TubulinTracker, a Novel *In Vitro* Reporter Assay to Study Intracellular Microtubule Dynamics and Cell Cycle Progression

I. Brandsma, N. Moelijker, M. Hoogenboom-Geijer, R.Derr and G. Hendriks

Toxys B.V., Leiden, The Netherlands

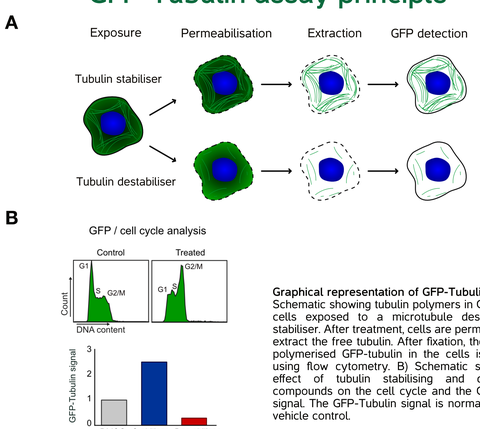
Introduction

Aneuploidy, the presence of an abnormal number of chromosomes, can be caused by any process that interferes with chromosome segregation during mitosis, including microtubule disruption or inhibition of the Aurora A/B/C cell cycle kinases. To detect aneugenicity as a result of chemical exposure, typically the micronucleus assay is used. However, both broken DNA fragments caused by clastogenic agents and mis-segregated chromosomes caused by aneugenic agents can lead to the formation of micronuclei.

Here, we established a reporter assay to study the effect of substances on tubulin stability, to provide more insight into the cause of aneuploidy. The use of a GFP-tagged tubulin reporter allows the direct visualisation of microtubuli, using either live cell microscopy to follow the dynamics during the cell cycle or flow cytometry.

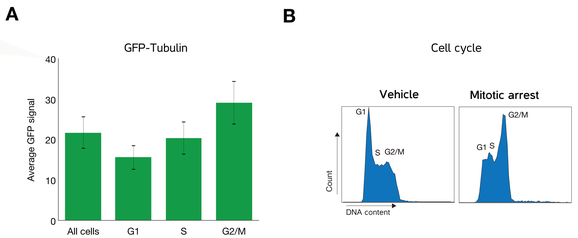
To measure the effect of substances on microtubule stability, cells were treated, fixed and the GFP signal was quantified using flow cytometry. In order to assess the effect of the compounds on the cell cycle distribution, a DNA staining (Hoechst) was included. The mechanistic insight into the MOA provided by this assay is important for hazard identification and part of weight of evidence approaches.

GFP-Tubulin assay principle



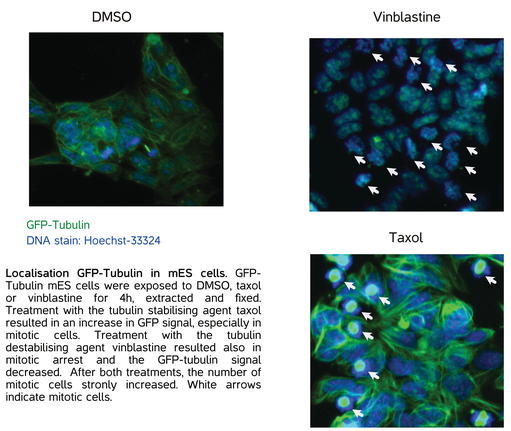
Graphical representation of GFP-Tubulin assay. A) Schematic showing tubulin polymers in GFP-Tubulin cells exposed to a microtubule destabiliser or stabiliser. After treatment, cells are permeabilised to extract the free tubulin. After fixation, the remaining polymerised GFP-tubulin in the cells is quantified using flow cytometry. B) Schematic showing the effect of tubulin stabilising and destabilising compounds on the cell cycle and the GFP-Tubulin signal. The GFP-Tubulin signal is normalised to the vehicle control.

Tubulin dynamics during the cell cycle



GFP-Tubulin signal during the cell cycle A) The GFP-Tubulin signal in vehicle treated cells changes during the cell cycle, as quantified by flow cytometry using the Hoechst DNA stain for cell cycle analysis. In G1, the GFP-Tubulin signal is lower than in S and G2/M phase cells. B) Mitotic arrest leads to an increase in cells in G2/M phase. This arrest can be due to microtubule disruption or inhibition of mitotic kinases. In both cases, the arrest will lead to an increase in the GFP-Tubulin signal. To be able to distinguish effects on tubulin itself from the cell cycle effect, the GFP-tubulin signal was quantified in G2/M phase cells.

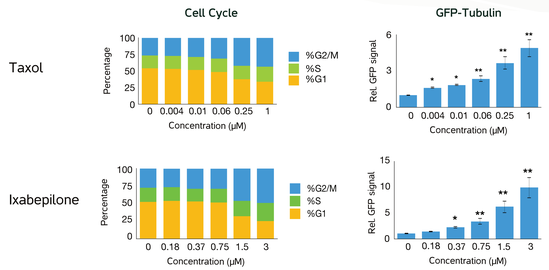
GFP-Tubulin localization



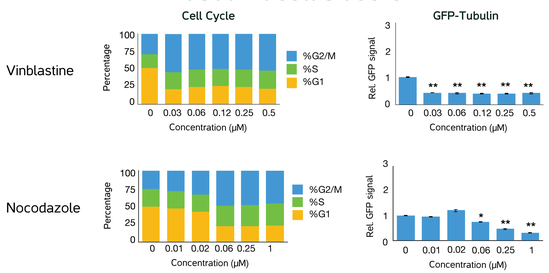
Localisation GFP-Tubulin in mES cells. GFP-Tubulin mES cells were exposed to DMSO, taxol or vinblastine for 4h, extracted and fixed. Treatment with the tubulin stabilising agent taxol resulted in an increase in GFP signal, especially in mitotic cells. Treatment with the tubulin destabilising agent vinblastine resulted also in mitotic arrest and the GFP-tubulin signal decreased. After both treatments, the number of mitotic cells stronly increased. White arrows indicate mitotic cells.

Tubulin poisons disrupt microtubule dynamics

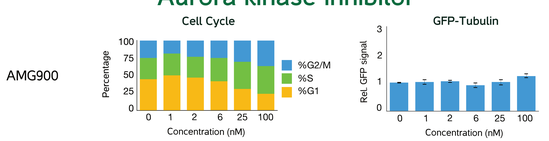
Tubulin stabilisers



Tubulin destabilisers

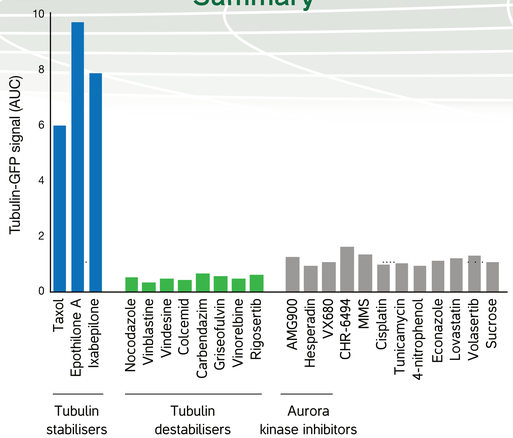


Aurora kinase inhibitor



Effect of compound exposure on the cell cycle and GFP-Tubulin signal. Left panels, show the percentage of cells at each cell cycle phase, after 4h of treatment with the indicated compounds. Right panels, the analysis of GFP-tubulin signal in G2/M cells only. Exposure to all substances lead to an increase in G2/M phase cells. Tubulin stabilisers significantly increased, while tubulin destabilisers significantly decreased the GFP-Tubulin signal. Bars represent the mean for 3 biological replicates, error bars show SEM. *p<0.05 **p<0.005 treated vs DMSO control (t-test).

Summary



Overview of Tubulin stabilising and destabilising compounds. The mES GFP-Tubulin assay was validated using 23 compounds: 3 microtubule stabilisers, 8 destabilisers and 12 agents not known to affect microtubuli. GFP-Tubulin cells were treated for 4h, using 10 concentrations of the test substances. To classify agents, the area under the curve (AUC) was calculated for the exposures, normalising both the exposure concentration as well as the fold-change in GFP-signal. Compounds stabilising microtubuli showed a large increase in AUC, while tubulin destabilising compounds showed a decrease in AUC. For compounds not affecting tubulin stability, the AUC was around 1 (dotted line).

Conclusions

- Microtubule dynamics were studied in the context of a living cell, using physiological expression levels of the GFP-Tubulin.
- Using a stable GFP-Tubulin reporter cell line microtubule poisons can be reliably identified.
- Exposure to other agents affecting mitotic progression or causing other types of damage does not affect the GFP-Tubulin signal.
- The assay provides insight into the MOA of aneugenic agents