Short communication

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Synthesis of novel taspine diphenyl derivatives as fluorescence probes and inhibitors of breast cancer cell proliferation

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ABSTRACT: Two novel taspine diphenyl derivatives (Ta-dD) were designed and synthesized by introducing different coumarin fluorescent groups into the basic structure of Ta-dD. The main advantage of these two compounds is that they can be used as fluorescence probes and inhibitors simultaneously. In the present study, the fluorescent properties of the probes were measured and their inhibition of four breast cancer cell lines was tested. Different concentrations of the fluorescence probe were added to MCF-7 breast cancer cells for fluorescence imaging analysis under normal conditions. The results suggested that both of the new compounds have not only fluorescence but also the ability to inhibit effects on different breast cancer cell lines, which indicates their possible further use as dual functional fluorescence probes in tracer analysis.

Keywords: taspine diphenyl derivatives; fluorescence probe; inhibitor; breast cancer cells

Introduction

Compared with normal cells, tumour cells appear to exhibit autocrine or paracrine-stimulated growth, avoiding the normal growth-suppressing signal (1). Breast cancer is a daunting disease and constitutes a continuing medical health problem for millions of women worldwide (2). Breast cancer cell lines have been the most widely used models to investigate how proliferation, apoptosis and migration become deregulated during the progression of breast cancer (3). As treatment of breast cancer with chemotherapy is often empirical, based on histological tumour parameters and in the absence of specific mechanistic understanding, it has been difficult to learn clearly from the success or failure of new drugs (4). Therefore, it is significant to develop a target-selective drug with fluorescence, which can be applied in tracer analysis.

In recent years, fluorescence analysis has been applied in medicinal preparations and research on drugs in body fluids (5,6). Fluorescence probes have become powerful tools for biochemical and cellular studies, because of their high sensitivity, visibility and low interference in living systems (7–10). Usually, a fluorescence probe consists of a fluorescence chromophore and an identifying group. Currently, two methods have been used, primarily in applying fluorescent probes to cancer detection and visualization. The most common technique is engineering the cancer cells to express fluorescence, such as green fluorescent protein (GFP) (11–13). Although this method tracks the cancer cells directly, it requires gene transfection of each type of target cancer cell line to express the fluorescent protein (14–16). Another method is to introduce novel telomerase-dependent and replication-competent adenovirus that can express the fluorescence to target the cancer cells (17). However, only the presence of highly active telomerase, such as that found in malignant tissues, causes bright fluorescence. Also, researchers usually conjugate a dye to a ligand, which targets the surface protein receptors expressed by tumour cells. All the methods evaluate the effectiveness of anticancer drugs by measuring their levels of fluorescence. The fluorescent probe itself does not take effect directly on the tumour cells. Therefore, it is important to develop dual-function fluorescence probes.

In a previous study, taspine was screened for the first time from *Radix et Rhizoma Leonticis* (Hong Mao Qi in Chinese; HMQ), using cell membrane chromatography (CMC) (18,19). HMQ has many pharmacological actions, particularly anti-cancer activity (20,21). Based on these data, our group designed and synthesized a series of taspine derivatives using a computer-aided drug design method. Through the CMC model selection, we found that one of the taspine diphenyl derivatives Ta-dD, can continue to maintain taspine's pharmacological activity, particularly in terms of tumour cell proliferation (22,23).

In this work, we used Ta-dD as the identifying group and synthesized two novel taspine diphenyl derivatives, 6-[2-(3-chloro-4-fluorophenylamino)-2-oxoethoxy]-5,5′-dimethoxy-N^2,$N^{2\prime}$-dimethyl-6′-[2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy)ethoxy] biphenyl-2,2′-dicarboxamide (F-Ta-dD-dOH) and 6-[2-(3-chloro-4-fluorophenylamino)-2-oxoethoxy]-5,5′-dimethoxy-N^2,$N^{2\prime}$-dimethyl-6′-[2-(4-methyl-2-ox-*o*-2*H*-chromen-7-yl-amino)-2-oxoethoxy] biphenyl-2,2′-dicarboxamide (F-Ta-dD-dNH_2), which were made by introducing different coumarin groups (R_1) into the structure

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of Ta-dD (Fig. 1). We studied the fluorescence properties of Ta-dD, the original coumarin groups, the modified coumarin groups and the target compounds simultaneously. We also explored the preliminary inhibitory effect of F-Ta-dD-dOH and F-Ta-dD-dNH$_2$ on four breast tumour cell lines.

Experimental

Instrumentation

Melting points were measured with an X-4 digital melting point apparatus. Infrared (IR) spectra were recorded on KBr pellets, using a Shimadzu FT-IR 440 spectrometer in the range 4000–500 cm^{-1}. Nuclear magnetic resonance (NMR) spectra were measured using a Varian INOVA spectrometer at 400 MHz in $CDCl_3$ with TMS as an internal reference. Fluorescence measurements were performed on a Hitachi F-4500 fluorescence spectrophotometer. The synthetic procedures were controlled by thin-layer chromatography (TLC) on plates precoated with silica gel (GF-254). The products were purified by recrystallization or column chromatography on silica gel (53–75 μm). The fluorescent images were detected on an inverted phase contrast microscope purchased from Nikon Instruments Co., Ltd. The microplate reader was purchased from Bio-Rad (USA). In this study, all optical measurements were carried out at room temperature under ambient conditions. The fluorescence intensities are reported in arbitrary units; the experimental parameters within a given experiment are the same, and the fluorescence intensities are comparable within a given experiment.

Chemicals and materials

All chemicals used were of analytical reagent grade. Methanol, dichloromethane, and *N*,*N*-dimethylformamide (DMF) needed further purification. $NaClO_2$ (80%) was purchased from Sigma-Aldrich. Isovanillin (99%), Br_2 (99.5%), 1-(chloromethyl)benzene (99%), chloroacetyl chloride (98.5%), H_2O_2 (≥ 30%), sodium phosphate monobasic dehydrate (≥ 99%), thionyl chloride (≥ 99%), methylamine water solution (25–30%), copper powder (99%), Pd/C (10%), 7-amino-4-methyl-coumain (95%), resorcinol (98%), 1,2-dibromoethane (98%) and dimethyl sulphoxide (98%) were purchased from Sinopharm Chemical Reagent Co. Ltd. Hydrogen (≥ 99.99%) and nitrogen (≥ 99.999%) were purchased from Xi'an Weiguang gases Co. Ltd. Human breast cancer cell lines were obtained from the Shanghai Institute of Cell Biology in the Chinese Academy of Sciences. MTT, RPMI 1640, DMEM and trypsin were purchased from Gibco (USA). Gefitinib (≥ 99%) was purchased from Nanjing Ange Pharmaceutical Co. Ltd.

Synthesis of the new fluorescence probes

The new fluorescence probes were prepared as shown in Scheme 1. On the basis of previous work, we used commercially available isovanillin as the starting material. The key intermediate Ta-dD was obtained via bromination, benzylation, oxidation, Ulmman reaction, catalytic hydrogenation and substitution reaction. Comprehensively utilizing the different activated substituents in the coumarin structure, including the hydroxyl group and amino group, we obtained the coumarin derivatives 7-(2-bromoethoxy)-4-methyl-2*H*-chromen-2-one (coumarin-OH-Br) and 2-chloro-*N*-(4-methyl-2-oxo-2*H*-chromen-7-yl) acetamide (coumarin-NH$_2$-Cl). The introduction of 1,2-dibromoethane or chloroacetyl chloride enhanced the reactivity between coumarin derivatives and Ta-dD.

Synthesis of F-Ta-dD-dOH

Ta-dD (5.45 g, 0.01 mol) was dissolved in anhydrous DMF (100 mL), then anhydrous K_2CO_3 (2.08 g, 0.015 mol) was added and the mixture was stirred at 80 °C for 30 min. Then we added coumarin-OH-Br (3.38 g, 0.012 mol). The reaction solution was stirred in the same temperature for 24 h. After the reaction, the mixture was allowed to cool and was poured into ice water. The suspension was filtered and chromatographed on silica gel (EtOAc:methanol, 20:1) to give F-Ta-dD-dOH as a white solid; m. p. 108–109 °C. Yield, 61%. IR (KBr) cm^{-1}, 3326, 2938, 1723, 1618, 1294, 1263, 1145, 1021. ^{1}H-NMR (400 MHz, $CDCl_3$) δ(ppm): 7.683 (s, 2H), 7.477 (d, J = 8.8 Hz, 1H), 7.306 (d, J = 8.8 Hz, 1H), 7.195 (d, J = 8.4 Hz, 1H), 7.106–7.062 (t, J = 8.8 Hz, 1H), 6.842–6.820 (m, 1H), 6.728 (d, J = 8.4 Hz, 1H), 6.640 (s, 1H), 6.425 (d, J = 4.8 Hz, 1H), 6.168 (s, 1H), 4.132 (s, 2H), 4.132–4.095 (d, J = 7.6 Hz, 2H), 4.006–3.970 (d, J = 7.2 Hz, 2H), 3.816 (s, 3H), 3.759 (s, 3H), 2.803 (d, J = 4.8 Hz, 3H), 2.629 (d, J = 4.8 Hz, 3H), 1.602 (s, 3H).

Synthesis of F-Ta-dD-dNH$_2$

As mentioned earlier, Ta-dD was treated using the same method. We added coumarin-NH$_2$-Cl (3.01 g, 0.012 mol) to the mixture. The reaction solution was stirred at 80 °C for 24 h. After the reaction, the suspension was filtered and chromatographed on silica gel (EtOAc:methanol, 25:1) to give F-Ta-dD-dNH$_2$ as a grey solid under the same post-treatment; m.p. 179–180 °C. Yield, 56%. IR (KBr) cm^{-1}: 3373, 2935, 1703, 1621, 1528, 1499, 1407, 1146, 1020. ^{1}H-NMR (400 MHz, $CDCl_3$) δ(ppm): 7.765–7.717 (m, 1H), 7.617–7.524 (m, 2H), 7.343–7.306 (m, 2H), 7.129–7.068 (m, 3H), 6.888 (d, J = 8.4 Hz, 2H), 6.211 (s, 1H), 4.457–4.405 (m, 2H), 4.204 (d, J = 14.8 Hz, 2H), 3.872 (s, 6H), 2.761 (d, J = 3.2 Hz, 6H), 2.420 (s, 3H).

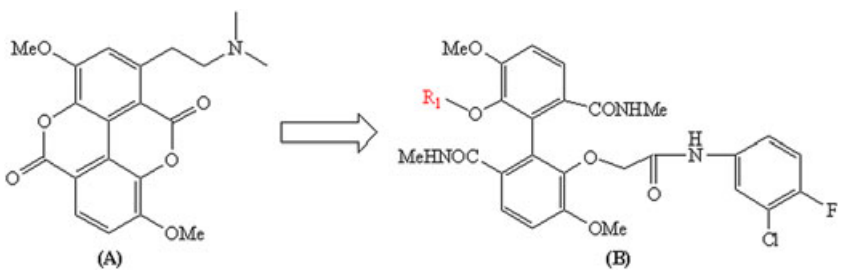

Figure 1. Structure of the compounds: (A) taspine; (B) basic taspine diphenyl derivative (Ta-dD).

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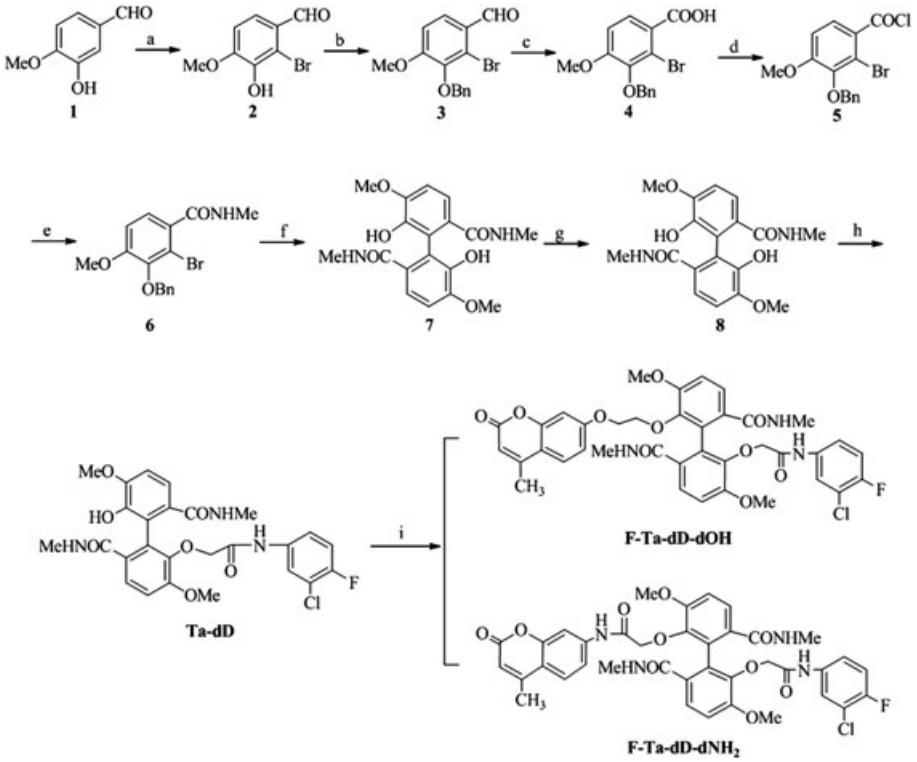

Scheme 1. General routes for the synthesis of the fluorescence probes. The reaction conditions were: (a) Br_2, HAc, Fe, NaAc, 23–25 °C; (b) BnCl, K_2CO_3, EtOH, reflux; (c) anhydrous THF, $NaClO_2$, NaH_2PO_4, 30% H_2O_2, rt; (d) CH_2Cl_2, $SOCl_2$; (e) CH_2Cl_2, CH_3NH_2, 0–5 °C; (f) anhydrous DMF, Cu, N_2, reflux; (g) H_2, Pd/C, rt; (h) anhydrous DMF, K_2CO_3, 80 °C; (i) anhydrous DMF, K_2CO_3, 80 °C.

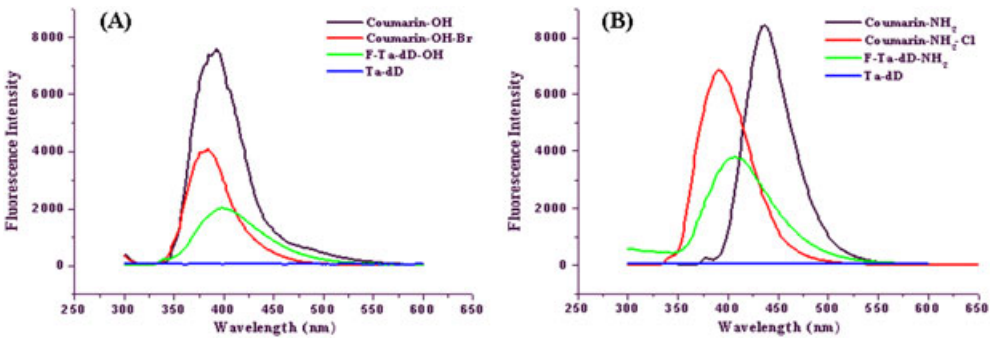

Figure 2. Fluorescence spectra of the analytes: (A) Ta-dD (blue), F-Ta-dD-dOH (green), coumarin-OH-Br (red), Coumarin-OH (black); (B) Ta-dD (blue), F-Ta-dD-dNH_2 (green), coumarin-NH_2-Cl (red), coumarin-NH_2 (black).

Results and discussion

Fluorescence characterization of the new fluorescence probes

The fluorescence spectra of the analytes were measured and the results are shown in Fig. 2. The results indicated the feasibility of adding fluorescence by conjugating with a fluorescence group. We assessed the fluorescence properties from the molecular structure. Due to the stabile conjugated π electrons and the lone electron pairs of oxygen or nitrogen atoms, coumarin-OH and coumarin-NH_2 themselves have the characteristic of strong stability and exhibit excellent fluorescence properties. Although their derivatives, coumarin-OH-Br and coumarin-NH_2-Cl, still maintain a certain fluorescence intensity, the conjugate effect reduces via connecting with 1,2-dibromoethane or chloroacetyl chloride. However, the introduction of these flexible link structures not only enhances the reactivity between coumarin derivatives and Ta-dD, but eases the influence of the original structure of Ta-dD on the fluorescent chromophore. Therefore, the fluorescence properties of the target compounds can be guaranteed.

Biological activity analysis

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay was used to quantify cell viability, measuring cell survival and proliferation spectrophotometrically. We examined the effects of the fluorescence probes F-Ta-dD-dOH and F-Ta-dD-dNH_2 on the growth of breast cancer cell lines MCF-7, MDA-MB-23, ZR-75-30 and SK-BR-3. As shown in Fig. 3, F-Ta-dD-dOH and F-Ta-dD-dNH_2 could

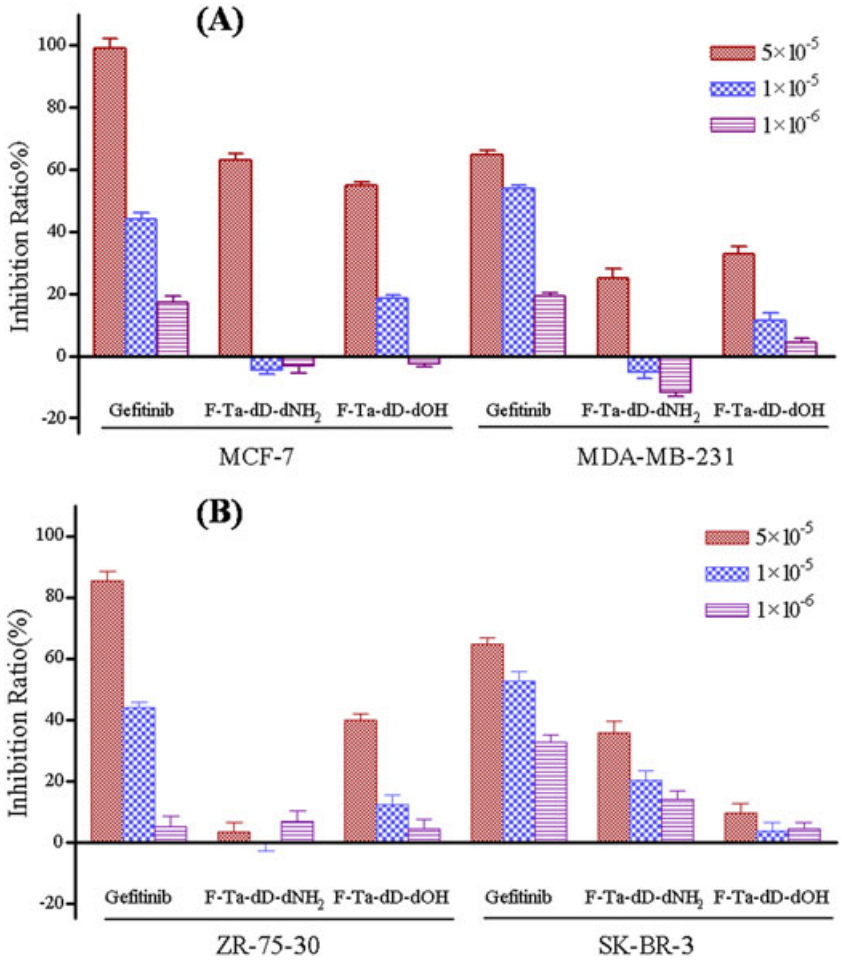

Figure 3. Inhibition of F-Ta-dD-dNH$_2$ and F-Ta-dD-dOH on breast tumour cell proliferation in different cell lines was evaluated using the MTT assay. Gefitinib was used as a positive control drug. (A) Breast tumour cell lines MCF-7 and MDA-MB-2; (B) breast tumour cell lines ZR-75-30 and SK-BR-3.

take effect with the target directly and inhibit cell growth to different degrees. The activity of F-Ta-dD-dOH against ZR-75-30 and the activity of F-Ta-dD-dNH$_2$ against SK-BR-3 were greatest. According to the above results, we can see that the introduction of the fluorescence chromophore not only retained the backbone structure of taspine biphenyl derivatives and pharmacological activity, but also produced target compounds with fluorescence properties.

Fluorescence micrographs of the breast cancer cells labelled by F-Ta-dD-dNH$_2$

We investigated the staining behaviour of the fluorescence probe F-Ta-dD-dNH$_2$ on the living breast cancer cell line MCF-7 and the normal cell line HEK 293. The measurements of fluorescence scanning and image collection were carried out after washing with PBS three times to get rid of excess compound. We did not find an obvious phenomenon of fluorescence quenching prior to clearing the excess probe from the cell. Fluorescence signal was detected using an inverted phase-contrast microscope. The images were taken using a fluorescence imaging system and analysed by NIS BR image processing software. All operations were carried out at room temperature. Figure 4 shows the staining capability of F-Ta-dD-dNH$_2$ for MCF-7 cells, where selective stainability was not recognized. Labelled MCF-7 cells were incubated *in vitro*. When the concentration of the probe was $< 5 \times 10^{-5}$ mol/L, a small number of tumour cells were labelled and had no obvious changes in shape (Fig. 4B, C). As the probe concentration increased, the visual field was brighter and the shapes of the MCF-7 cells appeared to have irregular defects (Fig. 4A). In addition, we simultaneously examined the staining capability of F-Ta-dD-dNH$_2$ for the normal cell line HEK 293. Compared with the control group (normal cells without probes), there was no obvious staining behaviour (Fig. 4D–F). The data demonstrated that the dual-function molecules had selectivity for cancer cells over normal ones. In short, combined with the MTT assay, it can be seen that F-Ta-dD-dNH$_2$ may label MCF-7 cells directly and has some inhibitory effect on this cell line.

Conclusions

In this study, two novel compounds, F-Ta-dD-dOH and F-Ta-dD-dNH$_2$, both able to fluoresce and inhibit the growth of breast tumour cells, have been synthesized. The fluorescence spectra of the target compounds and the change of relative fluorescence intensity were studied. The effects of F-Ta-dD-dOH and F-Ta-dD-dNH$_2$ on the growth of different cell lines of breast cancer were examined by an MTT assay. The staining capability of F-Ta-dD-dNH$_2$ for MCF-7 cells was also analysed, which would offer a new

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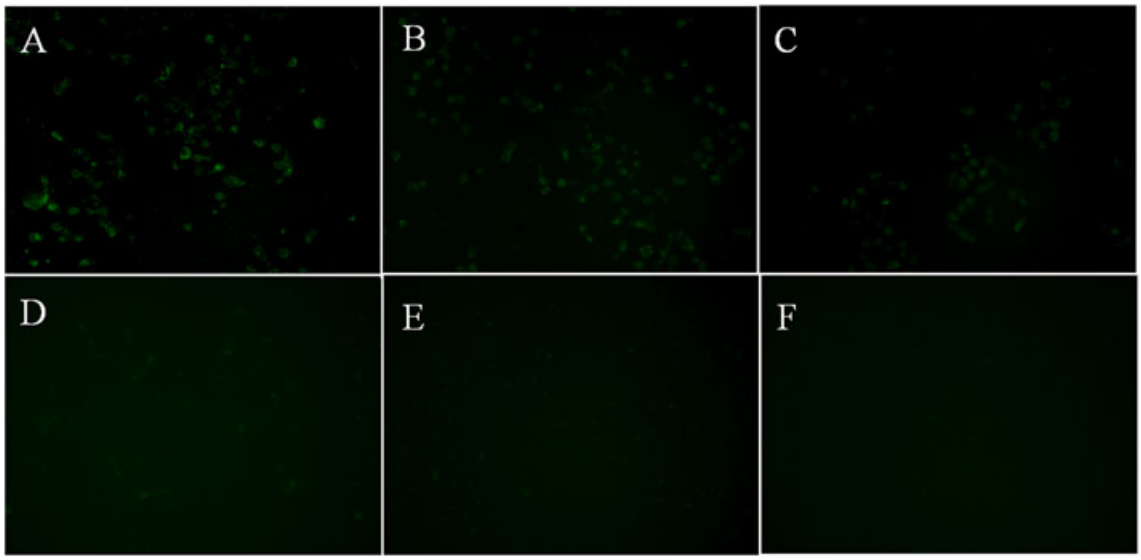

Figure 4. Fluorescence microscopy images of the breast cancer cell line MCF-7 and normal cell line HEK 293, each labelled with F-Ta-dD-dNH_2 in different concentrations: (A) MCF-7, 1×10^{-4} mol/L; (B) MCF-7, 5×10^{-5} mol/L; (C) MCF-7, 1×10^{-5} mol/L; (D) HEK 293, 1×10^{-4} mol/L; (E) HEK 293, 5×10^{-5} mol/L; (F) control (normal cells without probes). Magnification, ×200.

method in the study of mechanism of drug action. Although the new probes are not exceptionally bright, the introduction of these fluorescence chromophores does not affect the skeleton structure or the recognition of tumour cells.

Acknowledgements

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