***Sequential re-submission of inhibitors at low concentrations***

*Suppressing lactate generation*

In all dose-dependent tests, a gradual decrease in the inhibitory effect of the two compounds was observed after a certain period. The inhibitory potency appeared to disappear more rapidly at the lower concentrations, suggesting degradation of the compound in the medium or by cell metabolism. Therefore, the efficacy of selected inhibitors was evaluated by sequentially re-adding low concentrations at specific time points. In the experiment, the inhibitors were first added to the medium at lower concentrations at the start of inoculation and then added again at 24-hour intervals. As a control, identical concentrations of DMSO were sequentially added to the medium with the vehicle.

When lactate accumulation in **Jurkat cells** was monitored, convincing lactate suppression was observed with both inhibitors tested, even at the lowest inhibitor concentrations (10 µM). No significant increase in lactate concentration in the medium was observed within 72 hours after incubation (**Fig. 6A**).

Virtually no cytotoxic effects were observed in **Jurkat cells** after 72 hours from the inhibitors that were added sequentially. The following average percentages of dead cells were observed at the end of fermentation in the cells in the presence of a vehicle (3.21±0.28%), and the cells were sequentially treated with 10 µL cmpd No. 9 (3.24±0.25%) and cmpd No. 30 (3.64±0.23%).

Sustained inhibition of lactate formation was observed in **Caco-2 cells** with both inhibitors tested. Almost no dose-dependent effect of cmpd No. 9 and cmpd No. 30 was observed at all concentrations tested, indicating that effective suppression of lactate formation could be prevented at the lowest 10 µM concentrations (**S7 Fig**.).

No significant cytotoxic effects of the inhibitors were observed after 72 hours of incubation, although different concentrations of inhibitors were added sequentially. The following average percentages of dead cells were observed in the medium without added inhibitors (4.45±0,41%) and with the cells sequentially treated with 10 µM cmpd No. 9 (4.03±0.32%), and cmpd No. 30 (4.24±0.38%).

In **COLO 829 cells**, slightly weaker lactate formation was observed at 10 µM concentrations in the presence of both inhibitors, while extensive suppression was observed at 15 and 20 µM concentrations (**S8 Fig.).**

After 72 hours of incubation, no significant cytotoxic effect of the inhibitors could be detected, although different concentrations of inhibitors were added sequentially. The following average percentages of dead cells were observed in the medium without adder inhibitors (2.44±0.25%) and with the cells sequentially treated with 10 µM cmpd No. 9 (2.84±0.19%) and cmpd No. 30 (2.42±0.21%).

Similar results of suppressed lactate formation with inhibitors were obtained in **MDA-MB-231** cells (**S9 Fig**.).

No significant cytotoxic effect of the inhibitors could be detected after 72 hours. The following average percentages of dead cells were observed in the medium without added inhibitors (2.52±0.356%) and with the cells sequentially treated with 10 µM inhibitor No. 9 (2.35±0.09%) and inhibitor No. 30 (2.32±0.21%).

*Suppressing ROS/SOX generation*

Previously, effective suppression of lactate formation was observed by sequential re-administration of inhibitors at low concentrations, therefore the same strategy was used to evaluate the suppression of ROS and SOX by inhibitors in selected tumorigenic cells. First, cells were grown for 72 hours in RPMI 1640 GlutaMax medium containing phenol red. Since the fluorescent dyes used in the measurement kit could interfere with phenol red, the cells were transferred to a phenol red-free medium as described in Materials and Methods. Therefore, the ROS/SOX concentrations were only measured at the end of the incubation after 72 hours.

However, it is important to note that superoxide is a short-lived molecule and can be rapidly dismutated into the more stable hydrogen peroxide [39].

For the detection of ROS and SOX levels in the cells, the cells in 100 µL of medium were washed with PBS by standard centrifugation protocol and then placed into a well on the 96-well black wall/clear bottom plate. Suspension cells (Jurkat) were Washed by centrifugal with Wash Buffer to stick to the well. Positive control was estimated by treatment of ROS inducer Pyocyanin and negative control by treating with the ROS inducer N-acetyl-L-cysteine. To each well 100 µL ROS detection reagent (Green) and SOX detection reagent (Orange) were added as specified in the Cellular ROS/Superoxide Detection Assay Kit (ab139476 Abcam, Cambridge, UK). For measuring superoxide Cellular Superoxide Detection Assay Kit (ab139477 Abcam, Cambridge, UK) was used that contains “Orange” but not the “Green” reagent. Relative fluorescence was measured by a microplate reader (Biotek, Vermont, USA), using Ex/Em 525 nm for the “Green” and Ex/Em 550/620 for the “Orange” reagent.

Selected PFK1 inhibitors (cmpds No. 9 and 30), which are thought to reduce cytosolic NADH, primarily prevent SOX formation but not the formation of other oxygen stress reagents. Accordingly, the dose-dependent effect of the two inhibitors in reducing ROS formation was lower. While the 5 µM concentration showed an approximately 50% reduction, the 10 and 20 µM concentrations reduced ROS formation by approximately 65%. It appeared that the maximum reduction in ROS formation was achieved at a concentration of about 10 µM, which was characteristic of all tumorigenic cell lines tested. The following statistical significance differences between the vehicle and the cells treated with inhibitors were obtained: **Fig. 6B, (S10 Fig., S11 Fig., Fig., S12 Fig**.).

In terms of reduction in ROS formation, greater suppression of SOX levels was observed by the two selected PFK1 inhibitors in all tumorigenic cell lines. A reduction in SOX concentration of about 70 % was observed at a 5 µM concentration of both inhibitors, while at a concentration of 10 µM, virtually no SOX was present.

It is worth noting that in COLO 829 cells treated with both inhibitors, the most significant statistical differences were observed in the suppression of SOX generations compared to the other tumorigenic cells tested. However, the evaluation of lactate suppression by both inhibitors showed the least significant differences in COLO 829 cells compared to other tumorigenic cells tested.