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HIV-1 Tat is unconventionally secreted through the plasma membrane

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Abbreviations used: FGF, fibroblast growth factor; HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat.

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Abstract

The Tat protein is required for efficient HIV-1 transcription. Moreover, Tat is secreted by infected cells and circulating Tat can affect several cell types, thereby contributing to HIV-1 pathogenesis. We monitored Tat secretion by transfected CD4⁺ T-cells. A Tat chimera carrying an N-glycosylation site did not become glycosylated when expressed into cells, while the chimera was glycosylated when mechanically introduced into purified microsomes. These data indicate that secreted Tat does not transit through the endoplasmic reticulum. The use of pharmacological inhibitors indicated that Tat secretion pathway is unusual compared to previously identified unconventional secretion routes and does not involve intracellular organelles. Moreover, cell incubation at 16°C inhibited Tat secretion and caused its accumulation at the plasma membrane, suggesting that secretion takes place at this level.

Introduction

The human immunodeficiency virus type 1 (HIV-1) transactivating protein Tat is a small (86-102 residues) and basic protein that enables efficient transcription of viral genes and has thus a key role in virus replication (Jeang et al., 1999). Although devoid of signal sequence that allows targeting to the ER/Golgi secretion apparatus, Tat can be secreted by infected cells (Ensoli et al., 1990). Such unconventional secretion is also observed for some cell proteins. They include homeodomain transcription factors, HMGB1, a non-histone nuclear factor as well as numerous cytokines, including FGF-1, FGF-2 and IL-1 β (Nickel and Rabouille 2009). Their secretion pathways are collectively designed as nonclassical or unconventional export routes (Dupont et al., 2007; Nickel and Rabouille 2009). These processes are intriguingly diverse and, so far, there seems to be as many pathways as proteins. Some, such as FGF-1, FGF-2 and homeodomains, are directly released through the plasma membrane, although by different mechanisms (Dupont et al., 2007; Prudovsky et al., 2002; Temmerman et al., 2008). In other cases, secretion relies on intracellular organelles. This intermediate is a secretory lysosome for IL-1 β (Andrei et al., 1999; Gardella et al., 2001), and the ER or the Golgi apparatus for the Tax protein of the human T-cell leukemia virus type 1, another human retrovirus whose transactivating protein is secreted by infected cells (Alefantis et al., 2005; Jain et al., 2007). The overall secretion route has been identified for most proteins that are secreted in an unconventional manner but, despite the fact that accumulating evidence suggests that circulating Tat is involved in evolution to AIDS (Gallo 1999; Huigen et al., 2004; Rubartelli et al., 1998), this is not the case for HIV-1 Tat.

Here we show that Tat secretion does not rely on intracellular organelles but takes place at the plasma membrane.

Materials and Methods

Plasmids and transfection

Tat (BH10 isolate, 86 residues) was cloned into pBi Tet expression vector (Clontech) that was transfected together with pUHD 15.1 Neo (Clontech) at a ratio of 10 pBi /1 pUHD to ensure expression into mammalian cells. The pET11d vector (Novagen) was used for Tat expression in *E. coli*. Tat-PNVSG (termed Tat-Glyc) was obtained by PCR using appropriate primers. Its coding sequence entirely sequenced (Genome Express, Meylan). The Jurkat CD4⁺ clone E6-1 was obtained from the ATCC, grown following their recommendations and transfected using electroporation as described (Vendeville et al., 2004).

Reagents

The anti-Tat monoclonal antibodies for immunofluorescence and ELISA were from santa cruz biotechnology (sc-65912) and the Programme EVA Centre for AIDS Reagents (#ARP352), respectively. Rabbit anti-Tat (HXB2) antiserum was kindly provided by Erwann Loret (Marseille, France). Secondary antibodies were from Jackson ImmunoResearch. Chemicals and phalloidin conjugated to tetramethylrhodamine were from Sigma-Aldrich. Recombinant Tat and Tat-Glyc were produced in *E. coli* and purified as detailed earlier (Vendeville et al., 2004). Microsomes were obtained by centrifugation of postnuclear supernatant from homogenized Jurkat cells (Vendeville et al., 2004).

Secretion assays

Jurkat cells were transfected with pBi-Tat (20 µg), pBi-firefly luciferase (2 µg) and pUHD (2 µg). Two days after transfection, cells were washed and transferred (7.5×10^5 cells / well, in triplicate) to an ELISA plate that had been coated overnight at 4°C with monoclonal anti-Tat antibody (1 µg/ml) before saturation with 8% (w/v) skimmed milk in phosphate-buffered saline (PBS). Dilutions of recombinant Tat (20 pg – 1 ng/well) were also added to the plate for calibration purposes. After 6 h at 37°C (except when otherwise stated), cells were transferred to microtubes, centrifuged and the supernatant was frozen for later luciferase assay. Cells were then lysed and part of the lysate was used to assay intracellular Tat by ELISA, while the rest was frozen for the luciferase assay that was performed using a Promega kit. Secretion was calculated using the Tat (or luciferase) amounts present in the medium and intracellularly using the medium / (intracellular + medium) formula.

Immunofluorescence

Tat- or Tat-EGFP-transfected Jurkat cells were allowed to adhere to alcian-blue coated coverslips for 3 min at room temperature and were fixed in 3.7% paraformaldehyde before permeabilization with 0.1% saponin. Tat and cortical actin were then stained by immunofluorescence (Vendeville et al., 2004) and phalloidin-tetramethylrhodamine, respectively. Tat-EGFP was localized using EGFP fluorescence. Cells were mounted for observation using a Zeiss LSM 510 Meta confocal microscope and a 63 x objective. Median optical sections are shown.

Transactivation assays

Jurkat cells were cotransfected with 3 µg of a Tat or Tat-Glyc expressing vector, together with 7 µg of pGL3-LTR, which expresses firefly luciferase under control of the Tat-activated HIV-1-LTR promoter, and 1 µg of pRL-TK (Promega), which codes for renilla luciferase under the control of the herpes simplex virus thymidine kinase promoter. After 48 h, cells were lysed for dual luciferase assays (Promega) and transactivation activity was calculated using the firefly/renilla activity ratio (Vendeville et al., 2004).

N-glycosylation analysis

To assay cell-free N-glycosylation, Tat or Tat-Glyc (10 μ l at 2 mg/ml in 150 mM NaCl, 50 mM citrate, pH 7.1) were mixed with purified Jurkat microsomes (170 μ l, 15 mg protein/ml in 150 mM KCl, 2 mM MgCl₂, 2 mM DTT, 10 mM Pipes pH 7.1 supplemented with an antiprotease cocktail (Complete, Roche)) before sonication for 2 min and incubation at 37°C for up to 3 h. Where indicated, tunicamycin was used at 1 μ g/ml. Samples were then cooled on ice, treated with 0.1 M Na₂CO₃ to remove unimported proteins, before centrifugation for 10 min at 160,000 g and pellet resuspension in SDS/PAGE/tricine sample buffer. After Western blotting, Tat was detected using rabbit anti-Tat antiserum and the ECL⁺ reagent (GE Healthcare).

To examine whether Tat travels through the ER during export, Jurkat cells were transfected with Tat or Tat-Glyc. After 48 h, cells were lysed for Tat immunoprecipitation using ARP352 monoclonal antibody, followed by swine antimouse antibodies (Nordic Immunochemicals) and protein A sepharose (GE Healthcare). Immunoprecipitated proteins were resuspended in SDS/PAGE/tricine sample buffer. After Western blotting, Tat was detected using rabbit anti-Tat antiserum and the ECL⁺ reagent.

Results and Discussion

In this study we studied Tat secretion pathway using Jurkat cells. These CD4⁺ T-cells were cotransfected with a Tat-encoding plasmid and a luciferase vector that was used as a control for transfected cell lysis. To prevent Tat oxidation by air oxygen that causes aggregation (Chang et al., 1997) and makes it unreactive to antibodies, we directly plated transfected cells onto ELISA plates that have been coated with anti-Tat monoclonal antibodies before cell incubation in degassed medium. Antibodies were thus allowed to capture secreted Tat before it gets oxidized in the medium, and the plate was revealed using standard ELISA procedures. Cells were recovered, washed and lysed for the assay of intracellular Tat by ELISA, using the same antibodies. Using this experimental system, we found that Tat secretion was linear for up to 6 h at 37°C (Fig. 1A). After this time, 25-30% of Tat was found in the medium, indicating that transfected cells secrete ~5% of cellular Tat per hour. Hence, Tat secretion is an efficient process that takes place in the absence of cell lysis since no release of cotransfected luciferase was observed with time, whatever the temperature (Fig. 1B). As reported earlier for COS-1 cells (Chang et al., 1997), Tat secretion was temperature-dependent and was essentially blocked at 16°C (Fig.1A), a temperature that severely slows down membrane traffic and allows to accumulate transport intermediates in the exocytic (Presley et al., 1997) or endocytic (Magadan et al., 2006) pathways. In the search of such structures that might be key intermediates in Tat secretion, we examined by confocal microscopy Tat intracellular localization in cells kept at 16°C for 6 h. Whatever the temperature, most Tat concentrates in the nucleus of Jurkat cells (Fig.2). Nevertheless, while no plasma membrane labeling was detectable at 37°C, it became much more evident upon incubation at 16°C (Fig.2, arrows). This was observed using both immunofluorescence or a Tat-EGFP chimera in either fixed (Fig.2) or living cells (data not shown). These results indicated that Tat secretion takes place directly through the plasma membrane, and does not involve intracellular intermediates.

To further confirm this finding and characterize Tat secretion, we tried to interfere pharmacologically with this process using agents known to affect previously characterized unconventional secretion pathways. Drugs were used at concentrations that were found effective on T-cell lines (Alami et al., 1998; Vendeville et al., 2004). These concentrations only induced a minute luciferase release (< 0.8%; Fig.3), indicating that cell integrity was preserved throughout the experiment. Because Tat binds to microtubules (Chen et al., 2002) and FGF-1 secretion depends on the actin cytoskeleton (Prudovsky et al., 2002), we first

examined the effect of cytoskeleton poisons. Neither nocodazole, nor taxol that target microtubules significantly affected Tat secretion, while cytochalasin D that perturbs the actin cytoskeleton moderately inhibited secretion (by ~30%; Fig.3). FGF-2 secretion requires Na^+, K^+ -ATPase activity that is blocked by ouabain (Florkiewicz et al., 1998), but this inhibitor did not affect Tat secretion. Endosome neutralizing drugs such as bafilomycin A1, monensin and nigericin prevent IL-1 β import into late endocytic vesicles thereby inhibiting IL-1 β secretion (Andrei et al., 1999). None of these agents interfered with Tat secretion (Fig.3), indicating that Tat follows an original secretion pathway.

We then examined in more details whether, as described for HTLV-1 Tax (Alefantis et al., 2005; Jain et al., 2007), Tat transited by the ER and the Golgi apparatus on its way out. To this end we first used brefeldin A (BFA) that causes Golgi collapse and prevents exocytosis by the ER/Golgi pathway (Chardin and McCormick 1999), as well as Tax secretion (Alefantis et al., 2005). As reported earlier for COS-1 cells (Chang et al., 1997), BFA did not affect Tat export by Jurkat T-cells (Fig.3), indicating that Tat secretion does not involve the ER or the Golgi. To confirm this result using a different and drug-free approach, we prepared a Tat chimera bearing a N-glycosylation site (PNVSG) at its carboxyl terminus (Tat-Glyc). This tag should enable to trace the passage of a protein by the ER where the motif gets glycosylated (Johannes et al., 1997).

We first checked whether the glycosylation tag affected Tat biological activity. To this end, we used a transactivation assay. Indeed, this activity requires Tat interaction with several partners, *i.e.* proteins and RNA (Jeang et al., 1999) and therefore provides information regarding the preservation of Tat structure and reactivity. Jurkat cells were cotransfected with a Tat vector, a plasmid expressing *Firefly* luciferase under the control of HIV-1 LTR and another vector expressing *Renilla* luciferase behind a CMV promoter. Transactivation was quantified as the increase in the *Firefly* / *Renilla* luciferase activity ratio (Vendeville et al., 2004). Since the Glyc tag is only 5-residues long, it was not surprising to find that Tat-Glyc retained entire Tat transactivation activity (Fig.4A). We concluded that Tat conformation and reactivity was not affected by the presence of the Glyc tag that should not modify Tat secretion route.

We then examined whether the tag was correctly exposed so that Tat-Glyc was indeed glycosylated when reaching the ER lumen. We first tried to express Tat-Glyc behind a signal sequence to trigger Tat-Glyc import into the ER but this chimera was very poorly expressed into cells and was quickly degraded probably because, due to its basic nature, Tat could not be efficiently imported into the ER (data not shown). We thus prepared recombinant Tat-Glyc from *E.coli*, and microsomes from Jurkat cells (Vendeville et al., 2004). We then mechanically introduced Tat-Glyc into microsomes by sonication. Following incubation at 37°C, Tat-Glyc glycosylation was monitored by anti-Tat Western blot. An additional band (arrow) corresponding to a gain of ~5 kDa for Tat-Glyc appeared upon incubation (Fig.4B). This band was not observed in the presence of tunicamycin, a well established glycosylation inhibitor (Leavitt et al., 1977) or when Tat was used instead of Tat-Glyc. Hence, the glycosylation motif was functional in Tat-Glyc. Nevertheless, when Tat-Glyc was immunoprecipitated from transfected cells before Western blot no glycosylation was detectable (Fig.4C). Altogether with the absence of BFA effect on Tat secretion (Fig.3) and the accumulation of Tat at the plasma membrane at non-permissive temperature (Fig.2), these data indicate that Tat does not transit by the ER on its way out but is instead directly transported through the plasma membrane.

Conclusions

It should be noted that, although most cellular Tat is nuclear in cell lines such as Jurkat cells (Fig.2) and that Tat does not follow the conventional ER/Golgi secretion pathway, Tat secretion is an efficient process. Nevertheless, it is difficult to visualize it at export sites, *i.e.* on the plasma membrane. This is probably due to efficiency of the export process that prevents Tat accumulation at this level. The identification of Tat partner(s) enabling its recruitment at the plasma membrane should help to understand this unconventional secretion pathway. It would also be interesting to examine whether cellular or viral proteins regulate Tat secretion. For instance, Tat owns seven Cys residues and one of them could be palmitoylated, as is the case with many proteins that associate with the plasma membrane (Linder and Deschenes 2007).

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Author contribution

FR and SD performed secretions assays and some immunofluorescence experiments. AB prepared Tat-Glyc and performed glycosylation experiments. BB designed the study, performed some immunofluorescence experiments and wrote the paper.

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Figure Legends

Fig.1 Time and temperature dependence of Tat secretion. Jurkat cells were transfected with Tat and luciferase expression vectors. After 48 h, they were plated onto ELISA plates precoated with anti-Tat monoclonal antibody. Secretion was allowed for the indicated time and temperature before Tat ELISA and luciferase assay. Intracellular Tat and luciferase were also assayed and secretion was calculated as medium / (cell + medium) (%) for both Tat (A) and luciferase (B). Data are mean \pm SEM of three independent experiments performed in triplicate.

Fig.2. Tat is detected on the plasma membrane upon incubation at reduced temperature. Tat- or Tat-EGFP-transfected Jurkat cells were incubated at the indicated temperature before fixation, permeabilization and labelling with fluorescent phalloidin, which stains cortical actin just beneath the plasma membrane. Tat was detected using a monoclonal antibody and conventional immunofluorescence, while Tat-EGFP was localized using EGFP fluorescence. Representative medial confocal sections are shown. Bar, 10 μ m.

Fig.3. Pharmacological characterization of Tat secretion process. Secretion was allowed for 6 h in the presence of the indicated molecule. Drugs were used at the following concentrations. Taxol, 0.1 μ M; nocodazole (Noco), 10 μ M; brefeldin A (BFA), 5 μ g/ml; cytochalasin D (CytoD) 5 μ M; ouabain, 100 μ M; nigericin 0.1 μ M; monensin, 1 μ M; bafilomycin A1 (Bafilo), 100 nM. Higher drug concentrations were not more effective in inhibiting Tat secretion (data not shown). Data are mean \pm SEM of three independent experiments performed in triplicate.

Fig.4. A Tat chimera bearing a glycosylation signal does not get glycosylated in living cells. A, Cells were cotransfected with a Tat- or Tat-PNVSG (termed Tat-Glyc) expressing vector and a plasmid expressing *Firefly* luciferase behind HIV-1 LTR. A plasmid encoding *Renilla* luciferase behind a Tat-independent promoter was used for normalization purposes, and transactivation was calculated from the *Firefly* / *Renilla* activity ratio. Data are mean \pm SEM of two independent experiments performed in quadruplicate. B, Jurkat microsomes were sonicated with recombinant Tat-Glyc or Tat before incubation at 37°C in the absence or presence of tunicamycin (TM) before SDS/PAGE and anti-Tat Western blot. An additional band (arrow) of molecular weight corresponding to glycosylated Tat-Glyc appeared upon incubation at 37°C in the absence of tunicamycin. C, Jurkat cells were transfected with a Tat- or Tat-Glyc-expression vector. One day later, Tat was immunoprecipitated before anti-Tat Western blotting. * indicates the position of Tat dimers. Data are representative results of three independent experiments.

Fig.1, Rayne et al

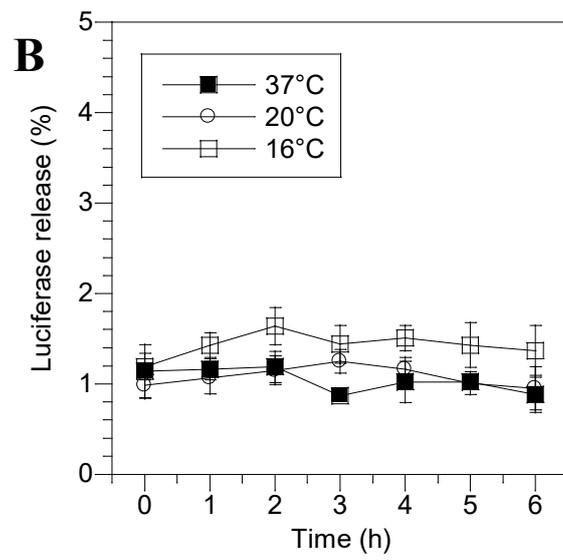
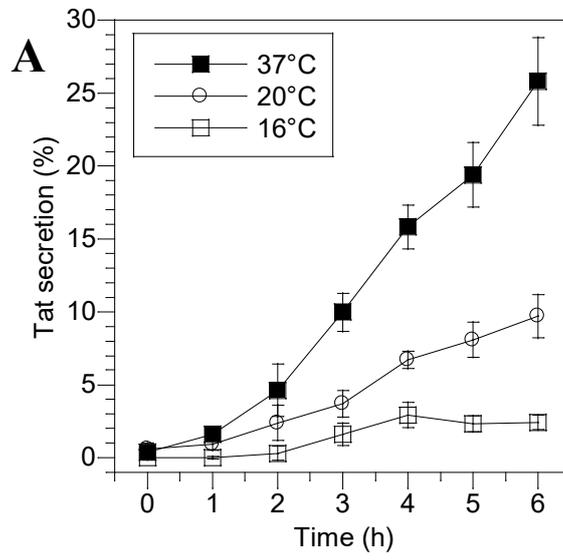


Fig.2, Rayne et al

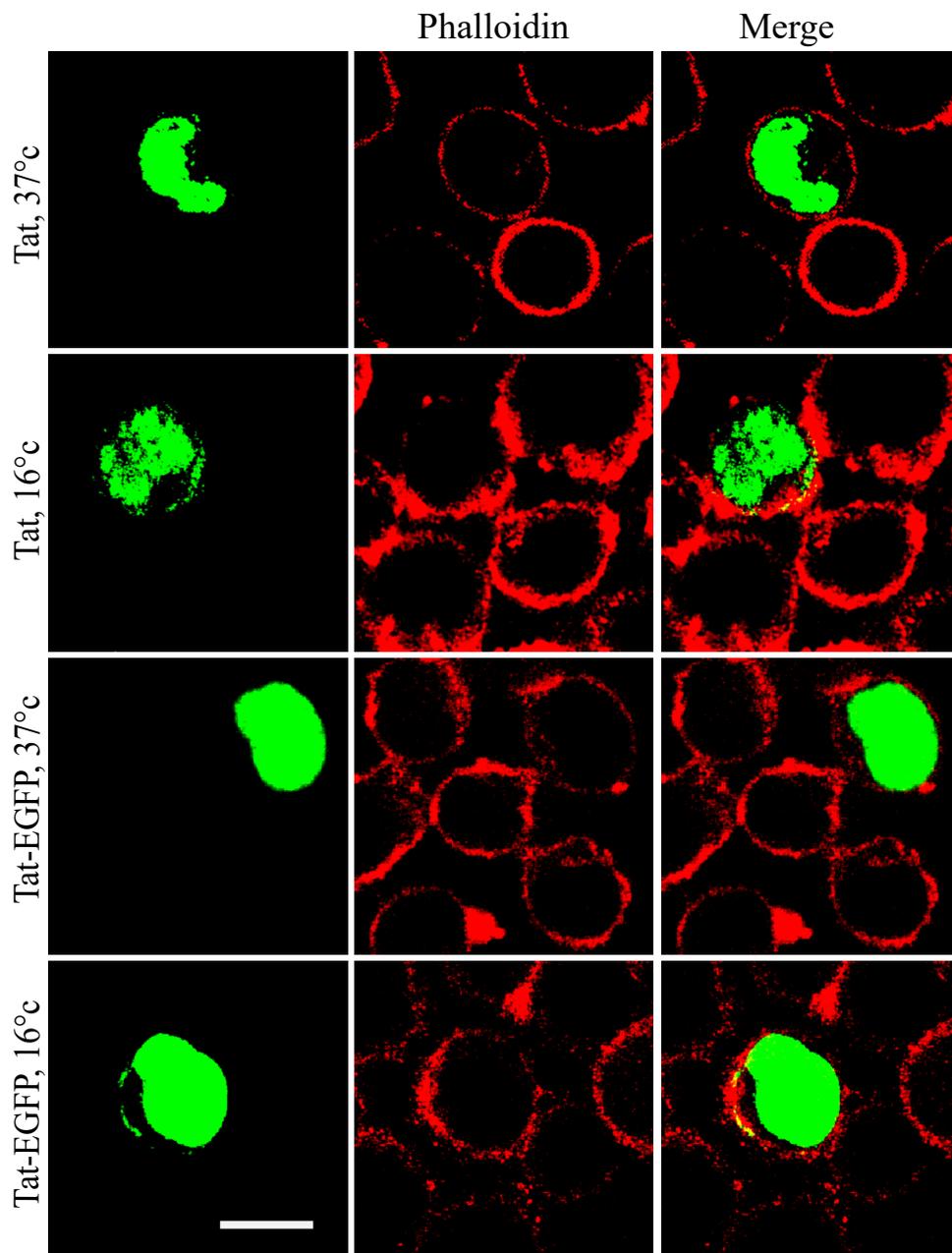


Fig.3, Rayne et al

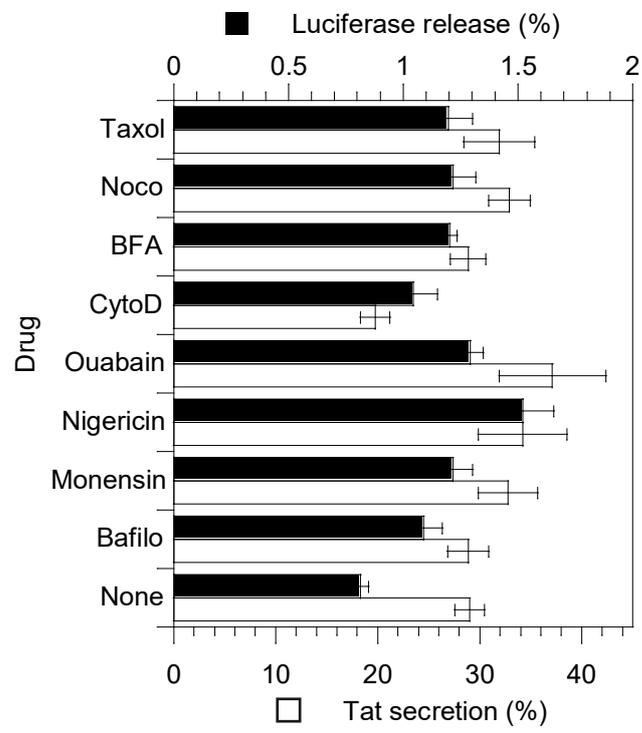


Fig.4, Rayne et al

