

# A structurally novel farnesylated dibenzodiazepinone, TLN-4601\*, inhibits wild-type, amplified and mutated EGFR glioma cell migration



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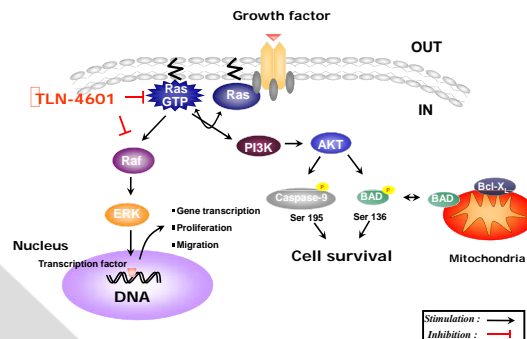
## BACKGROUND

The over-expression of PDGF receptor in low-grade gliomas and EGF receptor in glioblastoma multiform (GBM) suggest that these receptor-signaling pathways are critical for gliomagenesis. Receptor protein kinases signal through several effector arms, including Ras-MAPK, PI3K, PLC- $\gamma$  and JAK-STAT signaling pathways, which regulate cellular proliferation, survival, migration, calcium signaling and cytokine stimulation. The EGFR is frequently amplified (40-60%) in GBM and is associated with high levels of EGFR mRNA or proteins. In most cases, the gene is also rearranged during the process of amplification, resulting in several classes of variant EGFR transcripts. The most common is a genomic deletion of exons 2-7, resulting in an in-frame deletion of 801 bp of the coding sequence generating a mutant receptor with a truncation of its extracellular domain, referred to as de2-7 EGFR,  $\Delta$ EGFR or EGFRvIII. EGFRvIII protein is detected in 60% of GBMs and has also been detected in lung, breast and prostate cancer, but not in normal tissues. Both EGFR gene amplification and EGFRvIII expression has been associated with a poor prognosis in patients with GBM.

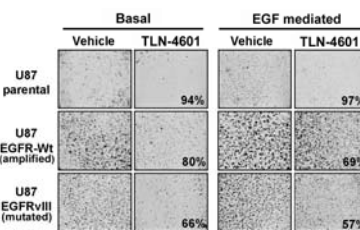
TLN-4601 is a structurally novel farnesylated dibenzodiazepinone (MW 462; US Patent 7,101,872) discovered using Thallion's proprietary Decipher® technology, a genomics and bioinformatics platform that predicts the chemical structures of secondary metabolites based on gene sequences obtained by scanning bacterial genomes. The compound has demonstrated *in vivo* antitumor activity in xenograft models, including rat glioma and human hormone-independent prostate and breast tumor models. TLN-4601 showed a good safety and tolerability profile in patients with advanced refractory solid tumors in a Phase I/II clinical trial.

## RATIONALE AND WORKING HYPOTHESIS

TLN-4601 is a targeted anticancer drug inhibiting Ras-MAPK signaling, resulting in an inhibition of cell proliferation and an induction of apoptosis. TLN-4601 also selectively binds to the peripheral benzodiazepine receptor (PBR), leading to intracellular accumulation in cancer cells. Inhibition of Ras-MAPK signaling occurs post Ras prenylation and prior to Raf-1 phosphorylation/degradation. More recently, we observed that TLN-4601 decreased activated (Ras-GTP). Since Ras-GTP interacts with several effector proteins, including the MAPK cascade which leads to ERK activation and, cell proliferation/migration and PI3K which targets AKT, leading to cell survival, we verified if TLN-4601 could inhibit Ras signaling in glioma cells harboring WT, amplified or mutated EGFRs. The human glioma U87 MG cell line was used concomitantly with U87 MG cells transfected with WT EGFR (to mimic amplified EGFR tumors) or with EGFRvIII (to mimic tumors harboring the mutated receptor). These stably transfected cell lines were obtained from Dr. Frank B. Furnari, UCSD, La-Jolla CA.



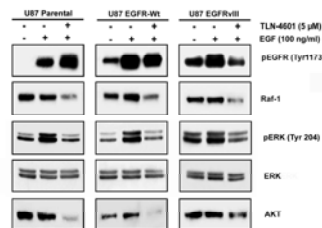
**Figure 1: TLN-4601 inhibits basal and EGF-induced migration of glioma cells harboring WT, amplified and mutated EGFRs**



Exponentially growing cells ( $5 \times 10^6$ ) were dispersed onto 1 mg/ml gelatin / PBS-coated chemotaxis filters (Costar; 8- $\mu$ m pore size) within Boyden chamber inserts. Migration proceeded for 18h at 37°C in 5% CO<sub>2</sub> in the presence or absence of 5  $\mu$ M TLN-4601. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate, colored with 0.1% crystal violet / 20% MeOH and counted by microscopic examination. Percent inhibition of TLN-4601 vs vehicle treated cells is indicated.

- Overexpression of WT EGFR (mimicking amplified) or EGFRvIII (mutated) resulted in a significant increase in cell migration, which was further increased by the addition of EGF
- TLN-4601 significantly inhibited both basal and EGF-mediated cell migration of these highly invasive glioma cell lines

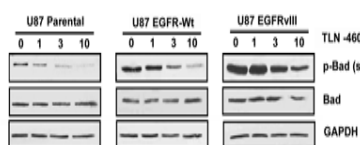
**Figure 2: TLN-4601 inhibits Ras-MAPK signaling pathway in glioma cells harboring WT, amplified and mutated EGFRs**



Exponentially growing cells were plated onto 100 mm<sup>3</sup> dishes in DMEM containing 10% FBS. 24h after plating, the media was removed and cells were treated with 5  $\mu$ M TLN-4601 for 18h in media containing 0.1% FBS. Cells were then stimulated for 1 min with 100 ng/ml EGF and harvested. Western blots analysis for p-EGFR, Raf-1, p-ERK, ERK and AKT were performed using specific commercial antibodies.

- While EGFR is not phosphorylated under basal conditions in the U87 MG parental cell line, it is phosphorylated in cells transfected with WT (mimicking EGFR amplification) and mutated (vIII) EGFRs.
- EGF stimulates receptor phosphorylation which is not affected by TLN-4601
- TLN-4601 exposure resulted in a decrease of total Raf-1 and decreased EGF induction of p-ERK as well as a reduction in AKT

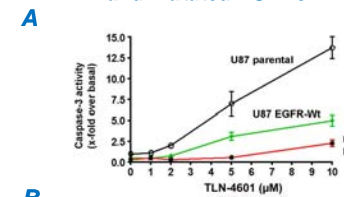
**Figure 3: TLN-4601 reduces AKT signaling**



Exponentially growing cells were plated onto 100 mm<sup>3</sup> dishes in DMEM containing 10% FBS. 24h after plating, the media was removed and cells were re-fed with serum-free DMEM and increasing concentrations of TLN-4601 for 18h. Cells were harvested and Western blot analysis for p-Bad and Bad were performed using specific commercial antibodies. GAPDH was used as a loading control.

- TLN-4601 exposure resulted in a dose-dependent decrease of p-Bad

**Figure 4: TLN-4601 induces caspase activation and PARP cleavage in glioma cells harboring WT, amplified and mutated EGFRs**



Cells were plated in 6 well plates in DMEM containing 10% FBS. The following day, cells were treated with increasing concentrations of TLN-4601 in serum-free medium. After an 18h incubation, caspase-3 activity was measured using a commercial kit.

Exponentially growing cells were plated onto 100 mm<sup>3</sup> dishes in DMEM containing 10% FBS. 24h after plating, the media was removed and cells were re-fed with DMEM containing 0.1% FBS and 20  $\mu$ M TLN-4601 (U87 MG) or 30  $\mu$ M TLN-4601 (U87 EGFR-WT and U87 EGFRvIII) at different times. Cells were harvested and Western blot analysis for PARP and GAPDH performed.

- TLN-4601 is cytotoxic towards parental U87 MG cells (measured by caspase-3 activation)
- Caspase-3 activation was also detected in glioma cells over-expressing WT and mutated EGFRs, although at a lower levels
- TLN-4601 exposure resulted in PARP cleavage a hallmark of apoptosis, in all three cell lines

## CONCLUSIONS

- TLN-4601 inhibits basal and EGF-induced migration of glioma cells harboring WT, amplified or mutated EGFRs
- TLN-4601 induces a decrease in Raf-1 protein levels and inhibits Ras-MAPK signaling in human glioma cells harboring WT, amplified or mutated EGFRs
- TLN-4601 exposure results in a decrease of AKT and phospho-Bad leading to apoptosis as indicated by caspase-3 activation and PARP cleavage

**Our results show that TLN-4601 inhibits Ras signaling in glioma cells harboring WT, amplified or mutated EGF receptors. These findings, together with Phase I/II clinical data showing good safety and tolerability, support a phase II clinical trial in GBM.**

\*Formerly ECO-4601.