Small molecule inhibitors of ICMT as potential cancer therapeutics

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Introduction

Posttranslational processing of proteins is a current focus in the field of cancer chemotherapeutics. The CAAX pathway is responsible for the covalent modification of >100 client proteins including key regulatory signaling proteins such as the Ras, Rho, and Rab GTPases and the nuclear lamins which terminate in a carboxyl-terminal CAAX motif. This sequence consisting of cysteine (C) followed by two aliphatic residues (AA) and a variable residue (X) undergoes a three-step posttranslational enzymatic modification where the cysteine residue is isoprenylated with a 15-carbon farnesyl or 20-carbon geranylgeranyl group followed by the proteolytic cleavage of the AAX amino acids. The third step involves the methylation of the newly formed C-terminal prenylcysteine by membrane-bound isoprenylcysteine carboxyl methyltransferase (ICMT) present in the endoplasmic reticulum[1] (Fig 1). This protein modification has been implicated in facilitating membrane attachments[2], catalysing protein-protein interactions[3], and increasing protein stability[4].

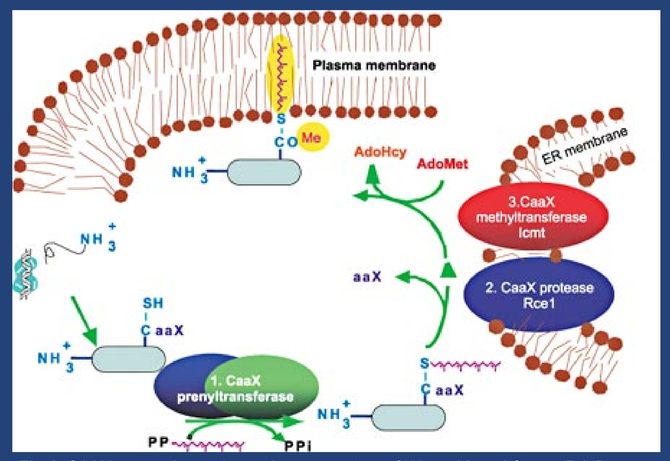


Fig 1. CAAX processing pathway. *Image courtesy of Wang, M. and Casey, P.J. Drugs Fut. 2006. 31, 437.*

As many of the CAAX motif client proteins regulate pathways important in oncogenesis the key enzymes involved in this posttranslational modification have emerged as potential anticancer targets. ICMT, as the sole enzyme responsible for the methylation of all CAAX proteins, is an particularly amenable drug target.

Selective inhibitors of ICMT are required to further our knowledge of this target and at the initiation of this programme the only existing small-molecule inhibitor was Cysmethynil. To identify novel small molecule inhibitors of ICMT we established a Scintillation Proximity Assay (SPA) and screened a CTx library of 151 381 compounds. Secondary enzyme kinetics assays were used to prioritise hits and determine mode of inhibitory action. High content imaging assays were established to assess the ability of newly identified inhibitors to disrupt the oncogenic potential of cancer cells in vitro. The results of our studies will be presented here.

Measuring ICMT inhibition in vitro

Transfected sf21 membranes containing human recombinant ICMT (0.5ug/well) were diluted in buffer (HEPES pH 7.4, 100mM NaCl, and 3mM DTT) and the reaction was initiated by the addition of a substrate mixture containing [^{3}H]-SAM (0.18 Ci/mmol, 1µM) unlabelled s-adenosyl methionine (SAM) (2µM) and Biotin-S-farnesyl cysteine (BFC) (3µM). After a 30min incubation at room temperature the reaction was terminated by the addition of SAM (50µM). SPA PVT Scintillation beads (10mg/mL) were added to capture the [^{3}H]-BFC methyl ester product. After 12hrs incubation at room temperature the radioactivity was counted on a Microbeta Trilux™ luminescence reader (Perkin Elmer) (Fig 2).

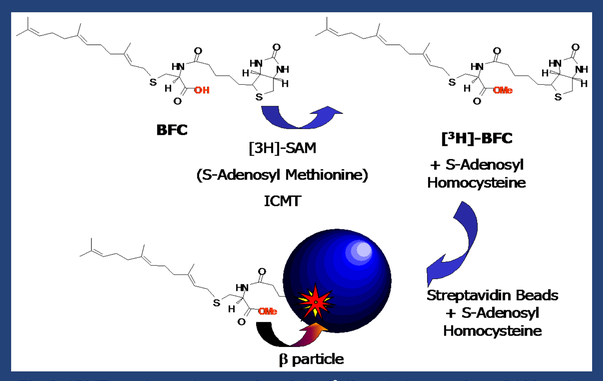


Fig 2. ICMT catalyses the transfer of the [^{3}H[-methyl group from SAM to the small molecule substrate BFC. [^{3}H]-methyl-incorporated BFC is captured on SPA beads and the radioactivity is measured by scintillation from the beads.

Identification of Hits

The library was screened in single point at a 10µM final assay concentration in 384-well microplate format using the ICMT SPA. Over the 7 day run compounds were screened in 2 batches per day using internal controls for data analysis. Data was expressed as % inhibition and each test result calculated as a percentage of the average internal plate MAX signal (Fig 3).

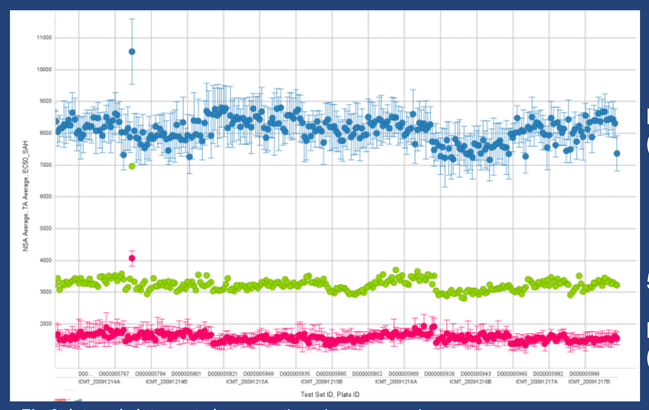


Fig 3. Internal plate controls across the primary screening run

Cysmethynil and s-adenosyl homocysteine (SAH), an inhibitory by-product of SAM methyl group transfer, were used as reference compounds (Fig 4).

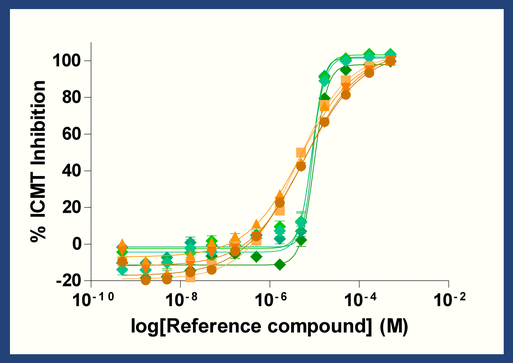
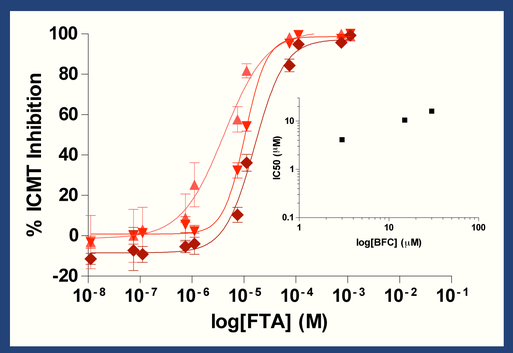


Fig 4. Batch 1 reference compound dose-response curves from day 6 of primary screening (n=4 plates). The Cysmethynil (green) and SAH (orange) reference compounds demonstrated IC_{50} = 9.1 ± 0.2µM and 5.0 ± 0.5µM respectively.

1931 compounds were identified with >50% inhibition (1.3% hit rate) and progressed to 3pt CRC reconfirmation (25, 10, 2.5µM). HTS was highly reproducible with Z' = 0.64 and S:B = 5.61. 515 compounds with an estimated IC_{50}<6µM were selected for cluster analysis before progressing.

93 actives with an estimated IC_{50}<2.5µM from 3pt CRC reconfirmation were prioritised for BFC substrate competition studies. Actives were tested against the ICMT SPA assay at BFC = K_m, 5x K_m, and 10x K_m in order to demonstrate that the IC_{50} increases proportional to substrate concentration for those compounds displaying characteristic competitive reversible inhibition. These compounds progressed to Lead Optimisation for further SAR (Fig 5). Actives competitive with the SAM methyl donor were deprioritised.



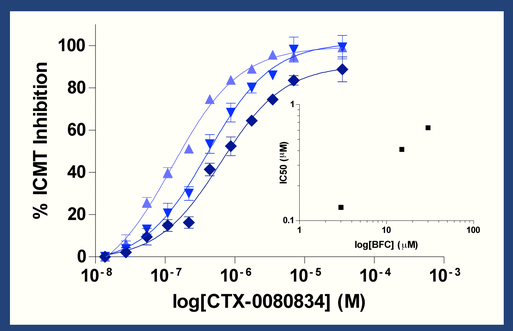


Fig 5. Competitive inhibition of ICMT by s-Farnesyl Thioacetic Acid (FTA), a known ICMT competitive inhibitor (top) and a reconfirmed active compound (bottom).

IC_{50} values increase with increasing BFC concentration (inset graphs)

Characterisation of ICMT inhibitors

ICMT-dependent inhibition of cell growth

Small-molecule ICMT inhibitors identified from Lead Optimisation (Fig 5) were assessed for their ability to inhibit cell growth via an ICMT-dependent process. Growth rates of mouse embryonic fibroblast (MEF) cells treated with CTX-0295348 were compared with untreated ICMT -/- MEF cells. At a 5µM dose CTX-0295348 was shown to inhibit the growth of MEF cells down to the level of ICMT -/- MEF cells. This compound had less of an effect on the growth rates of ICMT -/- cells indicating that inhibition of cell growth may be occurring via an ICMT-dependent mechanism (Fig 6).

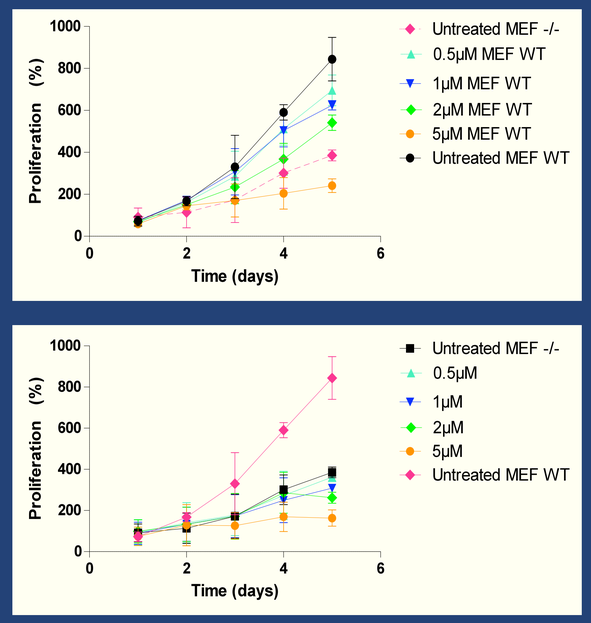


Fig 6. ICMT-dependent growth inhibition of mouse embryonic fibroblasts (top) and ICMT -/- MEF cells (bottom) over 5 days using CTX-0295348. Media and drug were replaced every 72hrs. Cell growth was detected with Alamar blue (535/595). Results are from n=3 independent experiments.

ICMT-dependent CAAX protein mislocalisation

Lamins are CAAX motif proteins crucial for the structural integrity of the nucleus[5] and inhibition of lamin processing may have a role in reducing tumourigenesis. High content imaging has shown an ICMT-competitive inhibitor, CTX-0118409, capable of disrupting the accumulation of Lamin B1 in the nuclear membrane resulting in this protein aggregating in the nucleus in a prostate cancer cell line (Fig 7).

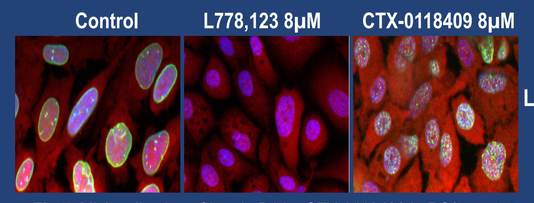


Fig 7. Mislocalisation of Lamin B1 by CTX-0118409 in PC3 prostate cancer cells

In contrast the farnesyltransferase inhibitor (L778,123), a member of a class of compounds able to block the farnesylation of prelamin A[6], was shown to inhibit this CAAX processing step leading to an accumulation of Prelamin A in the nucleus. CTX-0118409 did not affect Prelamin A processing (Fig 8).

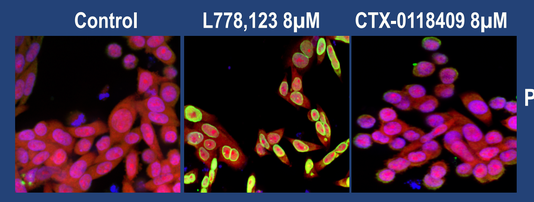


Fig 8. Accumulation of prelamin A by L778,123 in PC3 prostate cancer cells

Conclusions

- A highly reproducible high throughput screening campaign capable of identifying reversible competitive inhibitors of ICMT was successfully established.
- In vitro ICMT inhibitors identified from screening were shown to inhibit growth of wild type MEF cells over ICMT -/- cells and disrupt the localisation of specific CAAX motif proteins.

References

1) Lane, K.T.; Beese, L.S.; *J Lipid Res*. **2006**, 47, 681. **2)** Magee, A.I.; Seabra, M.C.; *Biochem J*. **2003**, 376, 3. **3)** Dietrich, A.; Scheer, A.; Illenberger, D.; Kloog, Y.; Henis, Y.I.; Gierschik, P.; *Biochem J*. **2003**, 376, 449. **4)** Hrycyna, C.A.; Clarke, S.; *Pharmacol Ther*. **1993**, 59, 281. **5)** Gerace, L.; Huber, M.D.; *J Struct Biol*.**2012.** 177, 24**. 6)** Adjei, A.A.; David, J.N.; Erlichman, C.; Svingen, P.A.; Kaufmann, S.H.; *Clin Can Res*. **2000**, 6, 2318.