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Short communication

Quantification intracellular levels of oligodeoxynucleotide-doxorubicin conjugate in human carcinoma cells in situ

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Abstract

Bifunctional oligodeoxynucleotide-intercalator conjugates provide new tools for a selective control of gene expression and may have potential applications as novel therapeutic agents. An 'in situ' analysis method has been developed to determine intracellular levels of oligodeoxynucleotide- doxorubicin conjugate which may be used to reverse the multidrug resistance in KB-A-1 cells. A linear response over a broad concentration range (0.05–6.0 μM, $r^2 = 0.97$) was obtained. The limit of detection and quantitation was set at 0.015 and 0.05 μM, respectively. Assay validation studies revealed that compared with HPLC, the method is rapid, high simple, high throughput and convenient for the determination of the conjugate in cells in situ. The uptake studies of the conjugate in cells using this method demonstrated that the cellular accumulation of the conjugate in KB-A-1 cells was dependent on a dynamic balance between influx and efflux processes.

Keywords: Oligodeoxynucleotide-doxorubicin conjugate; Uptake; Intracellular level; Determination

1. Introduction

Synthetic oligodeoxynucleotides (ODNs) can be used to specifically inhibit the expression of a gene through association with mRNA (the antisense approach), DNA (the antigene approach) or proteins (aptamer strategy) [1]. However, severe limitations, such as cellular uptake efficiency, affinity to the complementary sequence, and stability against nucleases, have impeded the use of ODNs in therapy [2]. Many attempts have been performed to circumvent these limitations. One major approach is chemical modifications of ODNs. There have been numerous reports demonstrated that the covalent attachment of intercalating agents to ODNs has been used to increase binding affinity to the complementary sequence, inhibit degradation by exonucleases, and facilitate cellular uptake [3–5]. These ODN-intercalator conjugates behave as bifunctional nucleic acid ligands—the ODN recognizes its complementary sequence and the intercalator provides an additional binding energy that leads to complex stabilization [6].

In our previous study, we synthesized a novel ODN-doxorubicin (DOX) conjugate and investigated its stability in medium using reverse-phase HPLC assay [7]. It has been found that the conjugate is efficiency in reversing multidrug resistance (data not in press). For future studies, developing convenient method detection the levels of the conjugate in cellular is necessary. The goals of this study were to develop a simple, reliable, reproducible, and high throughput fluorescent method to monitor the ODN-doxorubicin conjugate intracellular concentration in situ, and to examine the uptake and efflux of the conjugate targeted to mdr1 gene of human carcinoma DOX-resistance cells (KB-A-1). In general, the analysis of intracellular ODNs includes radio-labeled [8], and fluorescent-labeled [9] by chromatographic or flow cytometry methods [10]. In these methods there exist some defects such as inconvenience, cannot high throughput, and may be changing properties of ODN (using radio-labeled-biotin or fluorescent-labeled). Here, we used phosphorothioate modified ODNs to synthesize the ODN-DOX conjugate. We detected the conjugate's

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concentration in cells by measuring the intracellular fluorescence intensity using a fluorescence plate reader. We also compared this method with HPLC assay, and investigated the uptake and efflux of the conjugate in KB-A-1 cells.

2. Materials and methods

2.1. Materials

The sequence of ODN is 5′-TCCTCCATTGCGGTCCCCTT-3′, the 30-11 region of mdr1 gene resulting MDR in a human epidemic carcinomata cell line, which could control expression of the gene [11]. The phosphorothioate modified ODNs with phosphate group at the 3′-end were purchased from Shanghai Bioasia Biotech Co. Ltd. (Shanghai, China). DOX was a gift from Zhejiang Hisun Pharmacentical Co. Ltd. (Zhejiang, China). The conjugate of ODN-DOX was synthesized and purified in our laboratory. Fetal calf serum and heat-inactivation were purchased from Shanghai Bioasia Biotech Co. Ltd. (Shanghai, China). Other chemicals were obtained from commercial sources.

2.2. Preparation of standard solution

The conjugate standard solution was prepared by dissolving 30 OD of the ODN-DOX conjugate in PBS (Ph 7.4) and then was diluted to the range of 0.1–15 μM using lysed buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% sodium Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate). The solutions were storage at −20 °C and were defrosted before used. The calibration curve for using fluorimetry analysis was accomplished by plotting the ratio of the fluorescence intensity signals of the ODN-DOX conjugate to their concentration. The calibration curve for HPLC analysis was accomplished by plotting the ratio of the peak area of the ODN-DOX conjugate to their concentration.

2.3. Methods validation

The chromatographic conditions were as same as our previous studies [7] with the UV detector replaced by fluorescence detector. Briefly, the assay conditions were as follows: RP column (Lichrospher 5 μm, 4.6 × 125 mm, Merk). The mobile phase included two eluents. Eluent A: 5% CH3CN in 0.1 M aqueous triethylammonium acetate, pH 7.0. Eluent B: 80% CH3CN in 0.1 M aqueous triethylammonium acetate, pH 7.0. The initial mobile phase was eluent A. A linear gradient was stared from the initial conditions leading in 30 min to the final conditions containing of eluent A-eluent B (1:1) at 1 ml min^{-1}. Fluoresence detection was operated at excitation and emission wavelengths of 480 and 560 nm.

For fluorimetry analysis, the fluorescent intensity of the ODN-DOX conjugate was detected using a fluorescence plate reader (Thermo. Labsystem, Finland) with the same excitation and emission wavelengths.

Linearity was determined by analyzing the conjugate standard solution in a concentration range from 0.1 to 15 μM and establishing the concentration-response relationship. The limit of detection (LOD) in standard solution was defined by the concentration with a signal-to-noise ratio of 3. The limit of quantitation (LOQ) was defined by concentration with a signal-to-noise ratio of 10. Accuracy (between-day) and precision (within-day) of the method were determined by assaying three samples.

2.4. Cell line and culture conditions

A human epidermoid carcinoma, DOX-resistance cell line (KB-A-1) was kindly provided by Dr. Ira Pastan and Micheal M. Gottesman (National Institutes of Health, USA). KB-A-1 was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Inc. MA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA), 100 U ml^{-1} penicillin G, and 100 μg ml^{-1} streptomycin sulfate,. The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.5. Quantification of the ODN-DOX conjugate in cells using fluorescence plate reader and HPLC analysis

The intracellular concentration of the ODN-DOX conjugate was determined by detecting intracellular fluorescence intensity of DOX. Briefly, KB-A-1 cells from monolayer growing cultures were harvested and seeded in 96-well culture plates (Costar Corp., Combridge, MA) in 200 μl DMEM with 10% fetal bovine serum at concentration of 2×10^4 cells/well. After growing for 80% confluence, the medium was removed, and replaced with fresh medium containing 2.0 μM the conjugate. Cells treated with free ODN as the control. After incubation at 37 °C for 0.5, 2, 4, 8, 16 and 24 h, the culture medium was removed and cells were rinsed three times with cold PBS. The cells were lysed in 200 μl of a lysing buffer.

For fluorimetry analysis, total cellular ODN-DOX conjugate was directly determined by measuring the fluorescent emission of the solution ($\lambda_{ex} = 480$ nm, $\lambda_{em} = 560$ nm) in the cell lysate with a fluorescence plate reader. The conjugate concentration was given by the standard curve.

For HPLC analysis, the cell lysate was added with 200 μl solution containing an equal volume of phenol and chloroform to precipitate proteins. After centrifugation for 45 s at 3000 rpm, the supernatant solution was collected, and then 15 μl 100 mM $MgCl_2$ and 300 μl ice-cold isopropanol were added to the supernatant, allowed to stand for 6 h at 4 °C and centrifugation for 20 min in the cold. The supernatant was removed and 400 μl of ethanol (80%) was added into the nucleic acid pellet. Centrifugation was carried out for another 20 min, the supernatant was removed, the precipitates were resolved with 100 μl PBS (pH 7.4) and 40 μl was injected

Table 1
Analytical parameters for the determination of the ODN-DOX conjugate in lysed buffer solution

Method	RP-HPLC method	Fluorimetry method
Linearity range (μM)	0.01–15	0.05–6
r^{2*}	0.9921	0.9753
LD* (μM)	0.003	0.015
LQ* (μM)	0.01	0.05

LD*, the limit of detection; LQ*, the limit of quantitation; r^{2*}, coefficient of determination; Four samples were processed at each concentration.

as a sample for HPLC analysis. The conjugate concentration was given by the standard curve.

2.6. Quantitative uptake studies using fluorimetry analysis

For the time course study, KB-A-1 cells were incubated with 2 μM ODN-DOX conjugate for 0.5, 1, 2, 4, 8, 16 and 24 h. To examine the efflux processes, the culture medium containing 2 μM ODN-DOX conjugate was replaced by an ODN-free medium 8 h after incubation. Cells were harvested at 2, 4, 8, 16 and 24 h later, respectively. The fluorescence of ODN-DOX conjugate in cells was measured using fluorescence plate reader. The determination process was described as above. The conjugate concentration was given by the standard curve.

3. Results

3.1. Validation of the method

Analytical parameters of standard solution for the fluorescence intensity or the peak area ratio of varying amounts of the conjugate by two methods are shown as Table 1. By HPLC assay, the ODN-DOX conjugate was linear over 0.01–15 μM with coefficient of determination (r^2) values of greater than 0.99. By fluorescence plate reader determination, the conjugate was linear over 0.05–6 μM with r^2 values of greater than 0.97. The limit of detection was determined as the signal corresponding to three times the peak-to-peak noise for HPLC assay and to three times signal of the blank well for the fluorescence plate reader detected, respectively. The limit of quantitation was calculated as the signal corresponding to 10 times the peak-to-peak noise or 10 times signal of the blank well.

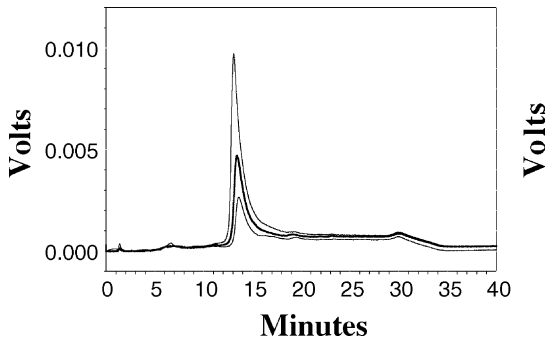

Fig. 1. RP-HPLC chromatograms of the purified ODN-DOX conjugate and intracellular conjugate with the chromatographic conditions: (a) Purified conjugate (0.6 μM); (b) The intracellular conjugate. Cells were treated with 2.0 μM conjugate for 24 h. (c) The intracellular conjugate. Cells were treated with 2.0 μM conjugate for 24 h and then incubated with conjugate-free medium for 24 h.

Precision and reproducibility were examined within-day and between-day in PBS and in plasma, respectively. The results are shown as Table 2. It was concluded that, for the ODN-DOX conjugate, there was no significant difference for the assays tested within-day and between-day between two methods. The results indicate that both methods are reliable for analysis of the ODN-DOX conjugate.

3.2. Quantitative uptake studies of the ODN-DOX conjugate

Using HPLC assay, the intracellular conjugate was shown to be intact without degradation after incubation with 2 μM conjugate for 24 h (Fig. 1). The intact conjugate was still present after 24 h incubation with conjugate-free medium. No breakdown of intact conjugate was observed by HPLC, indicating that the conjugate was stable and resistant to nucleases in the cellular environment.

Table 2
Results of within-day and between-day precision of ODN and ODN-DOX conjugate in lysed buffer solution

Method	Theoretical concentration (μM)	Within-day measured concentration (μM)		Between-day measured concentration (μM)	
		Mean	R.S.D. (%)	Mean	R.S.D. (%)
RP-HPLC					
	0.2	0.205	0.86	0.195	1.26
	1.0	1.03	0.77	0.991	1.38
	6.0	5.98	1.93	5.978	2.68
Fluorimetry method					
	0.2	0.197	0.64	0.196	0.78
	1.0	0.99	0.62	0.97	0.81
	6.0	5.89	1.67	5.71	2.87

Four samples were processed at each concentration.

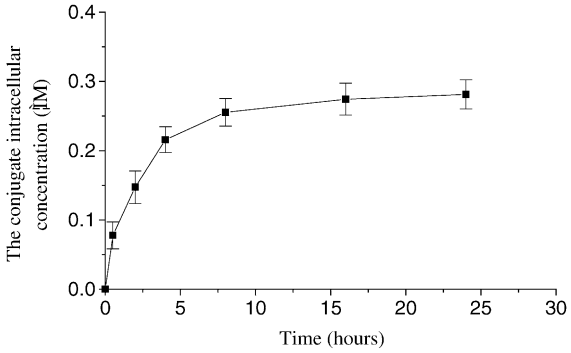

Fig. 2. Time course of cellular uptake of the ODN-DOX conjugate in KB-A-1 cells. After incubation of the cells with 2 μM conjugate, the intracellular conjugate was measured by fluorescence method using a fluorescence plate reader. Results are means ± S.D. of four different experiments.

The uptake studies of the conjugate were tested using a fluorescence plate reader by the method as described above (Section 2). The data demonstrated a biphasic increase in cellular levels over time, with an initial rapid accumulation over a period of 8 h followed by a more gradual increase over the next 16 h (Fig. 2). After incubation for 24 h at 37 °C, the intracellular accumulation of the conjugate was up to 0.28 μM.

In order to detect the efflux of the conjugate from the cells, we observed the intracellular conjugate levels for 24 h after replacement of conjugate contain medium with conjugate-free medium. Seventy-one per cent of the intracellular conjugate still remained in the cells after 24 h incubated with conjugate-free medium (Fig. 3). There was no breakdown of the intracellular conjugate shown by HPLC assay at 24 h. These results indicate that there are efflux processes occurring simultaneously with uptake influx processes during incubation with the conjugate.

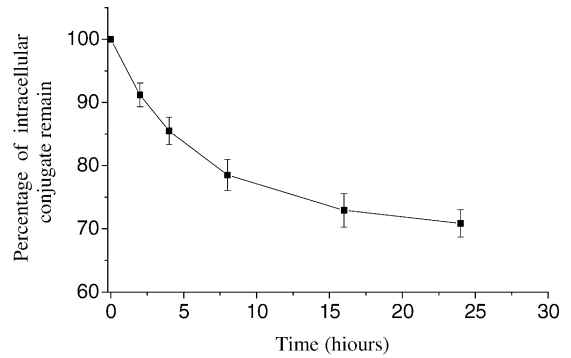

Fig. 3. The ODN-DOX conjugate efflux cure. Cells are incubated with 2 μM conjugate for 8 h at 37 °C. The medium was replaced by conjugate-free medium consequently for times. The amount of cellular conjugate was determined by fluorescence method using a fluorescence plate reader. Results are mean ± S.D. of four different experiments.

4. Discussion

DOX is an anthracycline antibiotic and possesses a unique fluorescence emission spectrum. Previous study demonstrated a good correlation between DOX concentration and the cellular fluorescence in the tumor cells as measured by HPLC [12]. In this study, we detected the intracellular concentration of the ODN-DOX conjugate in KB-A-1 cells through measuring the DOX moiety fluorescence intensity using a fluorescence plate reader and HPLC. From HPLC analysis, a major peak of the conjugate in cell extraction was observed. The results indicate that there is an endocytotic uptake mechanism by which the intact conjugate passes through the cell membrane and escape lysosomal nuclease degradation.

Compared with HPLC assay, the fluorimetry method using fluorescence plate reader is not as precise as HPLC, but it is very simple and convenience. Furthermore, it is high throughput assay also, 96 samples can be detected at once determination whereas only one sample could be test by HPLC at once determination. Our data show that the method could be used for examining the uptake of the conjugate in cells.

The uptake studies show that there were two separate phases in the conjugate uptake. Initaially there is rapid uptake during the first 8 h of incubation, then only a small increase until the end of the incubation (24 h). The mechanism of biphasic uptake remains unknown. However, the efflux occurs slowly and may explain why there is an initial rapid uptake followed by slower uptake. An efflux process suggests that there is a continuous dynamic balance between cellular uptake and exocytosis of the conjugate. At early time points (30 min to 4 h), rapid accumulation occurred since influx was much greater than the efflux process. At later time points (from 16 to 24 h), influx was opposed by efflux, but influx was still higher than efflux process. So a steady state or slow accumulation stage was achieved. Our data show a 29% decrease in intracellular conjugate after incubation with conjugate-free medium for 24 h, indicating a gradual release of the conjugate into medium. This is not due to degradation, because HPLC analysis of the conjugate from cell lysates showed no breakdown of the conjugate.

5. Conclusion

In summary, through detecting the fluorescence of the DOX moiety of the ODN-DOX conjugate using fluorescence plate reader, a high simple method has been developed and validated to determine intracellular levels of the conjugate in KB-A-1 cells in situ. The results of cellular uptake studies of the conjugate in KB-A-1 cells using this method indicated that the cellular accumulation of the conjugate was dependent on a dynamic balance between influx and efflux processes. The method developed appears accurate, reliable, reproducible and high throughput without complicated

sample treatment, and can be conveniently used for determining the intracellular levels of the conjugate in cells in situ.

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