**Co-culture of tumorigenic Jurkat cells and activated T-cells.**

*Isolating immune cells*

Peripheral Blood Monocyte Cells (PBMCs) were obtained according to the protocol approved by the National Medical Ethics Committee (0120-21/2020/4). Depletion of non-T cells from the PBMCs was conducted by Ficoll® Paque gradient centrifugation followed by Pan T Cell Isolation kit according to the manufacturer’s instructions (Miltenyi Biotec GmbH, Auburn, CA). Consequently, unharmed T-cells were isolated containing levels of enriched CD4 helper T-cells and CD8 cytotoxic T-cells. Isolated T-cells were grown in 12 well plates containing 1 mL medium. Growth stimulating medium contained in a total volume of 50 mL, X-Vivo 15 medium (Lonza, Basel, Switzerland) plus 5% FBS; 6.25 µL of 80 IU/mL IL-2; 70 µL/mL ImmunoCultTM human CD3/CD28 T-cell activator (Stemcell Technologies, Vancouver, Canada); and 0.17 µL 5 µM 2-mercaptoethanol. The T-cells were seeded in the medium at a concentration of 5.105 cells per mL. After 3 days of incubation when the number of stimulated cells reached 2-3.106 cells per mL, DMSO was added to the final concentration of 10 %, and the activated T-cells were stored at -80oC until use.

*The combined growth of Jurkat cells and activated T-cells.*

Before the start of the co-culture, the Jurkar cells were pre-grown in RPMI 1640 GlutaMax medium without sodium bicarbonate to enable pH alternation (Product number R6504, Sigma-Aldrich, Steinheim, Germany) with added FBS to the final concentration of 10% and incubated as specified above. The specific inhibitors were added to 10 µM concentration and sequentially reintroduced at 24-hour intervals. As a control, an equivalent amount of DMSO as used in inhibitors was supplemented to the medium. The tests started with 1x105 cells per mL. After 72 hours of Jurkat cell growth, immediately after the sequential reintroduction of inhibitors, the cells were collected by centrifuging, and the supernatant was saved for further experiments. An aliquot of precipitated Jurkat cells was added to 1mL of pre-used supernatant to reach a final cell number of 1.104 cells per mL. The pre-used medium has been taken to maintain the pH value of the medium during the co-culture experiment. Finally, to each well with Jurkat cells an aliquot of activated T-cells was added (5.104 per mL). As recommended previously [38] the co-culture was incubated under standard conditions for 18 hours.

*Measuring apoptosis in a co-culture of Jurkat cells and activated T-cells.*

Early and late-stage apoptosis of Jurkat cells in a co-culture were detected after washing cells with PBS and adding fluorescently labeled apoptotic dye Annexin V and viability dye 7-AAD. eBioscience Annexin V Apoptosis Detection Kit eFluor 450 (Cat. No 88-8006) (Thermo Fisher Scientific, Waltham, MA, USA) was used for all experiments according to the manufacturer’s instructions.

Fluorescence was measured using a Spectral Flow Cytometer (Aurora Cytometer, Cytek Biosciences, Amsterdam, The Netherlands) and analyzed using FloJo software (Tree Star Inc., San Carlos, CA, USA). Annexin V+/7-AAD+ (late apoptotic) and annexin V+/7-AAD- (early apoptotic) cells were quantified by the frequency of fluorescently labeled cells and statistical significance was assessed by the two sample T-test (independent variable).

**Apoptotic assays in a co-culture of tumorigenic Jutkat cells and activated T-cells**

Acidification of the tumor matrix by extracellular lactate accumulation may be an important factor in evading destruction by the immune system [19], as it is thought to interfere with the normal functionality of immune cells in destroying cancer cells [18]. It is hypothesized that reduced formation and transmembrane transport of lactate could prevent a drop in pH in the tumor microenvironment while restoring normal immune cell activity to induce apoptotic death of cancer cells. To test this, tumorigenic Jurkat cells were incubated together with activated human T lymphocytes in a co-culture with treated or untreated cells. To maintain acidosis, a medium without bicarbonate or other buffers was used in the experiments. 10 µM of the tested inhibitor or the equivalent of DMSO (vehicle) was added to a co-culture. After 18 hours of co-culture incubation, limited and extensive apoptotic cell death was assessed by detecting early-stage (AnnexinV+/7-AAD-)(Q2-R4) and late-stage (AnnexinV+/7-AAD+)(Q2-R2) apoptosis. The flow cytometer results showed a significantly increased percentage of late apoptosis in cells treated with cmpds No. 9 or 30 at 46.83% (**Fig. 8b**) and 44.63% (**Fig. 8c**), respectively, while only 16.38% late apoptotic cells were observed in untreated Jurkat cells in a co-culture with activated T cells (vehicle) (**Fig. 8a**). These observations clearly show for the first time that this class of PFK1 inhibitors can accelerate apoptosis and result in a faster progression of late apoptosis than in untreated cells.