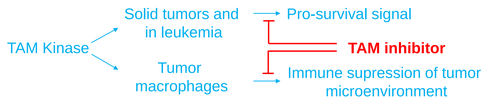
Exploring the functions of TAM kinases: Assay development and characterization of potential therapeutics

Alokta Chakrabarti, Daniel Feger, Frank Totzke, Orysya Stus, Sarah Umber, Marianne Birkle, Oliver Siedentopf, Marcel Pathe,

Thomas Weber, Michael Kubbutat , Jan Ehlert. ProQinase, Freiburg, Germany

Introduction

TAM kinases have gained interest as therapeutic targets in cancer, chronic inflammatory and autoimmune diseases. Comprising of TYRO3, AXL and MERTK, TAMs need an extracellular lipid-protein complex for their activation which is unique to this family of receptor tyrosine kinases. For example, they bind to phosphatidyl serine bound gamma-carboxylated proteins like Gas-6 on apoptotic cells as ligands. TAMs function as regulators of the immune system such as controlling innate immunity or differentiation of natural killer cells and also in hematopoiesis. Defective TAM signaling is often strongly associated with cancer progression, metastasis and resistance to targeted therapies. Several TAM kinase inhibitors are under (pre-)clinical investigation, e.g., R428, an AXL inhibitor, is currently in Phase 1 trials for mono- or combination therapy and the dual MERTK-FLT3 inhibitor MRX2843 has received FDA Investigational New Drug (IND) status.



Here, we report on the establishment of TAM kinase-specific cellular phosphorylation assays, as well as of phenotypic assays relevant for TAM kinase biology. Having these systems at hand would allow to directly compare several TAM kinase inhibitors side-by-side.

In vitro activity of TAM inhibitors

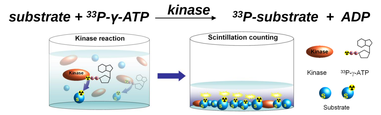


Figure1: Biochemical ^{33}PanQinase™ Assay principle. In the kinase reaction of the ^{33}PanQinase™ assay, ATP carries a radioactive isotope of phosphate ^{33}P which gets transferred onto the substrate. After the incubation time and several washing steps, the amount of ^{33}P-substrate is measured via scintillation counting.

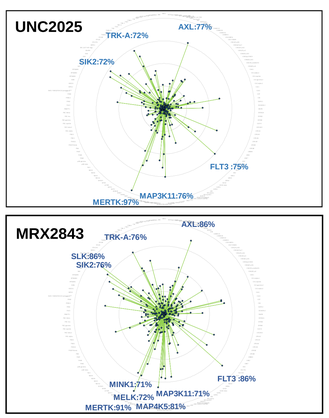


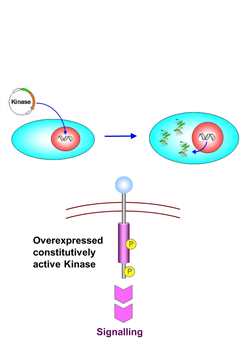
Figure 2: ProQinase Wild-Type Profiler assay with TAM inhibitors helps to identify off-target kinases.

UNC2025 and MRX2843 were screened at 100 nM against 320 kinases. Kinase IDs are shown for kinases inhibited >70%. Circular lines represent 25% increment in inhibition steps.

Table 1: Inhibitors potently target TAM kinases, but also have a significant side activity on FLT3. Shown are IC50 values determined in the biochemical ^{33}PanQinase™ assay.

Kinase	IC50 [M]			
	LDC1267	R428	UNC2025	MRX2843
AXL	4.2E-08	1.3E-09	5.6E-09	3.9E-09
MERTK	1.3E-07	4.7E-08	2.9E-09	2.4E-09
TYRO3	6.3E-08	1.4E-07	7.6E-08	5.7E-08
FLT3	2.5E-06	4.7E-07	2.9E-08	1.6E-08

Similar inhibition profile in cellular MEF-AXL and Rat1-AXL assays



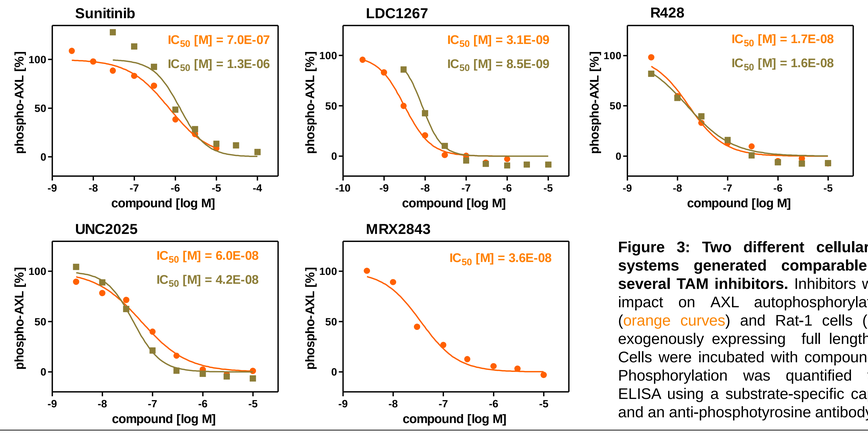


Figure 3: Two different cellular AXL assay systems generated comparable results for several TAM inhibitors. Inhibitors were tested for impact on AXL autophosphorylation in MEF (orange curves) and Rat-1 cells (green curves) exogenously expressing full length human AXL. Cells were incubated with compounds for 90 min. Phosphorylation was quantified via sandwich ELISA using a substrate-specific capture antibody and an anti-phosphotyrosine antibody.

TAM inhibition in A549 colony formation assay

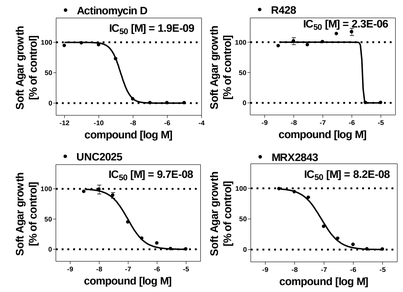
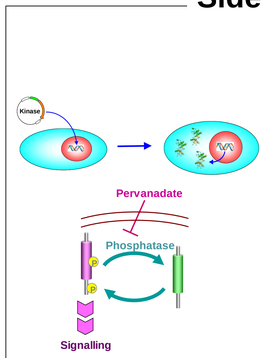


Figure 7: Inhibition of colony formation in A549 cells. Cells were cultured in semi solid soft agar for 10 days before the assay was developed with Alamar Blue staining. UNC2025 and MRX2843 proved significantly more potent then R428.

Side by side comparison of cellular TAM kinase inhibition



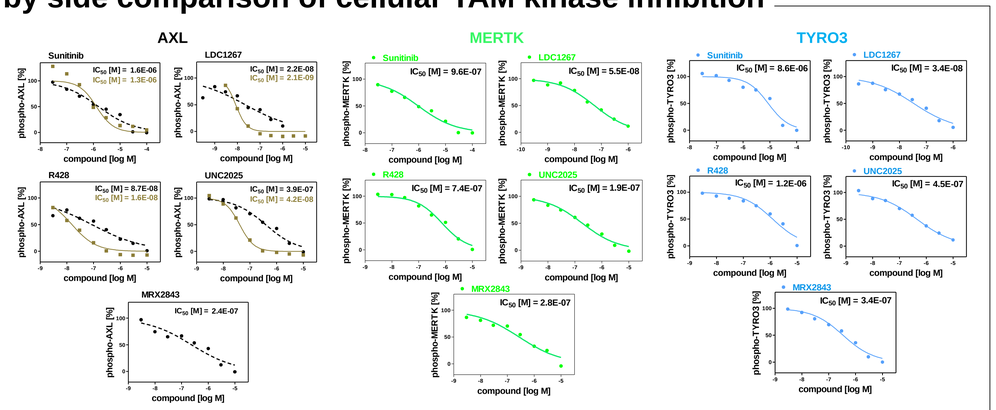


Figure 4: Inhibition of TAM kinases in transduced Rat-1 cells stimulated with phosphatase inhibitor. Boosting the AXL phosphorylation signal by pervanadate stimulation increased the margin of detection but generally resulted in ~ 5x loss of inhibitor potency. Under these conditions, we succeeded to establish phosphorylation assay systems for MERTK and TYRO3 which allowed for side-by-side comparison of TAM kinase inhibitors. For AXL, curves from stimulated (**black**) and non-stimulated (green) assays are both shown.

TAM as immuno-oncology targets

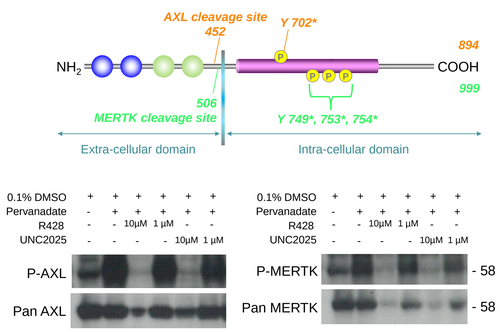


Figure 8: TAM inhibitors block AXL and MERTK activation in cell line NK-92. Cells were treated with DMSO or compounds for 90 min followed by Pervanadate stimulation. Observed bands at 55-58 kDa correspond to the intracellular domain. The lack of bands corresponding to the fullsize receptor indicate the relevance of proteolytic TAM processing in NK cells.

TAM inhibitors in proliferation assay

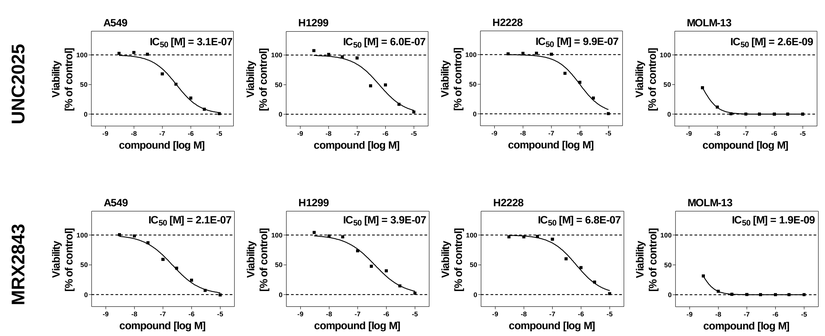


Figure 5: Inhibition of proliferation of cancer cell lines by TAM inhibitors.
An important function has been reported for TAM kinases with respect to the viability of the cell lines A549, NCI-H1299 and NCI-H2228.
Tested inhibitors indeed show submicromolar inhibitory activity. Interestingly, a curve saddle indicates a biphasic effect of the inhibitors, suggesting an even more potent activity with the first phase.
No correlation was observed between sensitivity to tested inhibitors and mRNA expression of either TAM kinase (data not shown). Not surprisingly, cell lines knowingly addicted to FLT3 activity such as MOLM-13 proved highly sensitive to inhibitors with strong FLT3 side activity.
Proliferation was assessed 72h after compound addition by CellTiterGlo.

Conclusions

- We have successfully established phosphorylation and phenotypic assays for the analysis of TAM kinase functions which allowed for side-by-side testing of several inhibitors.
- Comparing biochemical and cellular potency revealed that the respective kinase preference of the inhibitors remained similar. However, while UNC2025, MRX2843 and R428 showed reduced potency in the cell assay, potency of LDC1267 was increased.
- While inhibitors inhibited growth of reportedly TAM-sensitive cell lines in proliferation and soft agar assays, activity did not necessarily correlate with TAM mRNA expression levels.
- Inhibitors effectively reduced TAM phosphorylation in natural killer cell line NK92. Further studies will focus on the establishment of NK cell assays to study TAM kinase function and inhibition in immuno-oncology.