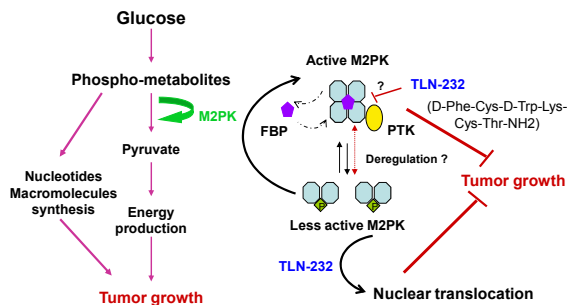


## Background

Pyruvate kinase isoenzyme type M2 (M2PK) is one of the four isoforms that catalyzes the last step of glycolysis. These isoforms have a tissue specific expression and differ in their kinetic characteristics and regulation mechanisms. M2PK is highly expressed in tumor cells and in undifferentiated and proliferating normal cells. During tumorigenesis, the tissue specific isoform is down regulated and replaced by the M2 isoenzyme (Staal and Rijkse, 1991; Steinberg et al., 1999). M2PK, the only isoform allosterically regulated, forms a tetramer (active form) or a dimer (less active form) depending on cellular phosphometabolite levels (Eigenbrodt et al., 1992). This regulation of M2PK activity promotes a shift towards aerobic glycolysis and enables the availability of macromolecules for anabolic processes (Ferguson and Rathmell, 2008). Furthermore, M2PK-mediated aerobic glycolysis promotes tumor growth and constitutes an important factor in the regulation of the glycolytic phenotype of cancer cells (Christofk et al., 2008). In contrast to the M1 isoform, M2PK activity is inhibited by tyrosine kinase signaling (Christofk et al., 2008).

## Rationale

TLN-232 is a synthetic cyclic heptapeptide and a somatostatin structural derivative having broad *in vitro* and *in vivo* antitumor activity (Tejeda et al., 2003, Tejeda et al., 2007). Cytotoxic activity of TLN-232 appears to be mediated by the nuclear translocation of M2PK (Stetak et al., 2007). This re-localization of M2PK may result in alterations of aerobic glycolysis (the Warburg effect), a process that has been linked to malignancy and aggressiveness of tumors. Given the key role of M2PK in the regulation of the Warburg effect, this alteration in the distribution and/or activity of M2PK could represent a potential mechanism by which TLN-232 induces apoptosis in tumor cells. In order to advance our understanding of the molecular mechanism of TLN-232, we evaluated the role of M2PK expression in TLN-232 mediated cell death using RNAi gene silencing. The human glioma cell line SF-188 was used as it exhibits a high rate of aerobic glycolysis.

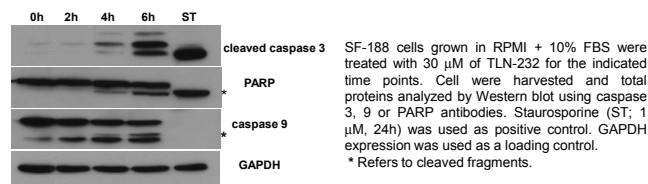


TLN-232 may modulate M2PK activity through:

1. Alteration of protein association (e.g. PTK)
2. Changes in M2PK cellular localization/activity

## Results

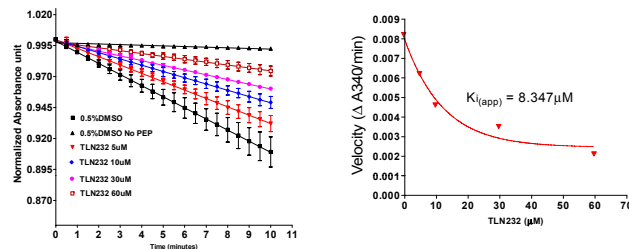
**Figure 1. TLN-232 exposure results in caspase activation and PARP cleavage in SF-188 cells**



SF-188 cells grown in RPMI + 10% FBS were treated with 30  $\mu$ M of TLN-232 for the indicated time points. Cell were harvested and total proteins analyzed by Western blot using caspase 3, 9 or PARP antibodies. Staurosporine (ST; 1  $\mu$ M, 24h) was used as positive control. GAPDH expression was used as a loading control. \* Refers to cleaved fragments.

TLN-232 induces apoptosis in SF-188 as indicated by caspases 3 and 9 activation and PARP cleavage

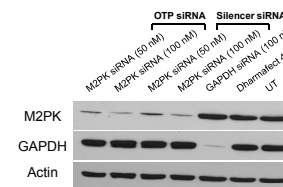
**Figure 3. TLN-232 treatment causes a dose response inhibition of M2PK enzymatic activity in SF-188 cells**



SF-188 cells were grown overnight in RPMI media containing 10% FBS. Cells were collected and protein concentration determined. Measurement of pyruvate kinase activity was performed using 2  $\mu$ g of total proteins in a lactate dehydrogenase coupled assay. Different concentrations of TLN-232 were added to the reaction mixture and absorbance at 340 nm was recorded at 25 °C every 30 sec for 10 min.

TLN-232 inhibits M2PK enzymatic activity with a  $K_i$  (app) of 8.35  $\mu$ M

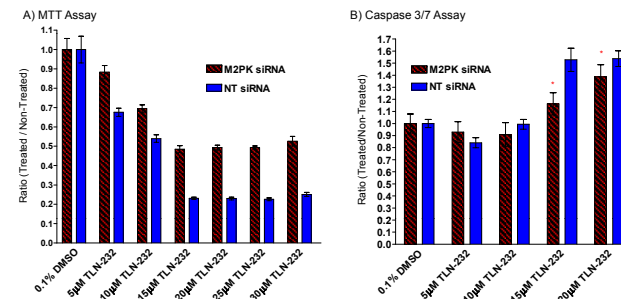
**Figure 2. RNAi down regulation of M2PK expression in SF-188 cells**



Exponentially growing SF-188 cells (RPMI + 10% FBS) were transfected with 50 nM or 100 nM of ON-Target Plus Smart Pool (OTP) siRNAs (a mixture of 4 different siRNAs duplexes; Dharmacon RNAi Technologies, Lafayette, CO) or Silencer siRNA (single siRNA duplex; Ambion Applied Biosystems, Austin, TX). GAPDH siRNA (Dharmacon RNAi Technologies, Lafayette, CO) was used as a positive control. Cells were harvested 72h post transfection, and Western blot analysis performed using M2PK,  $\beta$ -actin and GAPDH specific antibodies. UT represents untransfected controls.

M2PK expression was decreased by ~95 % 72h post-transfection using both systems

**Figure 4. M2PK expression contributes to the cytotoxicity effect of TLN-232 in SF-188 cells**



$P < 0.001$  between the two groups between both conditions for all doses tested

\*  $P < 0.001$

Exponentially growing SF-188 cells (RPMI + 10 % FBS) were transfected with 50 nM of OTP siRNAs. 48h later, cells were trypsinized and seeded in 96 well plates and allowed to attach for 6h before treatment with different doses of TLN-232.

siRNA knockdown of M2PK expression attenuates the effect of TLN-232 on cell cytotoxicity and caspase activation

## Conclusions

- ❖ TLN-232 induces cleavage and activation of caspases 3, 7, 9 and PARP
- ❖ RNAi silencing of M2PK expression attenuates TLN-232 cytotoxicity in SF-188 cells
- ❖ TLN-232 inhibits M2PK enzymatic activity in a dose-dependent manner
- ❖ TLN-232 cytotoxicity is mediated via M2PK inhibition