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Research Article

Targeting The Tlk1-Nek1-Mediated Activation of Yap1 Attenuates *PD-L1* and Anti-Inflammation set for Prostate Cancer Immunoevasion

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Plans for future development of the phenothiazine

Identification of J54 as a powerful inhibitor of TLK. In 2013, we published that several structurally similar Phenothiazines (PTH) in use as antipsychotics were identified as *in vitro* inhibitors of TLKs in a screen of 3 compound libraries (~6000 compounds) using a site-targeted Rad9 peptide [1]. Interestingly, already then we had shown that several similar PTH (some also used as antipsychotics) were not good inhibitors of TLK [1], but we could not approach the reasons for this as no TLK structural models existed at the time and no docking studies were possible. A medicinal chemist, Sivapriya Kirubakaran and her student Siddhant Bhoir (IIT Gandhinagar), and two molecular modelers, Rupesh Chikhale and Richard Bryce (U. of Manchester) contacted our lab because of their interests in TLKs in DDR, and we have been able to synthesize, model, and test a number of custom-made PTH inhibitors [2,3]. The *TLK1* docking model was further refined by 1 μs Molecular Dynamics (MD). J54 and THD were docked into the final MD-refined model. In the docked pose of compound J54, good interactions with the hinge region residues of *TLK1* were exhibited; the morpholino head forms hydrogen bonds with Asp122, with a distance of 1.68 Å from the Asp122 carboxylate Oδ to the ligand NH group [3]. J54 inhibits the IVK autophosphorylation in dose- dependent fashion similar to the effect in cells (already was shown in [1]) and it (as well as some other PTHs) block access to the peptide binding site when tested by Fl-anisotropy with the Fl-Rad9 peptide (Figure 1SI4). Note that we tested over 30 other PTH in all [1,2]) and found some to be inactive as TLK inhibitors, but several others worked well, with some derivatives that are 2-3 times more potent than J54 or are more H_2O-soluble [2] but these still lack the extensive testing already carried out on J54 (e.g., Kinomescan, PD/PK, and DR antagonism). The main issue for J54 was low solubility, although this was largely overcome with saline solution containing 10% Polysorbate-80 (PS80). However, structural modification to improve it solubility while still maintaining biological potency are ongoing (Figure 2SI4). We previously carried out a Kinome study (DiscoverX at 15 μM) which revealed that only TTK out of 463 kinases (including LATS1/2, MST1/2) was inhibited beside *TLK1* and *TLK2* [3], although TTK was not inhibited at 5μM, while TLKs is fully inhibited at ~1μM. All future derivatives will also be processed through kinomescans. Also, competition assays with radiolabeled dopamine onto recombinant DR2 (human D1 and D3) revealed that J54 is weakly competitive compared to THD: Ki~450 nM for purified receptor protein compared to ~20 nM (carried out by XenoTech-Sekisui, which for THD was consistent with the literature [4]). Such a level of inhibition would be expected to be minimal *in vivo* at circulating levels of J54, but modifications that are predicted to prevent BBB passage (like compound 9) are being consider.

A direct comparison of THD *vs* J54 as suppressors of pNek1-T141 revealed similar potency but the effect of J54 lasted longer (3 days vs. 1 day for THD - not shown), which suggests prolonged inhibition of TLK and likely up to the protein turn-over period. J54 also had reasonable pharmacokinetics, where plasma concentrations of 100ng/ml were reached 2h after IP injection (10 mg/kg) and ~6ng/ml was still detected after 24hr [3] - better than reported for THD [5,6]. Moreover, while the additional targets and possible side-effects of PTHs are generally known (e.g., DR2 and hERG channel), other compounds we tested with indole scaffold modification [2], while more H_2O-soluble and even more potent than J54, have less medical literature and records of FDA approval, so we plan to optimize PTHs derivates of J54 by testing inhibition of pNEK1(Figure 7). Further plans are rather standard:

Lead optimization

- Solubility and Bioavailability: Continue SAR (Structure-Activity Relationship) studies to improve aqueous solubility and oral bioavailability while retaining potency. Consider PEGylation or prodrug strategies.
- BBB Permeability Control: Prioritize compounds with poor BBB permeability for cancer indications outside the CNS to reduce neuropsychiatric side effects (e.g., compound 9), or

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intentionally design CNS-penetrant analogs for glioma and brain metastases.

Mechanism of action and biomarker development

• Downstream Target Validation: Further validate NEK1-pT141 and additional downstream substrates of *TLK1/2* as biomarkers of J54 activity.

• Resistance Mechanisms: Screen for resistance mutations or compensatory signaling in cell lines to anticipate clinical resistance and guide combination strategies.

Expanded preclinical evaluation

• Patient-Derived Xenografts (PDX): Test J54 and analogs in *TLK1/2*-expressing PDX models from cancers with high replication stress (e.g., prostate, ovarian, or TNBC).

• 3D Organoid Cultures: Leverage patient-derived organoids for ex vivo drug testing and biomarker correlation

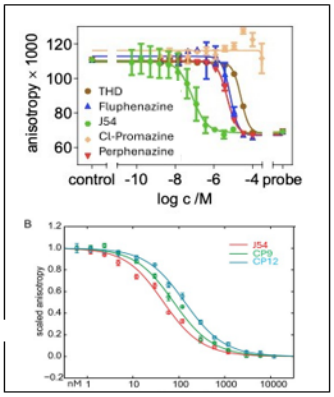

Figure 1SI4: A. Direct physical evidence for binding of J54 and MB to *TLK1*. Recombinant, native *TLK1*B (0.5 ug/per reaction) was tested by Flanisotropy: fluorescein-labelled Rad9 peptide was added to a serial dilution of inhibitors in an assay with 200 ng *TLK1*B. After 4h incubation, the Fl anisotropy (λ excitation 482 and λ emission 530) was recorded in a Synergy-4 plate reader. Likely some PTHs act to block peptide access to the catalytic site; not as ATP mimics. B. Compounds (CP) 9,12 are compared to J54 in the same assay.

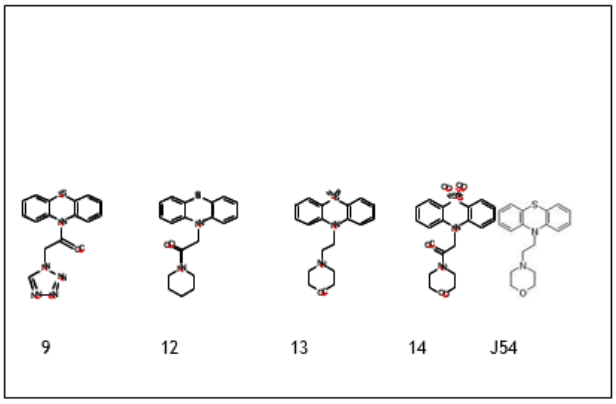

Figure 2SI4: Comparison of 3 PTH derivates of J54 published in [2] as inhibitors of *TLK1* in LNCaP cells (1day treatment). pNek1-T141, a highly specific readout of *TLK1*, was monitored following a 1h incubation with J54 or the analogs. Note that the *TLK1* inhibition occurs at sub-micromolar concentrations – much lower doses than the growth inhibitory effects observed in Figure: 3A,B in PCa cell lines (in the absence of ANSI or DDR activation). Notably, compound 9 is predicted to not cross the BBB at all, unlike other PTHs.

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