A biochemical and cell-based screening approach to identify new RET kinase inhibitors

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INTRODUCTION

RET (Rearranged during transfection) is a transmembrane receptor tyrosine kinase which is involved in various downstream cell signalling events, e.g. activating the Ras/MAP kinase and PI3K/AKT pathway. Thereby playing an important role in development as well as mediating cell survival (1). Dysregulation of RET kinase activity is also linked to various types of cancer, promoting tumour growth and invasion and thus disease progression (2,3).

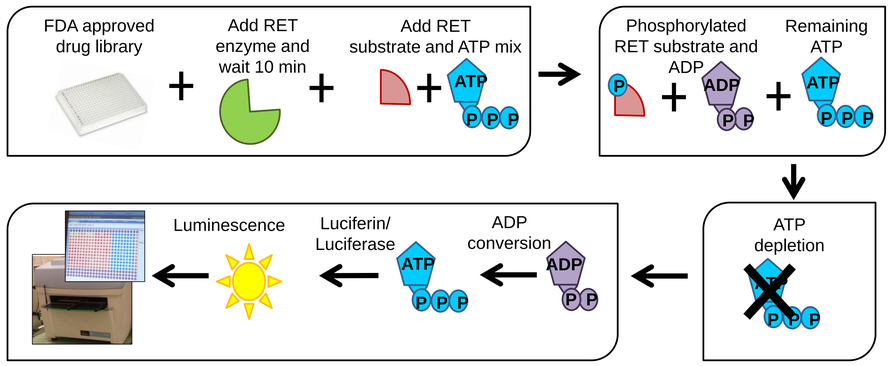
Upregulated RET protein expression, increased glial cell line-derived neurotrophic factor (GDNF) stimulation or RET mutations result in increased RET activity (3). A mutation associated with increased RET activity is the point mutation G691S, which has been shown to increase cell proliferation and invasiveness in pancreatic cancer cells (2). Pancreatic cancer is among the most common causes of cancer death worldwide and patients show a very low survival rate due to often late diagnosis and limited therapeutic options (4). Targeting the RET kinase presents a promising therapeutic strategy for cancer patients showing RET overexpression and/or RET fusion or single point mutations (3). Current FDA approved inhibitors for RET, such as vandetanib and ponatinib, are all multikinase inhibitors, thus lack RET selectivity and have multiple off-target effects on other kinases (5).

In this study, we screened a library of FDA approved drugs (742 compounds) to identify compounds with an inhibitory effect on RET and determined their activity against the wildtype RET and G691S mutated RET protein. We also assessed RET-selectivity using a target-driven cell-based assay. We then optimised conditions for an automated high content based imaging assay with pancreatic cancer cells expressing wild-type RET (PanC-1 cells) and the mutated G691S protein (MiaPaCa2 cells) to be able to assess phenotypic changes associated with invasion, such as expression of invasion markers, e.g. vimentin and cell morphology after treatment with RET inhibitors.

METHODS – SCREENING CASCADE

1) Biochemical assay to identify RET inhibitors

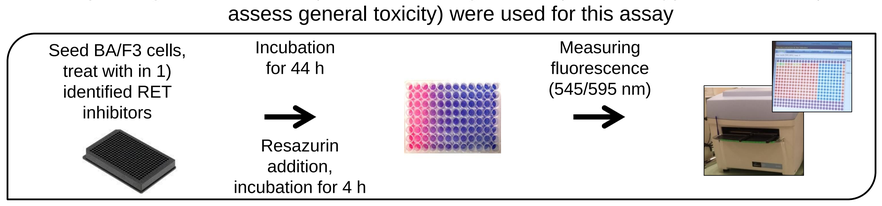
(384 well plate format, 10 µL reaction volume)



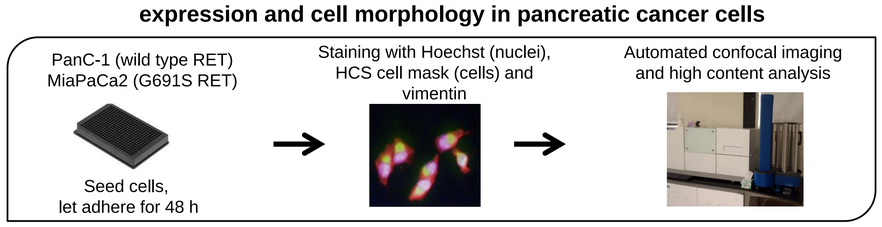
2) Target driven cell-based assay to assess RET selectivity and toxicity

(384 well plate format, 50 µL assay volume)

Murine BA/F3 cells depending on RET kinase (to confirm RET inhibition), BA/F3 cells depending on KDR kinase (to assess off-target effects) and wild type BA/F3 cells (to assess general toxicity) were used for this assay



3) Establishing a high content imaging assay to determine invasion marker expression and cell morphology in pancreatic cancer cells



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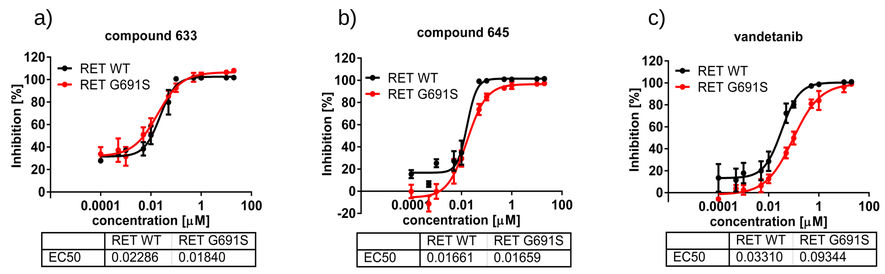
RESULTS AND DISCUSSION

Fig 1: Inhibitory effect of compounds on RET wildtype (WT) and RET G691S activity



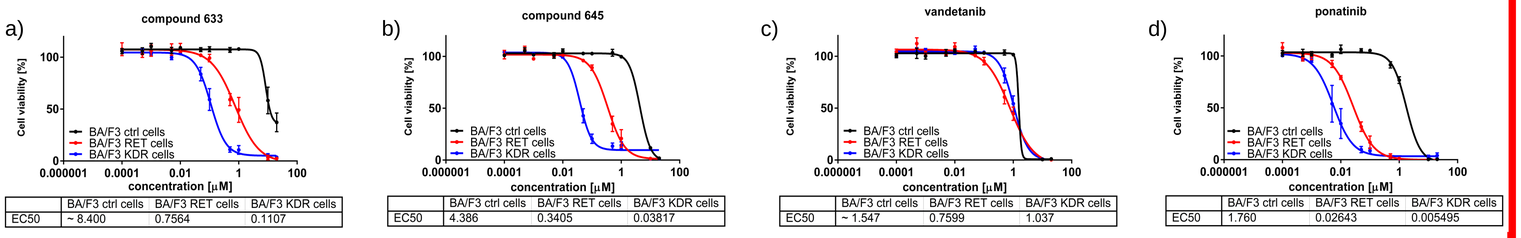
	RET WT	RET G691S
EC50	0.02286	0.01840

	RET WT	RET G691S
EC50	0.01661	0.01659

	RET WT	RET G691S
EC50	0.03310	0.09344

Fig 1.: To identify new RET inhibitors, a library of 742 FDA approved drugs was screened against wildtype RET protein. For this assay the ADP-Glo™ Kinase Assay (Promega) was used and conditions were optimised for a 384 well plate, low volume format, compatible with automation. Ten (of 742) compounds with RET inhibitory activity were identified. These compounds were then tested in 11 pt CRCs against wild type RET and G691S RET (Fig 1). Data was normalised to 1 µM vandetanib/DMSO, n=2 +/- SEM in duplicates. Eight of 10 compounds were reconfirmed. The 2 most active compounds showed IC50s in the low submicromolar range, similar or even lower compared to vandetanib (Fig 1). Compounds 633 and 645 also showed similar activity against wildtype RET and G691S mutated RET (Fig 1a,b).

Fig 2: Effect of identified compounds on cell viability



	BA/F3 ctrl cells	BA/F3 RET cells	BA/F3 KDR cells
EC50	~ 8.400	0.7564	0.1107

	BA/F3 ctrl cells	BA/F3 RET cells	BA/F3 KDR cells
EC50	4.386	0.3405	0.03817

	BA/F3 ctrl cells	BA/F3 RET cells	BA/F3 KDR cells
EC50	~ 1.547	0.7599	1.037

	BA/F3 ctrl cells	BA/F3 RET cells	BA/F3 KDR cells
EC50	1.760	0.02643	0.005495

Fig 2.: The 2 most active compounds identified in (1) were tested for their in-cell activity against RET in the BA/F3 RET cell line, for off-target effects (BA/F3 KDR) and general toxicity (BA/F3 ctrl cells) (n=2 +/- SEM in duplicates). Both compounds showed in-cell activity against RET with an IC50 of 0.8/0.3 µM (Fig 3 a,b, in red). Also, both compounds showed lower activity against BA/F3 control (ctrl) cells (>10 µM, 4 µM), indicating that these compounds act through RET inhibition and not only general cytotoxicity (Fig. 3a,b, black). These compounds were then tested against BA/F3 cells expressing KDR, a kinase associated with general toxicity and/or off-target effects of multikinase inhibitors (6). Both compounds showed activity with a lower IC50 against KDR, indicating off-target effects on at least one other kinase. The activity of 633 and 645 was then compared with known RET inhibitors, the control vandetanib and another RET inhibitor, ponatinib (7) (Fig 3). Both compounds showed similar/better activity compared to vandetanib (Fig 3c) in BA/F3 RET cells. However, both compounds were less active than ponatinib (Fig 3d). The 2 new identified RET inhibitors, didn't show increased selectivity, rather showing a more potent inhibitory effect on KDR than RET similar to the known broad spectrum RET inhibitors. The identified 2 compounds showed similar/better properties than vandetanib, but no improvement in activity and also showing off-target effect on KDR. In future experiments these compounds should be investigated further for their effect on a panel of kinases, to determine if these compounds show overall less off-target effects than current RET drugs (5). Furthermore these compounds should be screened against additional RET mutants to test their activity as alternative to current drugs with RET resistance (7).

Fig 3: Assay optimisation for an automated confocal high content imaging assay

To assess parameters associated with invasion in PanC-1 and MiaPaCa2 cells

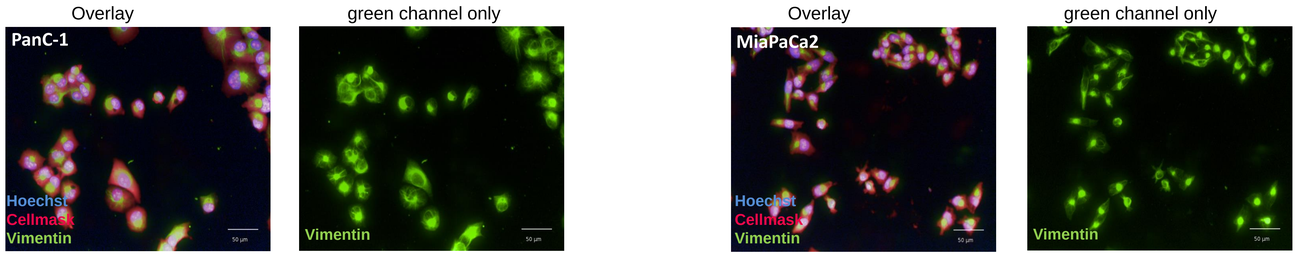


Fig 3.: To be able to identify phenotypic changes after RET inhibition in pancreatic cancer cells, an automated high content imaging assay was established. The images show PanC-1 (left) and MiaPaCa2 cells (right) 48h after seeding, stained with HCS cellmask (complete cell, red), Hoechst (nuclei, blue) and vimentin (green). Using this assay, cell number, cell morphology as well as expression level and localisation of proteins associated with invasion, e.g. vimentin can be measured. This assay format will now be applied to assess the effect of RET inhibition on phenotypic changes associated with invasion.

CONCLUSION AND FUTURE EXPERIMENTS

- We showed that our screening cascade is able to identify RET inhibitors using a biochemical assay, compatible with automation for two RET isoforms tested.
- This screening cascade also allows to assess compound selectivity, off-target effects and cell toxicity in a cell-based *in vitro* assay.
- We identified 8 new RET inhibitors, active against wildtype/G691S RET isoforms. The 2 most active compounds also showed in-cell activity and a dose dependent inhibitory effect on RET. However, both compounds showed off-target effect on KDR.
- We also established a high content imaging assay, with pancreatic cancer cells expressing wildtype and G691S RET, to assess vimentin expression, cell shape and cell number. This assay will be used in future experiments to assess phenotypic changes after treatment with RET inhibitors.
- This assay set up will be used to screen other compound libraries to identify more selective RET inhibitors.

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