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Taxol and 10-deacetylbaccatinIII induce distinct changes in the dynamics of caveolae

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Abstract **Taxol treatment of HeLa cells resulted in a transient recruitment of Caveolin-1 to the cell surface followed by internalization. Interestingly, 20 min after 10-deacetylbaccatinIII (10-DAB) treatment, the caveolae displayed faster 'kiss and run' dynamics while BaccatinIII (BacIII) did not induce any change. Sustained phosphorylation of Caveolin-1 is observed upon treatment and between Taxol and 10-DAB, the former shows phosphorylated Raf-1, ERK1/2 and hyperphosphorylated Bcl-2 while the later showed much less magnitude of the same. BacIII treatment did not induce phosphorylation of Raf-1 or Bcl-2. It is possible that Taxol might act on multiple targets and the side chain may be crucial.**

Keywords: Caveolae; Kiss and run dynamic; TIRFM; Taxol; 10-DeacetylbaccatinIII; BaccatinIII; Cytotoxicity

1. Introduction

Taxol has shown considerable promise as an effective anti-tumor drug against various human cancers [1]. With the development of the chemical method(s) for the synthesis of the Taxol and its derivatives it became clear that it is the side chain which apparently plays a significant role in its cytotoxic activity [2]. Our aim of the present study was to understand the role of the side chain of Taxol with respect to its functionality. Hence, we compared Taxol, 10-deacetylbaccatinIII (10-DAB) and BaccatinIII (BacIII) which differ only in the side chain. All three drugs have been shown to induce apoptosis but might follow apparently different pathways [3]. Recently it has been reported that Taxol induces expression of Caveolin-1, a marker protein of the caveolae, in MCF-7 cells and that the Caveolin-1 phosphorylation at tyrosine-14 is necessary to enhance Taxol mediated cytotoxicity [4]. Considering that Taxol is known to bind to microtubules and not caveolae per se, the requirement and role of Caveolin-1 is not clear. Moreover, Caveolin-1, as part of caveolae, is not an entirely static entity as the caveolae undergo a continuous cycle of 'kiss and run' (K&R) dynamics with the plasma membrane [5]. In view of the dynamic nature of caveolae, we examined the distribution and change in dynamics of Caveolin-1 (or caveolae) in context with the cytotoxicity in HeLa cells expressing GFP-Caveolin-1 by total internal reflection fluorescence microscopy (TIRFM).

2. Materials and methods

Taxol, BacIII and 10-DAB, purchased from Sigma Chemical Co., USA, which were dissolved in dimethylsulfoxide (DMSO) to 1 mg/ml stock solution.

2.1. Cell culture and transfection with GFP-Caveolin-1

HeLa cells were cultured in DMEM medium supplemented with 10% (v/v) fetal calf serum and penicillin and streptomycin. All drug treatments were done at 60–70% cell confluency. HeLa cells were transfected with GFP-Caveolin-1 construct (given by Dr. A. Helenius) after seeding them at a density of 2×10^5 cells/ml in 35 mm culture dish with fuGENE 6 kit using 1 μg of plasmid at 6:1 reagent to plasmid ratio. After 6 h of transfection, the medium was replaced with complete medium.

2.2. TIRFM

The cover slip containing the HeLa cells transfected with GFP-Caveolin-1 construct after 12 h were mounted in an Atto chamber containing DMEM without phenol red and 1 mg/ml BSA. The total internal reflection angle was adjusted to observe the dynamics of GFP-Caveolin-1 in regions of the cells in an Olympus IX-81 microscope equipped with an Argon laser (488 nm line). All recordings were performed with 100×-TIRFM (1.45NA) objective with Cascade 512B camera at 10 Hz at 10 ms exposure time for about 90 frames for each recording. The data were recorded before treatment and after the indicated drug treatment. All the videos submitted were recorded with 25 frames per second.

2.3. Confocal microscopy

HeLa cells, after 12 h of transfection, were treated with Taxol or BacIII or 10-DAB for a given time followed by washing with PBS. Cells were then fixed with 3.7% paraformaldehyde (pH 7.4) in PBS, pH 7.4, for 10 min and permeabilized with 0.1% Triton X-100 for 20 min. Non-specific binding was blocked by pretreatment with 3% BSA in PBS for 30 min. The cells were then incubated with anti-Tubulin antibody (at 1:1000 dilution) for 1 h at room temperature followed by washing and incubation with anti-mouse Alexa-568 secondary antibody (1:1000). The cells were then visualized in Zeiss LSM 510 Confocal microscope. Fluorescence emission was detected in 0.5 μm optical sections.

2.4. MTT assay

To assay the viability of cell after treatment with Taxol, BacIII or 10-DAB treatment, HeLa cells were seeded at a density of 5×10^3 cells per well in a 96-well plate and treated with various concentrations of

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Taxol, BacIII or 10-DAB (10–0.15 μM) for 48 h in triplicates. At the end of the treatment, the media was removed and 50 μl of MTT (1 mg/ml) in DMEM (without phenol red) was added to each well and incubated for another 4 h at 37 °C. Formazan crystals were solublized in 50 μl of *iso*-propanol with shaking at room temperature for 10 min. Absorbance was measured at 570 nm using 630 nm filter. Absorbance given by cells treated with DMSO was taken as 100% viability.

2.5. Immunoblotting

HeLa cells (2.0×10^5) were seeded in six well plates till they attained 70% confluency. Cells were treated with the three drugs for indicated times. The wells were washed with PBS and the cells were scraped from the wells to recover the cell pellet by centrifugation. The cells were then resuspended in lysis buffer containing protease inhibitors (10 μg/ml leupeptin, 2 mM phenylmethylsulfonylfluoride (PMSF) and 2 mM Na_3VO_4). Equal amount of protein was loaded on SDS–PAGE (10–15%) and electrophoresed proteins were transferred to a nitrocellulose membrane using Tris–Glycine pH 8.0 and were probed with anti-phosphotyrosine (PY20) (Santa Cruz, sc-508), p-Raf-1 (cell signaling, #9421), p-ERK1/2 (Santa Cruz, sc-7383), ERK1/2 (Santa Cruz, sc-94), p-P38 (Santa Cruz, sc-7973), Bcl-2 (Santa Cruz, sc-7382), anti-mouse IgG, HRP-linked antibody (cell signaling, #7076), anti-rabbit IgG, HRP-linked antibody (cell signaling, #7074), HRP detection reagent, LumiGLO™ reagent and peroxide (cell signaling #7003).

3. Results and discussion

The aim of the present work is to understand the role and dynamics of Caveolin-1 in Taxol induced cytotoxicity of mammalian cells. HeLa cells have shown both very high and moderate expression for GFP-Caveolin-1 and for the present work, we selected the cells that moderately expressed GFP-Caveolin-1. Firstly, treatment with the three drugs did not induce any membrane damage within 1 h of their addition as seen by trypan blue staining of drug treated cells. The number of viable cells remained same in comparison to untreated cells as shown in Fig. 1A. There are several reports indicating that Taxol, 10-DAB and BacIII induce cell death by apoptosis [3,6–8]. The data is Fig. 1B is consistent with the published literature which shows that the HeLa cells exhibited susceptibility to all the three drugs. Different concentrations, starting from 0.15 μM to 10 μM for 48 h, have clear cytotoxic affect on HeLa cells. It is interesting to see that at highest concentration of Taxol (10 μM), BacIII and 10-DAB showed almost same percentage of cell death (around 80%), as compared with the solvent control (DMSO), but at lower concentrations (5–0.15 μM) only Taxol was effective. Moreover, after 24 h of treatment, only Taxol was cytotoxic to HeLa cells (data not shown).

Caveolin-1 of caveolae is not entirely a static entity in mammalian cell membranes as the caveolae undergo K&R dynamics with the plasma membrane. In view of the K&R dynamics of caveolae, we have employed total internal reflection fluorescence microscopy, which is an excellent tool for the study of events that occur at/beneath plasma membrane. It was interesting to see that Taxol and 10-DAB elicited distinct behavior of the GFP-Caveolin-1 dynamics [videos SV1–SV5 and Fig. 2A (IV–IX)]. The HeLa cells expressing GFP-Caveolin-1, before treatment, exhibited a characteristic K&R events which are identical to the observations reported by Pelkmans and Zerial (see video SV1 and Supplementary Video 1 in Ref. [5]). With the addition of Taxol (5 μM) there was an immediate cell surface recruitment of GFP-Caveolin-1 (Fig. 2A-V, and video SV2). One could clearly see the formation of several new clusters of GFP-Caveolin-1 at the cell surface in comparison to untreated cells (videos SV2 vs. SV1; Fig. 2A-V vs. 2A–IV). However, after 1 h of Taxol (5 μM) treatment there was a significant withdrawal of the GFP-Caveolin-1 from the cell surface (Fig. 2A-VI, and video SV3). While no such immediate cell surface recruitment was seen in case of 10-DAB (Fig. 2A-VIII) and BacIII (Fig. 2A-XI). A very interesting observation was made incase of 10-DAB (5 μM) where a several fold increase in the radial velocity of the GFP-Caveolin-1 was observed after 20 min of addition (video SV5), as compared to the movement of the GFP-Caveolin-1 before addition of the 10-DAB (video SV4). The motion was analyzed by plotting the velocity histograms of Caveolae spots of several videos. In brief, the velocity of each caveolae spot seen in videos before and after treatment was measured by obtaining the net distance traveled by the caveolae spot divided by the time taken to reach the end point of observation. The representative velocity histogram, shown in Fig. 2B, highlights the normal

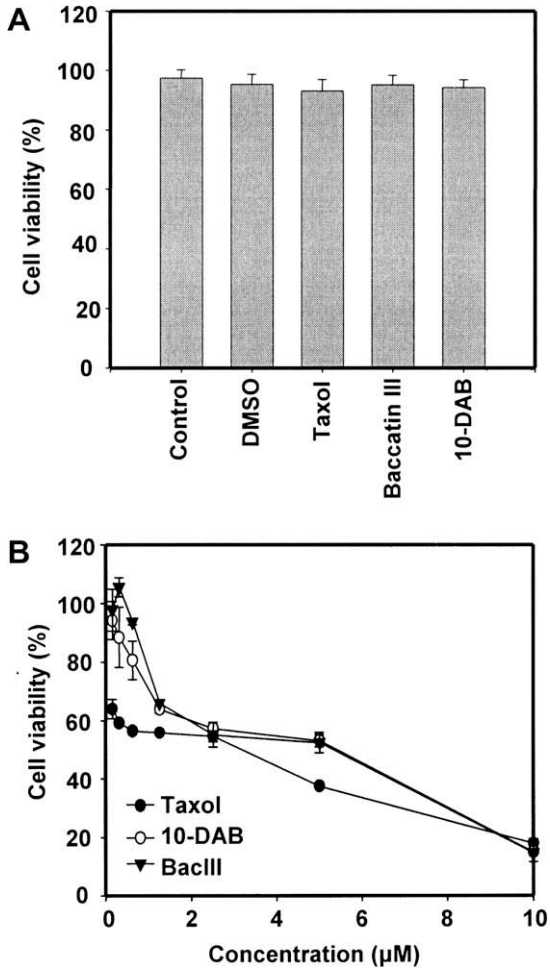

Fig. 1. (A) Trypanblue staining of HeLa cells post drug treatment: Viable cells after treatment with drugs (1 h) was assayed by trypan blue staining and the percentage of unstained cells were plotted. Data shown are in both panels are an average of three independent experiments. (B) Cell viability assay by MTT. HeLa cells were seeded at a density of 5×10^3 cells per well into 96-well plate and incubated with various concentrations of Taxol, 10-DAB and BacIII ranging from 10 μM to 0.15 μM for 48 h and then MTT was added. Absorbance of DMSO treated cells was taken as 100% and the viable cells for drug treatment were plotted.