Effect of Rhodamine-taxol conjugate on Caveolin dynamics

1. Summary
In the present study, Rhodamine- taxol conjugate treatment resulted in a transient recruitment of Caveolin-1 to the cell surface followed by internalization. Conjugate accumulation on the cell surface was predominantly seen in cell lines expressing Caveolin-1-GFP such as HeLa and A431, while cells such as 293T which have very less expression of Caveolin-1 did not show any sign of accumulation. Further these interactions were supported by localization studies of the conjugate and Caveolin-1-GFP by confocal microscopy. Localization was observed as early as 5 minutes and reached saturation by 9 h. These observations thus infer that there is some interaction between taxol and Caveolin-1.

2. Introduction
Taxol, a diterpene, first isolated from *Taxus brevifolia*, is a promising drug against various human cancers (Magri and Kingston, 1998). In a recent report it is shown that taxol induces expression of Caveolin-1, a marker protein of the caveolae in MCF-7 cells and also that the Caveolin-1 phosphorylation at tyrosine-14 is necessary to enhance taxol mediated cytotoxicity (Shajahan et al, 2007). With respect to earlier studies, taxol’s mode of action involves binding to microtubules, stabilizing them further and inducing apoptosis (Manfredi and Horwitz, 1984), but its role in enhancing Caveolin-1 expression in not clear. Moreover, Caveolin-1 is a dynamic entity, as the caveolae undergo a continuous cycle of 'kiss and run' dynamics with the plasma membrane (Pelkmans and Zerial, 2005). Hence in this study, we examined the dynamics of caveolae, their distribution and change in dynamics of taxol vis-à-vis cytotoxicity in HeLa cells expressing Caveolin-1-GFP when treated with Rhodamine-taxol conjugate by total internal reflection fluorescence microscopy (TIRFM) and also compared the accumulation of taxol fluorescent conjugate signal on cells with low expression of Caveolin-1. Most importantly to observe any interaction between taxol and Caveolin-1 confocal study was done to see localization.
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3. Materials
HeLa cells were cultured in DMEM medium supplemented with 10 % (v/v) fetal calf serum and penicillin and streptomycin. Rhodamine-taxol conjugate treatment was done at 60-70 % cell confluency. HeLa cells were transfected with Caveolin-1-GFP construct (gifted by Dr. A. Helenius) after seeding them at a density of 2x10^5 cells/ml in 35mm 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.

4. Methods
To study the interactions, two taxol conjugates having FITC and Rhodamine as labels were synthesized. Preliminary studies to find the compatibility between taxol conjugate signals and GFP signals using TIRFM system filters was done. These studies showed that FITC-taxol conjugate could not be used as it is not suitable for TIRF single spot study. Thus, further studies were done using Rhodamine-Taxol conjugate (RTc).

4.1. TIRFM (Total Internal Reflection Fluorescence Microscopy)

4.1.1. Studies on HeLa with and without GFP transfection
HeLa cells, grown on coverslips, were mounted in an Atto chamber containing DMEM medium without phenol red and 1 mg/ml BSA. The total internal reflection angle was adjusted to observe the dynamics of Rhodamine-taxol in regions of the cells in an Olympus IX-81 microscope equipped with a 532 nm laser line. All recordings were performed with 100X-TIRFM, 1.45NA, objective with Cascade 512B camera at 10 hertz 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 examined were recorded with 25 frames per second. Rhodamine-taxol concentrations used for the study were ranging from 1-10 nM. Various concentrations were used to study the change in the dynamics. In contrary to non transfected cells, Caveolin-1-GFP dynamics and Rhodamine-taxol signals were recorded simultaneous using respective filters in transfected cells.
4.1.2. Studies on A431 and 293T cell lines

Dynamics of RTc were recorded on both the cell lines under the same conditions as described above. None of these cell lines were transfected with Caveolin-1-GFP as these studies would act as control systems. However, recordings on 239T cell lines were taken even after 3 h while in case of A431 they were taken up to 2 h.

4.2. Confocal microscopy

HeLa cells, after 12 h of transfection, were treated with RTc 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. RTc concentrations used were 2-7 nM. The cells were then visualized in Zeiss LSM 510 Confocal microscope. Fluorescence emission was detected in 0.5 µm optical sections.

4.3. MTT assay

To assay the viability of cell after treatment with RTc, HeLa cells were seeded at a density of 5x10³ cells per well in a 96 well plate and treated with various concentrations of RTc 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 isopropanol 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.

5. Results

The aim of the present work is to understand the role and dynamics of Taxol with respect to the dynamics of Caveolin-1 to examine any possible direct interactions involved between the two. Cytotoxicity against HeLa and A431 cell line was found to be similar with the treatment of unlabeled taxol and RTc.
5.1. TIRFM

5.1.1. HeLa cells without GFP transfection

RTc (at concentration 2-7 nM) signal appeared on the cell surface within 5 minutes and the intensity increased with the increase in time. Fluorescence appeared as single bright spots with dynamic movement throughout the membrane. The tiny bright fluorescence spots firstly formed clusters and diffused into membrane with increase in time. Fluorescence appeared to be unevenly distributed i.e. spots did not appear evenly spread throughout the membrane, instead, were seen dense at the periphery and sparse at the center. After about 30 minutes the fluorescence reached saturation and these spots started appearing diffused. Cell membrane and morphology did not change with treatment at these concentrations (1 nM and 2.5 nM) of RTc, but when high concentration (7 nM) was used membrane appeared damaged and fluorescence appeared immediately on the cell surface (FIG 1).
FIG 1: TIRFM images of Rhodamine-taxol conjugate on HeLa cells. Still frame images from videos showing the distribution pattern at 2.5 nM concentration. Arrows showing cluster formation which was seen after treatment with RTc. a) Immediately after the addition of Rhodamine-taxol, b) after 5 min, c) after 10 min, d) after 20 min and e) after 30 min of addition.
5.1.2. HeLa cells with GFP transfection

HeLa cells after Caveolin-1-GFP transfection showed very high and moderate expression of Caveolin-1-GFP. For TIRFM studies, moderately expressed Caveolin-1-GFP cell which showed distinct bright spots with dynamic movement were selected. Fluorescence of Caveolin-1-GFP and Rhodamine-taxol conjugate was recorded simultaneously using the respective filters. Signal spillage and leaking was fool proofed by observing GFP signal using Rhodamine filter and vice versa. With the addition of RTc, Caveolin-1-GFP cell surface recruitment was clearly seen. Kiss and run dynamics of Caveolin-1-GFP was observed. New cluster of Caveolin-1-GFP appeared with progression of time which was not seen before treatment. The other most important observation was the velocity with which the spots appeared to move through out the membrane after the treatment, which was very less before treatment. After about 30 minutes the Caveolin-1-GFP withdrawal from the cell surface was seen (FIG 2).

RTc fluorescence on the other hand also showed similar dynamics in terms of amount of signal appearing on the cell surface and the time taken to appear. RTc distribution appeared exactly as that of the Caveolin-1-GFP confining its density towards the periphery of the cell membrane or to some areas on the surface. Wherever clusters of Caveolin-1-GFP appeared clusters of RTc were also seen indicating the possible interactions involved between the two. The signal strength appeared to increase and then diffuse with increase in time. Saturation in the fluorescence was observed by 1 h after which diffusion of the signal was seen (FIG 2).
FIG 2: TIRFM images showing distribution of Rhodamine-taxol conjugate on the HeLa cell surface (Arrows in white). Clusters formation was seen at many points on the cell membrane (shown by orange arrows). a) Caveolin-1-GFP distribution on HeLa cell. b) Immediately after the addition of Rhodamine-taxol. c) Rhodamine-taxol signal under GFP filter. d), e) & f) Rhodamine-taxol conjugate localization at 5, 15 and 30 min.
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5.1.3. A431 and 293T cell line

Caveolin-1-GFP and Rhodamine-taxol conjugate dynamics appeared very similar with A431 cell, as observed in case of HeLa cells. But the dynamics observed on 293T cell line were very interesting. With the treatment of 1 nM RTc the cells did not show any fluorescence spots on the surface even after 20 minutes (FIG 3) and with the treatment of 2.5 nM concentrations RTc signal was seen but was not confined to the cellular area (FIG 4). Indeed the RTc signal appeared diffused throughout the focused area without any discrimination between cellular and non cellular area. Even when treated with high concentrations such as 7 nM there was no significant localization of RTc was seen on the cell surface (FIG 5), while HeLa cells when treated with same concentrations showed an immediate localization of RTc on cell surface.
FIG 3: TIRFM images showing even distribution of Rhodamine-taxol conjugate through out the cover slip not being confined to cellular area. This did not increase with increase in time. a) Phase contrast micrograph of 293T cells, b) Before addition of RT conjugate (1nM), c) Cells after addition at zero minutes, d), e) & f) after addition, at 10 min, 20 min and 2 h respectively.
FIG 4: TIRFM images showing 293T cells with no prominent localization of Rhodamine-taxol conjugate on cell membrane with concentration (2.5 nM). a) Phase contrast micrograph, b) Before addition of Rhodamine-taxol conjugate, c), d), e), & f) at 10 min, 20 min, 1 h and 2 h after addition.
FIG 5: TIRFM images showing 293T cells with no prominent localization of Rhodamine-taxol conjugate on cell membrane even with high concentration such as 7 nM. a) Phase contrast micrographs of 293T cells, b) Before addition of Rhodamine-taxol conjugate, c) & d) after addition of RT conjugate at 10 min and 1 h.
5.2. Confocal studies:
RTc and Caveolin-1-GFP localization was clearly studied using confocal microscopy. With the treatment of RTc, Caveolin-1-GFP started forming clusters within 5 minutes and RTc started localizing on the cell surface with respect to Caveolin-1-GFP localization (FIG 6). Clusters of RTc were seen prominently than that of the individual spots. This probably infers the early recruitment of Caveolin-1 and internalization of the caveolae. However the intensities of RTc observed were not that prominent as that of the Caveolin-1-GFP.

After about 1 h RTc localization was seen very prominently through out the periphery of the cells wherever Caveolin-1-GFP was localized. An important observation found in confocal imaging was the cluster formation which was prominently seen even after 3 h and receded after 9 h (FIG 6). This probably infers the surface recruitment of Caveolin-1-GFP followed by internalization. RTc reached saturation after 3-4 h which was inferred by the diffused state of signal.
**FIG 6**: Localization of Caveolin-1-GFP and Rhodamine-taxol conjugate on HeLa cells as examined by confocal microscope. Panel A shows Caveolin-1-GFP localization, Panel B shows phase contrast images, Panel C shows RTc localization and Panel D shows merged images of Caveolin-1-GFP signal and RTc signal.
6. Discussion

Our studies suspected a possible interaction between taxol and Caveolin-1 and probably suggest that the taxol side chain may play a role in these interactions (Neesar et al., 2008). It is reported that taxol side chain can interact with the aromatic amino acids of Caveolin-1 (97YWFYRL102) which have been projected to insert into cytoplasmic side of the cell membrane. Thus the present study was to extend more focus on the interactions. The modifications done on the taxol molecule to couple with Rhodamine were exclusively done on OH group at 7th carbon (Magri and Kingston, 1998; Mellado et al, 1984). These modifications did not result in any change in its cytotoxic activity and its ability to interact with Caveolin-1 which was quite evident by the localization of Rhodamine-taxol conjugate on cell surface. The cell lines used for the study were chosen based on their expression abilities of Caveolin-1. As HeLa cell show high expression of Caveolin-1, in A431, it is moderately expressed and in 293T cell line, its expression is very low (Koleske et al, 1995). This helped us to understand the role of Caveolin-1 in taxol mediated cytotoxicity.

Previous studies showed that taxol upregulates the expression of Caveolin-1 in MCF-7 cell line (Yang et al, 1998). Further our studies also showed that Caveolin-1 surface recruitment is seen when HeLa cells were treated with taxol followed by withdrawal, which infers the internalization of caveolae. With these studies it is known that there is some peculiar Kiss and Run dynamics seen when the cells are treated with taxol and the possible interactions involved between taxol and Caveolin-1. With the treatment of low concentrations of RTc (2-7 nM) similar dynamics of Caveolin-1 was seen on cell membranes of HeLa and A431 cell lines as studied by TRIF microscopy. The method employed is a good tool to examine the events that occur at/beneath plasma membrane. RTc started localizing on the cell surface after about 5 minutes and reached saturation after 2-3 h. New cluster formation of the RTc was seen similarly as observed in Caveolin-1 forming caveolae. A large amount of Rhodamine-taxol seems to accumulate at cell membrane.

The HeLa cells expressing Caveolin-1-GFP, before treatment, exhibited characteristic K&R events which are identical to the observations reported by Pelkans and Zerial. With the addition of RTc there was immediate surface recruitment Caveolin-1-GFP. As
the RTc started localizing on the cell surface, it was interesting to see the formation of Caveolin-1-GFP clusters and RTc clusters simultaneously using respective filters. These RTc clusters, however, were seen diffusing with increase in time inferring the possible internalization of RTc by caveolae.

Interesting observations were acquired when 293T cells were treated with Rhodamine-taxol conjugate. There was no defined accumulation of RTc on the cellular surface of 293T cells. Even with high concentrations of RTc, there was no localization on the cell membrane and we could not observe any clusters on the cell surface after about 3 h of treatment. This clearly indicates that in localization of taxol on the cell surface there is a definite role played by Caveolin-1. This also suggests that taxol enhances expression of Caveolin-1 and might be engulfed through caveolae showing the “Kiss and Run” dynamics before it actually triggers the apoptosis. These observations helped us understand the interactions involved between taxol and Caveolin-1. These observations lead to the conclusions that Taxol probably interacts with Caveolin-1, caveolae helps in the internalization of taxol and the amount of taxol binding to the cell surface is greater than the cytotoxicity shown.

7. References


induce distinct changes in the 'kiss and Run' dynamics of caveolae. FEBS Letters.


