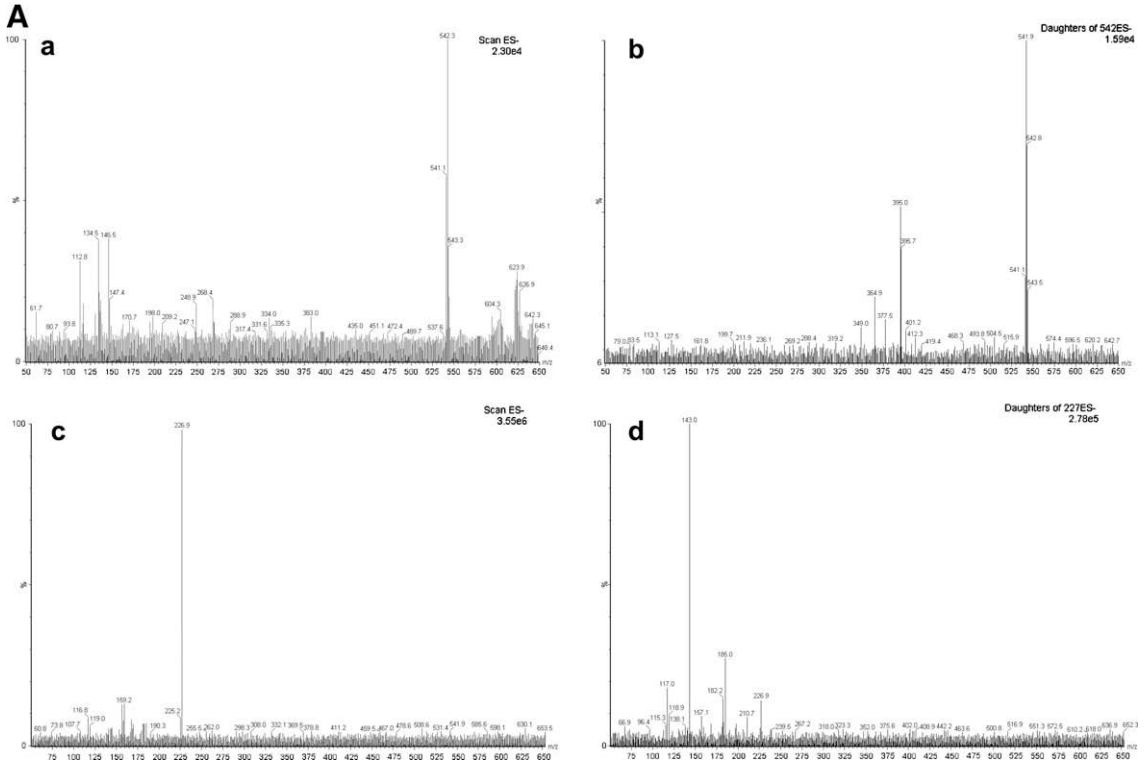

Figure 4. Intracellular DOX levels assayed by LC/MS/MS. Precursor/product ion mass spectra of DOX and the internal standard (resveratrol) are listed in panel A. (top left) DOX, *m*/*z* 542; (top right) fragment of DOX, *m*/*z* 542→395; (bottom left) resveratrol, *m*/*z* 226.9; (bottom right) fragment of resveratrol, *m*/*z* 226.9→142.8. MRM chromatograms of DOX and the internal standard (resveratrol) in the cell sample are shown in panel B. The retention times for DOX and resveratrol were 2.77 min and 2.40 min, respectively. The intracellular DOX levels (c) were measured by **LC/ESI-MS/MS** after free DOX and DOX–LNA were incubated with MCF-7 (top), MDA-MB-231 (middle) or HepG2 cells (bottom) for 48 h.

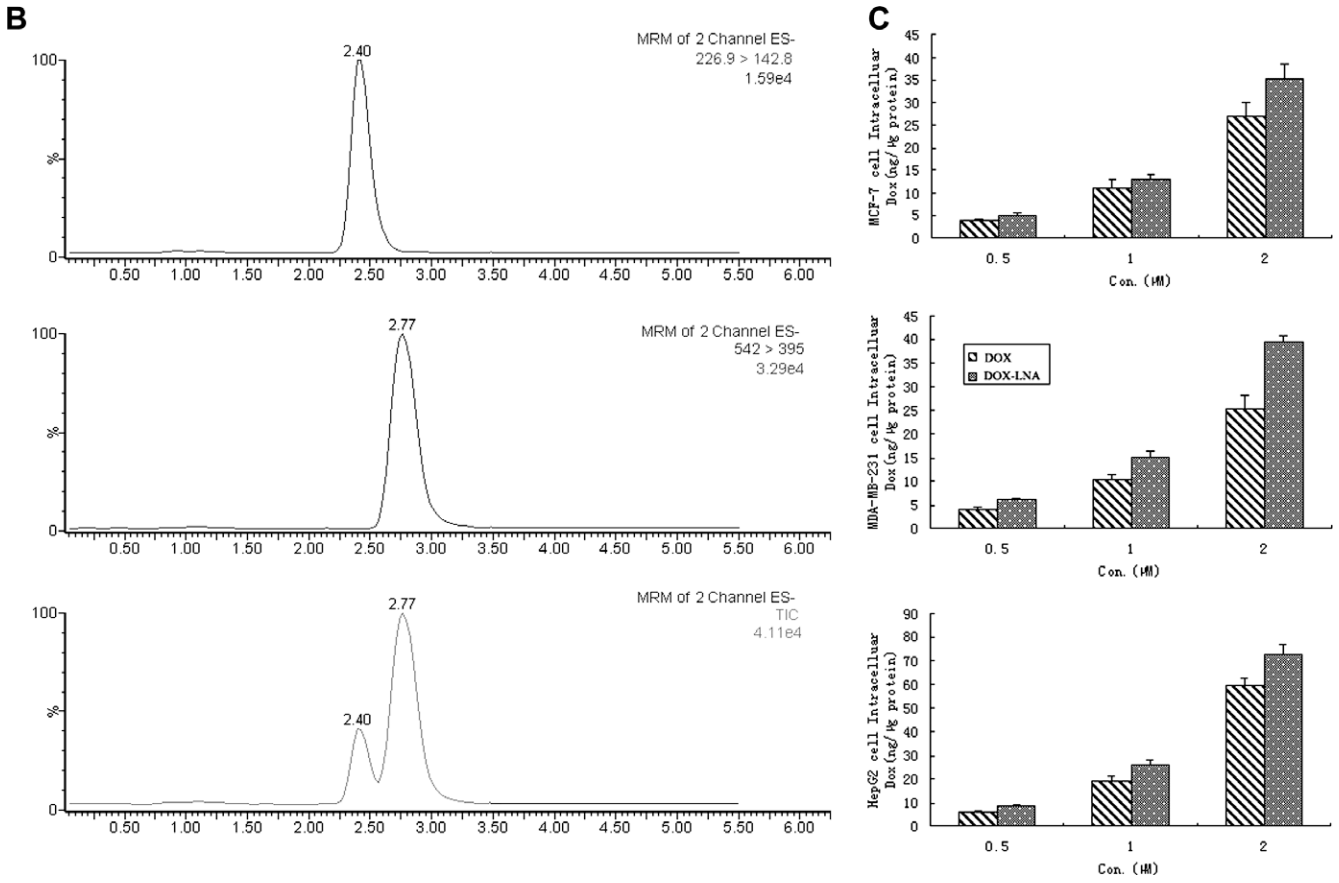

Fig. 4 (*continued*)

Table 1
In vitro cytotoxicity of DOX and DOX–LNA

Drug	IC_{50} (μM)		
	MCF-7	MDA-MB-231	HepG2
DOX	8.3 ± 0.2	10.2 ± 0.3	14.3 ± 0.4
DOX–LNA	4.7 ± 0.1	3.1 ± 0.2	6.8 ± 0.1

MCF-7 or HepG2 cells. Conjugating with LNA increased the bioavailability of the whole complex and its internalization by cancer cells, whereas an increase in the DOX content in the prodrug enhanced anti-cancer activity of the prodrug. The LNA targeting moiety accelerated internalization of the prodrug by cancer cells and substantially enhanced its cytotoxicity.

Our results demonstrate that DOX, a model anti-tumor drug, was successfully conjugated with LNA.[14,15] The in vitro anti-tumor activity studies indicated that the DOX–LNA conjugate was more cytotoxic than free DOX. Thus, LNA may be useful as a DOX carrier for more effective anti-tumor activity. Furthermore, the LNA–DOX conjugates are very promising and clinically suitable candidates for anti-cancer therapy. This prodrug has great potential to become a new dosage form, of DOX and the methodology can also be applied to other anti-cancer drugs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.016.

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11. *The HPLC and mass condition*: Collision-induced dissociation was achieved using argon as the collision gas. A standard solution of DOX (1 μg/mL) and IS (resveratrol, 1 μg/mL) was applied to optimize the detection conditions in the presence of mobile phase. The compounds were separated on a reversed-phase column (Symmetry C18 column, 50 × 2.1 mm, 5 μm) with an isocratic mobile phase consisting of 0.1% acetic acid and acetonitrile (30:70, v/v). The column temperature and flow rate were 25 °C and 0.2 ml/min, respectively. The mass transitions used for DOX and IS (resveratrol) were *m/z* 542→120 (cone voltage, 120 eV; collision energy, 15 eV; dwell time, 400 ms) and 226.9→142.8 (cone voltage, 40 eV; collision energy, 30 eV; dwell time, 400 ms), respectively.
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14. *Synthesis of DOX–LNA conjugate*: The 3′-amino group on free DOX was transformed to an amide bond by acylation with LNA through DCC activation methods. Briefly, DOX·HCl (312 mg) was converted to the free DOX base by triethylamine (TEA) (DOX/TEA molar ratio = 1/1.2) and then dissolved in dichloromethane (5 mL). To this solution, LNA (100 mg), DCC (222 mg) and 4-DMAP (132 mg) were added. The conjugation reaction was performed

overnight under a nitrogen atmosphere in the dark at room temperature. The product was filtered to remove dicyclohexylurea and then purified by column chromatography, eluting with petroleum ether/ethyl acetate (2:1) to generate a red powder with a yield of 73%. In addition, the purity of the conjugate was analyzed using a reverse-phase HPLC system (Waters Corp., Milford, MA) with a model 2996 photodiode array detector and a symmetry C_{18} column (250 × 4.6 mm, 5 μm). The mobile phase was 0.1% acetic acid/acetonitrile (30:70, v/v). The column temperature and flow rate were 25 °C and 1.0 ml/min, respectively. DOX·HCl had a retention time of 3.79 min by UV absorbance at 254 nm. Under the same conditions, the DOX–LNA conjugate showed one sharp peak at 6.11 min at 254 nm. The purity of the conjugate was 97%.

15. *Characterization of DOX–LNA conjugate*: The synthetic DOX–LNA conjugate was characterized by ^{1}HNMR using dimethyl sulfoxide (DMSO)-d_6 as a solvent. The spectra were recorded on a 400-MHz spectrophotometer using DMSO-d_6 as a solvent. The spectrum of the DOX–LNA conjugate exhibited typical peaks at δ 7.8 (2H, d, CH-2 and CH-4), 7.4 (1H, d, CH-3), 5.50 (1H, s, CH-1′), 4.08 (3H, s, H_3C-O-C-1) resulting from DOX, and the peaks at δ 5.28–5.49 (6H, m, CH-9,10,12,13,15 and 16), 2.81–2.78 (4H, t, CH_2-11 and CH_2-14) were signals from LNA, thus confirming the synthesis of the conjugate. The molecular mass of the conjugate was determined by electrospray ionization tandem mass spectrometry with a negative ion $[M-H]^-$ model. From the spectrum, we identified the stable molecular ion peak at m/z = 802.8, in agreement with the calculated molecular mass of the LNA–DOX conjugate.