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Short communication

Synthesis and immunofluorescence assay of a new biotinylated paclitaxel

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Abstract

7-(5′-Biotinylamidopropanoyl)paclitaxel was synthesised by chemical methods; its immunofluorescence assay and the cell uptake experiments were performed by use of human leukemia U937 cells. The results indicate that paclitaxel is arresting cell cycle at the G_2M phase only. © 2002 Published by Éditions scientifiques et médicales Elsevier SAS.

Keywords: Biotin; Immunofluorescence; Photo-affinity; Paclitaxel; G_2M-phase cell

1. Introduction

Paclitaxel is a complex polyoxygenated diterpene, which is isolated from the Pacific Yew, *Taxus brevifolia* [1]. It is currently used as an anti-cancer agent for treatment of cancers of ovarian, metastatic breast, lung, head, and neck [2]. Paclitaxel also possesses a unique mode of action in which it promotes microtubules polymerisation and subsequent stabilisation [3,4]. We planned to synthesise fluorescent-labelled paclitaxel derivatives and studied their mode of action [5].

Some photo-affinity labelled paclitaxel derivatives are prepared and studied in microtubles [6–15]. Horwitz and coworkers [16] developed a method for the linkage of a photo-affinity analog in the N-terminal 31 amino acid part of the β-tubulin subunit. Recently, Nogales et al. [17–19] have reported the structure of tubulin at a resolution of 3.7 Å using electron crystallography on crystalline sheets formed in the presence of zinc. Guénard and coworkers [20] also synthesised biotinylated docetaxel derivatives and studied its binding with avidin. They succeeded in attaching biotin on docetaxel with 6-aminocaproyl linker. Nevertheless, 3-aminopropanoyl linker is not applicable because of the instability of 7-(3′-aminopropanoyl)docetaxel intermediate. Herein we report our results on successful introduction of biotin to naturally occurring paclitaxel with 3-aminopropanoyl linker and its immunofluorescent properties.

2. Chemistry

2.1. Synthesis of 7-(5′-biotinylamidopropanoyl)paclitaxel (**5**, Fig. 1)

For the synthesis of biotinylated paclitaxel **5**, paclitaxel (**1**) was first silylated with chlorotriethylsilane to give the resultant silyl ether **2** [21,22] in 81% yield. This compound was first reported by Chen et al. [21]. Treatment of **2** with *N*-(4-methoxytrityl)-β-alanine, dicyclohexyl-carbodiimide (DCC), and 4-(dimethylamino)-pyridine (DMAP) in CH_2Cl_2 at room temperature gave the corresponding C(7)-ester derivative **3** in 66% yield. Detritylation and desilylation of **3** in situ with acetic acid in a mixture of THF and water at 50 °C for 24 h afforded 7-(β-alanyl)paclitaxel (**4**) in 59% yield. Very recently, Wandless and coworkers [23] have reported a different method for its preparation.

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Reaction of **4** with biotinyl-*N*-hydroxysuccinimide [24] in the presence of *N*-ethylmorpholine and dry DMF gave the target biotinylated paclitaxel derivative **5** in 62% yield. Its ^{1}H-NMR spectrum showed that a broad triplet with $J = 7.0$ Hz appeared at 3.27 ppm, which was associated with $-CH_2NC(=O)-$. The results clearly indicate that a new amide bond was successfully generated; it connected biotin moiety onto 7-(β-alanyl)paclitaxel (**4**). Furthermore, two strong fragments showed up in its mass–mass spectrum with m/z at 888.2 (100%) and 828.2 (29%). These diagnostic ion peaks came from the decarboxylation of **5** ($[M + Na]^+ = 1173.4$) at C-13 position (i.e., $[M + Na - PhCONHCHPhCH(OH)COOH]^+$) and a sequential deacetylation (i.e., $[M + Na - PhCONHCHPhCH(OH)COOH - MeCOOH]^+$), respectively [25].

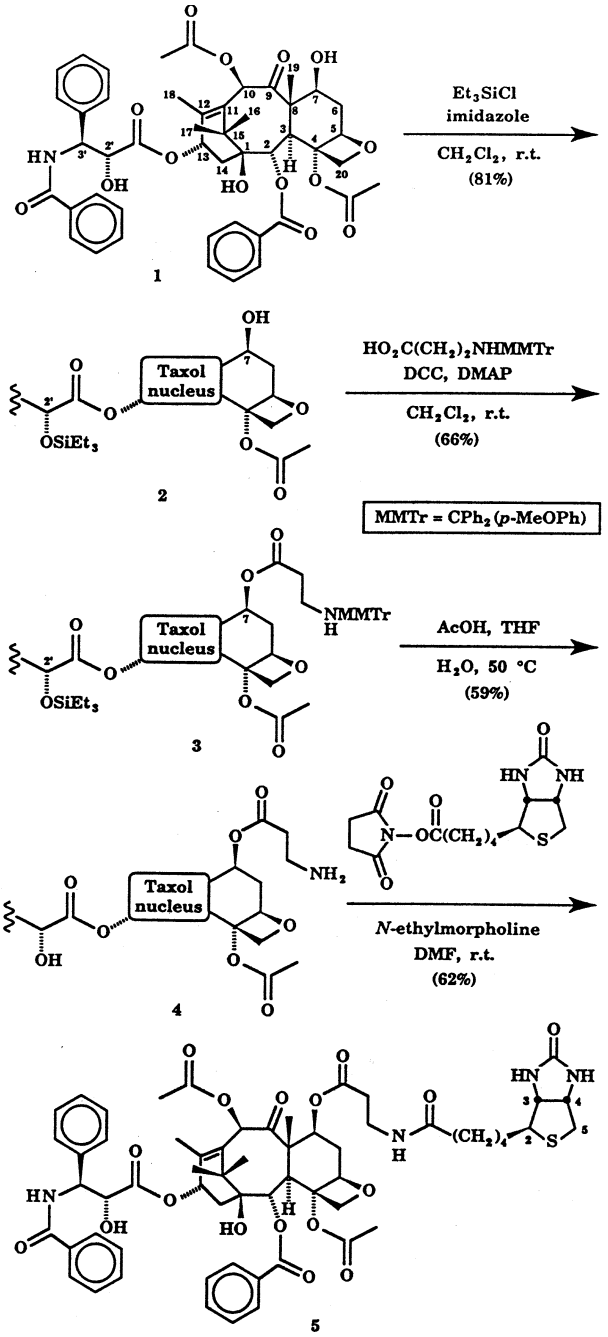

Fig. 1. Chemical synthesis of 7-(5′-biotinylamidopropanoyl)paclitaxel (**5**) from paclitaxel (**1**) in four steps.

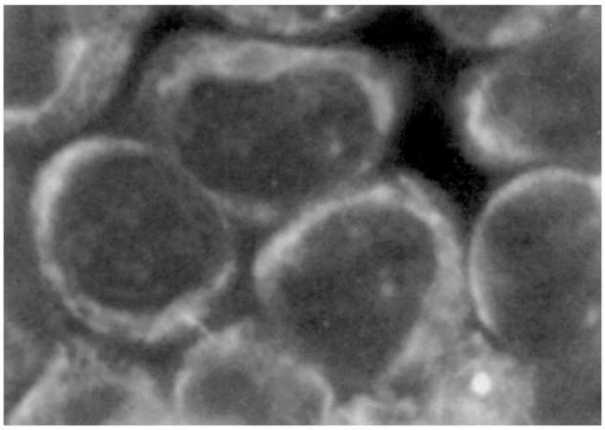

Fig. 2. The human leukemia U937 cells were stained with biotinylated paclitaxel **5** and the intracellular microtubules were visualised by FITC-linked avidin.

3. Result and discussions

3.1. Immunofluorescent studies

The biotinylated paclitaxel **5** retained its biological activity as the parent paclitaxel; the IC_{50} values were measured 4.0 nM for **5** and 4.5 nM for paclitaxel (**1**). We treated human leukemic U937 cells with biotinylated paclitaxel **5** at 37 °C for 6.0 h. The intracellular microtubules were labelled with biotinylated paclitaxel and visualised by the fluorescin isothiocyanate (FITC)-linked avidin. In the interphase cells, the yellow fluorescence at the edge of the cell was observed corresponding to microtubule filaments (Fig. 2).

3.2. Cell uptake fluorescence studies

To study the cell uptake fluorescence experiments, we incubated the human leukemia U937 cells with biotinylated paclitaxel **5** at different time intervals (i.e. 15 min, 30 min, 1.0 h and 2.0 h). After incubation with **5** for 15 min, the cells were observed by fluorescence microscope. We found that the biotinylated paclitaxel entered into the cell, especially G_2M-phase cells (Fig. 3C). Thus paclitaxel is mostly arresting cell cycle at the G_2M-phase.

After the biotinylated paclitaxel **5** was incubated for 30 min, fluorescent results show that more paclitaxel entered into the cell and little in G_1S phase cells (Fig. 3D). Similarly, biotinylated paclitaxel **5** was incu-

bated for 1.0 and 2.0 h, separately. The fluorescent results indicate that more paclitaxel entered into the cells (Fig. 3E and F).

4. Conclusion

A biotinylated paclitaxel with a short linker was synthesised and its immunofluorescence assay was performed on human leukemic U937 cells. Results from cell uptake experiments indicate that after 15 min of treatment with biotinylated paclitaxel **5**, it entered preferentially into the G_2M-phase cells.

5. Experimental

5.1. Chemistry

Proton NMR spectra were obtained on Varian Unity-400 (400 MHz) and Gemini Unity-300 (300 MHz) spectrometers. Chloroform-*d* was used as solvent; Me_4Si (δ 0.00 ppm) was used as an internal standard. Carbon-13 NMR spectra were recorded on a Gemini Unity-300 (75 MHz) spectrometer. Chloroform-*d* was used as solvent; the centre of the $CDCl_3$ triplet (δ 77.00 ppm) was used as an internal standard. All NMR chemical shifts are reported as δ values in

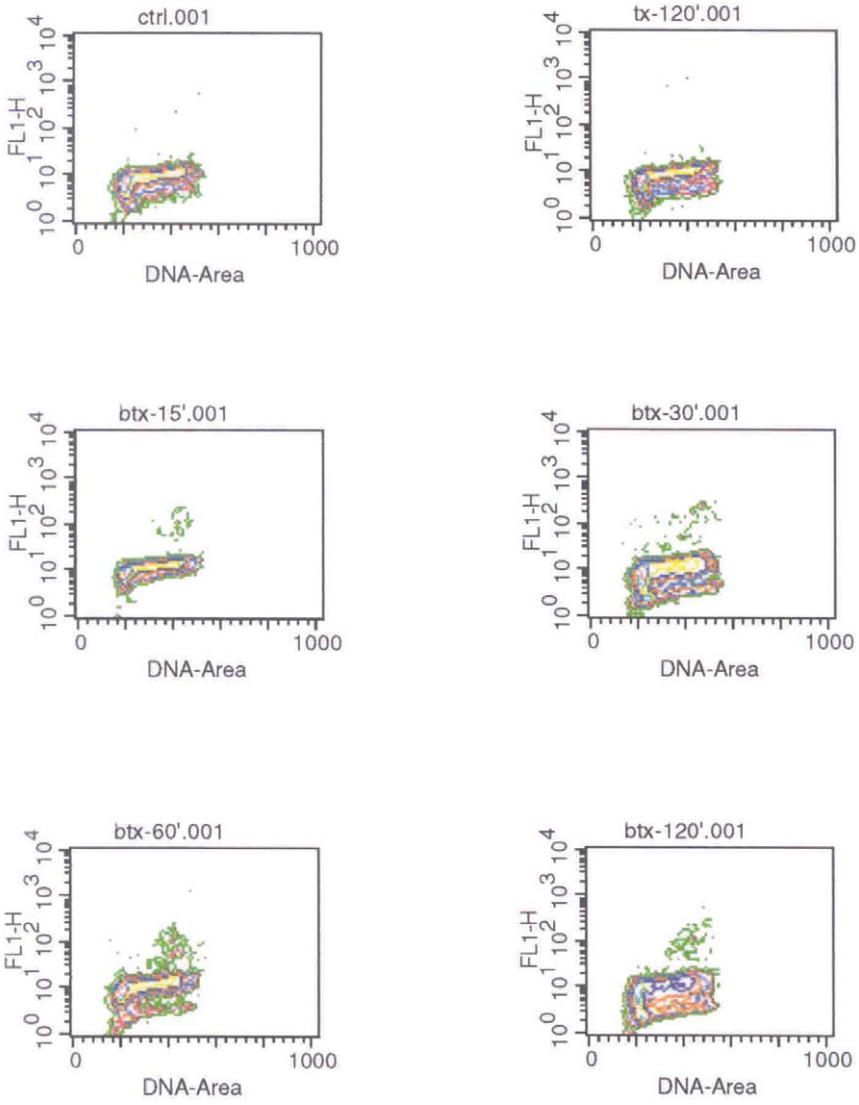

Fig. 3. Cell uptake fluorescent studies by incubation of human leukemia U937 cells with biotinylated paclitaxel **5** at different time intervals: (A) control; (B) non-labelled paclitaxel; (C) after 15 min; (D) after 30 min; (E) after 1.0 h; and (F) after 2.0 h.

parts per million (ppm) and coupling constants (J) are given in Hertz (Hz). The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, unresolved multiplet; and dd, doublet of doublets. Electrospray ionisation mass spectrometry (ESI–MS) analyses were performed on a quadrupole ion trap mass analyser fitted with an electrospray ionisation source (Finnigan LCQ, Finnigan MAT, San Jose, CA). A three-point calibration was carried out with a standard mixture of the peptide MRFA, caffeine, and Ultramark. Samples were injected at 5.0 μL min^{-1}. Ions were produced with a spray voltage of 4.25 keV, with the heated capillary set at 200 °C. Spectra were collected in the positive ion mode. Each spectrum was an average of 10–20 individual scans, which were composed of three microscans.

Purification on silica gel refers to gravity column chromatography or preparative thin layer chromatography on Merck silica gel. Analytical thin layer chromatography was performed on precoated plates purchased from Merck (silica gel 60 F_{254}).

2′-(Triethylsilyl)paclitaxel (**2**) was prepared according to the literature method [21,22]. The immunofluorescence assay was performed by use of a Zeiss Axiophot microscope.

*5.1.1. 2′-(Triethylsilyl)-7-[N-(4-methoxytrityl)-β-alanyl]paclitaxel (**3**)*

To a solution of 2′-(triethylsilyl)paclitaxel (**2**) (18.3 mg, 0.0189 mmol, 1.0 equiv.) and *N*-(4-methoxytrityl)-β-alanine (7.7 mg, 0.022 mmol, 1.2 equiv.) in CH_2Cl_2 (5.0 mL) were added DCC (20.1 mg, 0.0974 mmol, 5.2 equiv.) and DMAP (3.10 mg, 0.0254 mmol, 1.3 equiv.). The reaction mixture was stirred at room temperature (r.t.) under nitrogen atmosphere for 36 h. After filtration, the filtrate was washed with water (2 × 10 mL), saturated NaCl aq. solution (5.0 mL), dried over $MgSO_4$ (s), filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (20% EtOAc in hexanes as eluant) to give **3** (16.1 mg, 0.0124 mmol) as a colourless solid in 66% yield: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.45 (q, $J = 8.0$ Hz, 6H, 3 × $SiCH_2$), 0.83 (t, $J = 8.0$ Hz, 9H, 3 × CCH_3), 1.16 (s, 3H, C(17)H_3), 1.21 (s, 3H, C(16)H_3), 1.79 (s, 3H, C(19)H_3), 1.96 (s, 3H, C(18)H_3), 2.10 (s, 3H, C(4)OCOCH_3), 2.16–2.22 (m, 3H, $COCH_2$ + C(6)H_β), 2.35–2.50 (m, 4H, NCH_2 + C(14)H_2), 2.54 (s, 3H, C(10)OCOCH_3), 2.58–2.62 (m, 1H, C(6)H_α), 3.76 (s, 3H, OCH_3), 3.92 (d, $J = 7.3$ Hz, 1H, C(3)H), 4.22 (d, $J = 8.1$ Hz, 1H, C(20)H_β), 4.34 (d, $J = 8.1$ Hz, 1H, C(20)H_α), 4.70 (d, $J = 1.8$ Hz, 1H, C(2′)H), 4.96 (d, $J = 8.1$ Hz, 1H, C(5)H), 5.58 (dd, $J = 9.5$, 6.2 Hz, 1H, C(7)H), 5.70 (m, 2H, C(2)H + C(3′)H), 6.23 (br, t, $J = 8.3$ Hz, 1H, C(13)H), 6.25 (s, 1H, C(10)H), 6.79 (d, $J = 8.9$ Hz, 2H, ArH), 6.84 (d, $J = 7.8$ Hz, 1H, NH), 7.08–7.62 (m, 23H, ArH), 7.75 (d, $J = 8.0$ Hz, 2H, ArH), 8.14 (d, $J = 7.6$ Hz, 2H, ArH); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 4.32, 6.47, 10.84, 14.45, 20.61, 21.34, 22.92, 26.35, 33.36, 35.42, 35.55, 39.01, 43.32, 46.77, 55.12, 55.69, 55.94, 70.32, 71.36, 74.51, 74.69, 75.12, 76.36, 78.55, 80.94, 83.98, 113.02, 126.07, 126.43, 127.01, 127.71, 127.98, 128.53, 128.68, 128.74, 129.07, 129.77, 130.17, 131.74, 133.69, 134.08, 138.24, 138.36, 140.85, 146.34, 157.75, 167.09, 168.77, 169.80, 171.59, 172.04, 201.75.

*5.1.2. 7-(β-Alanyl)paclitaxel (**4**) [23]*

A solution of tritylated paclitaxel **3** (39.8 mg, 0.0306 mmol) containing a mixture of AcOH, H_2O, and THF (6:3:1, 5.0 mL) was heated at 45–50 °C with stirring for 24 h. The reaction mixture was cooled to r.t., neutralised with 10% $NaHCO_3$ aq. solution, and extracted with EtOAc (2 × 30 mL). The combined organic layers were washed with water (20 mL), saturated NaCl aq. solution (20 mL), dried over $MgSO_4$ (s), filtered, and concentrated under reduced pressure. The residue was purified by use of the preparative thin layer chromatography on silica gel (10% MeOH in CH_2Cl_2 as eluant) to afford **4** (16.8 mg, 0.0182 mmol) as a colourless solid in 59% yield: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.10 (s, 3H, C(17)H_3), 1.17 (s, 3H, C(16)H_3), 1.76 (s, 3H, C(19)H_3), 1.81 (s, 3H, C(18)H_3), 2.13–2.20 (m, 3H, $COCH_2$ + C(6)H_β), 2.15 (s, 3H, C(4)OCOCH_3), 2.35–2.43 (m, 2H, C(14)H_2), 2.38 (s, 3H, C(10)OCOCH_3), 2.43–2.58 (m, 1H, C(6)H_α), 2.65 (br, t, $J = 7.3$ Hz, 2H, NCH_2), 3.32 (br, s, 2H, NH_2), 3.84 (d, $J = 6.9$ Hz, 1H, C(3)H), 4.14 (d, $J = 7.8$ Hz, 1H, C(20)H_β), 4.27 (d, $J = 7.8$ Hz, 1H, C(20)H_α), 4.86 (d, $J = 2.8$ Hz, 1H, C(2′)H), 4.92 (d, $J = 8.8$ Hz, 1H, C(5)H), 5.58–5.73 (m, 3H, C(2)H + C(3′)H + C(7)H), 6.09 (br, t, $J = 8.0$ Hz, 1H, C(13)H), 6.12 (s, 1H, C(10)H), 6.85 (d, $J = 7.2$ Hz, 1H, NH), 7.26–7.63 (m, 11H, ArH), 7.82 (d, $J = 7.1$ Hz, 2H, ArH), 8.08 (d, $J = 7.68$ Hz, 2H, ArH); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 10.83, 14.48, 20.81, 21.15, 22.53, 26.37, 31.08, 33.07, 35.58, 35.67, 43.09, 46.84, 55.58, 55.89, 71.52, 71.98, 73.87, 74.68, 75.08, 76.22, 78.58, 80.72, 83.63, 127.27, 127.34, 127.87, 128.55, 128.78, 129.12, 130.15, 131.76, 132.38, 132.94, 133.48, 138.30, 141.39, 166.79, 167.60, 170.66, 170.96, 171.04, 172.96.

*5.1.3. 7-(5′-Biotinylamidopropanoyl)paclitaxel (**5**)*

A solution of biotinyl-*N*-hydroxysuccinimide (15.1 mg, 0.0442 mmol, 2.0 equiv.) in dry DMF was added to a stirred solution of 7-(β-alanyl)paclitaxel (**4**) (20.4 mg, 0.0221 mmol, 1.0 equiv.) and *N*-ethylmorpholine (4.40 mg, 0.038 mmol, 1.7 equiv.) in CH_2Cl_2 (5.0 mL) at 0 °C. After the solution was stirred at r.t. for 10 h, the solvent was removed under reduced pressure. The residue was dissolved in EtOAc (50 mL), washed with 10% citric acid solution (10 mL), water (10 mL), dried over $MgSO_4$ (s), and concentrated under reduced pres-

sure. The residue was purified by use of the preparative thin layer chromatography on silica gel (5% MeOH in CH_2Cl_2 as eluant) to give **5** (15.9 mg, 0.0138 mmol) as colourless solid in 62% yield: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.15 (s, 3H, C(17)CH_3), 1.21 (s, 3H, C(16)CH_3), 1.31–1.64 (m, 6H, $3 \times CH_2$), 1.80 (s, 3H, C(19)CH_3), 1.88 (s, 3H, C(18)CH_3), 2.08–2.25 (m, 5H, $2 \times COCH_2 + C(6)H_\beta$), 2.18 (s, 3H, C(4)OCO$CH_3$), 2.36–2.41 (m, 2H, C(14)H_2), 2.38 (s, 3H, C(10)OCOCH_3), 2.43–2.52 (m, 1H, C(6)H_α), 2.65–2.72 (m, 1H, biotin H(5)$_\beta$), 2.87–2.92 (m, 1H, biotin H(5)$_\alpha$), 3.08–3.21 (m, 1H, biotin H(2)), 3.27 (br, t, $J = 7.0$ Hz, 2H, CH_2N), 3.84 (d, $J = 6.8$ Hz, 1H, C(3)H), 4.17 (d, $J = 8.8$ Hz, 1H, C(20)H_β), 4.28–4.32 (m, 2H, C(20)H_α + biotin H(3)), 4.48–4.51 (m, 1H, biotin H(4)), 4.79 (d, $J = 2.6$ Hz, 1H, C(2′)H), 4.93 (d, $J = 8.4$ Hz, 1H, C(5)H), 5.31 (br, s, 1H, NH), 5.52–5.56 (m, 1H, C(7)H), 5.59 (br, s, 1H, NH), 5.68 (d, $J = 6.8$ Hz, 1H, C(2)H), 5.74 (dd, $J = 8.8$, 2.6 Hz, 1H, C(3′)H), 6.21 (br, t, $J = 8.2$ Hz, 1H, C(13)H), 6.25 (s, 1H, C(10)H), 6.79 (d, $J = 8.0$ Hz, 1H, NH), 7.30–7.63 (m, 11H, ArH), 7.81 (d, $J = 7.3$ Hz, 2H, ArH), 8.10 (d, $J = 7.2$ Hz, 2H, ArH). ESI–MS: m/z (positive mode) 1151.1 $[M + H]^+$, 1173.4 $[M + Na]^+$. ESI–MS–MS: m/z (relative intensity) 1173.4 (12), 1113.7 (8), 1030.4 (4), 974.4 (3), 905.6 (7), 888.2 (100) $[M + Na - C_{16}H_{15}NO_4]^+$, 887.4 (6), 828.2 (29) $[M + Na - C_{18}H_{19}NO_6]^+$, 646.2 (8), 628.2 (10), 588.1 (7).

5.2. Biological evaluations

5.2.1. Immunofluorescence assay

Human leukemic U937 cells were treated with biotinylated paclitaxel **5** (10 μM) at 37 °C for 6.0 h. After treatment, the cells were washed with Hanks' buffered saline solution twice, and were cytospun onto a glass slide. The cell spot was fixed with 100% MeOH at −20 °C for 15 min, rinsed with phosphate buffer saline (PBS), and then blocked with 10% fetal bovine serum (FBS) in PBS for 30 min. Subsequently, the cell spot was incubated with 50 μL of avidin conjugated with FITC (1:20 dilution in 10% FBS) in the dark for 30 min. After incubation, the cells were rinsed with PBS three times and then observed under fluorescent microscope.

Acknowledgements

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