Published in final edited form as:

J Dermatol Sci. 2017 July ; 87(1): 88–91. doi:10.1016/j.jdermsci.2017.04.001.

ITK inhibition for the targeted treatment of CTCL

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Study conducted in Sacramento, CA, USA.

Conflict of interest

The authors state no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jdermsci.2017.04.001>.

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Cutaneous T cell lymphomas (CTCLs) comprise a heterogeneous group of uncommon non-Hodgkin lymphomas with primary manifestations in the skin [1]. Though CTCL treatment strategies are rapidly evolving, novel CTCL-targeted therapies are still needed, especially for patients with advanced disease. Highly targeted therapies have been developed for other immune system malignancies. For example, Bruton's tyrosine kinase (BTK) is implicated in the development of a variety of B-cell malignancies [2] and the targeted BTK inhibitor, ibrutinib, is now FDA approved for treatment of mantle cell lymphoma and chronic lymphocytic leukemia [3]. Although the cause of CTCL is variable, genetic studies have revealed that this cancer acquires mutations that promote T cell growth and survival; however, no single mutation is present in the majority of patients [4]. Therefore, rather than targeting a specific mutation, broad inhibition of T cell-specific signaling is a promising therapeutic approach for CTCL. Like Bruton's tyrosine kinase (BTK), the IL-2-inducible T cell kinase (ITK) (Fig. 1A) is a member of the Tec family of non-receptor protein tyrosine kinases. Tec kinases, like BTK and ITK, help to transduce signals originating from cell surface receptors. ITK is directly downstream of LCK in the T cell receptor (TCR)-mediated signal transduction cascade. It plays an important role in T cell activation, proliferation, differentiation, and effector cytokine production [5] (Fig. 1A). In contrast to BTK, ITK inhibitors have not been extensively studied for their anti-cancer properties, but it is known that ITK is expressed in all T cell malignancies, including CTCL. Because ITK is only expressed by T cells, NKT cells, and to a much lesser extent, mast cells, ITK-targeted therapy would be highly specific for T cell malignancies. Herein we demonstrate that ITK inhibition is a potential avenue for the development of CTCL-targeted therapies.

Through screening a 9600-diversity combinatorial solution-phase small-molecule library, compounds with inhibitory activities against ITK were isolated. Subsequent molecular modeling and extensive structure-activity relationship studies led to the identification of CTA191 (Fig. 1B). Docking studies of CTA191 with ITK indicate that CTA191 binds well to the kinase domain of ITK, fitting into the ATP-binding pocket (Fig. 1C). Binding appears to be mediated through hydrogen bonds and π - π hydrophobic interactions with residues in the ATP-binding pocket (Fig. 1C). The synthetic scheme of CTA191 is depicted in Fig. 1B.

Following inhibitor-kinase molecular modeling studies, kinase specificities were directly tested *in vitro* for the most promising ITK inhibitors. Fig. 1D demonstrates selective and

potent ITK inhibition of CTA191 by thin layer chromatography (TLC). To measure CTA191's ability to inhibit intracellular ITK, the CTCL cell lines HTB176 and CRL2105 were incubated with or without CTA191 (1000 nM) and autophosphorylation of ITK was measured by Luminex. Phosphorylation of ERK and I κ B (downstream kinases of ITK) were measured for comparison. Results demonstrate that CTA191 was able to significantly inhibit ITK autophosphorylation and I κ B phosphorylation at 1 and 5 min (Fig. 2A). Some downstream inhibition of ERK was detected in HTB176 but not CRL2105 cells (Fig. 2A, bottom panel).

To compare CTA191 to other known ITK inhibitors, HTB176 and CRL2105 cell lines were incubated with increasing concentrations of ITK inhibitors 34 [6], CTA056, and CTA191. Cell viability/growth was then measured using the WST1 assay with cell numbers extrapolated from WST1 OD values using a standard curve made from freshly plated cells in trypan blue-verified live CRL2105 and HTB176 cells. Compared to other ITK inhibitors, significantly fewer viable cells were noted following incubation with CTA191 ($p < 0.0001$ ANOVA for both CRL2105 and HTB176) (Fig. 2B). Next, cell death due to ITK inhibition was directly measured using the Calcein AM propidium iodide "live dead" flow cytometry assay. Results demonstrate that CTA191 readily induced cell death in both CTCL lines (Fig. 2C), but primary T cells incubated with or without CTA191 (2000 nM) and with or without anti-CD3/CD28 (Dynabeads) stimulation were resistant to killing (Fig. 2D).

To determine the mechanism by which ITK inhibition induces malignant T cell apoptosis, CTA191-induced gene expression was measured in HTB176 and CRL2105 cells using a quantitative real-time PCR array (PrimePCR, Bio-Rad). Results demonstrated that CTA191 induced upregulation of both pro-apoptotic (BCL-2L11) and anti-apoptotic (LMNA and BCL-2) genes (Fig. 2E). Given these mixed results, caspase-3/7 activity was then directly measured via fluorescent microscopy after CTA191 treatment. HTB176 and CRL2105 were preincubated with the caspase-3/7 green detection reagent CellEvent (ThermoFisher) and cultured with CTA191 for 6 and 8 h. Fig. 2F clearly demonstrates that caspase-3/7 becomes strongly activated in both CTCL lines following incubation with CTA191.

Advancements in combinatorial chemistry and high-through-put screening approaches have ushered in a wave of kinase-targeting drugs to the market, and the recent success of BTK inhibition highlights the selectivity of this approach. We have recently developed methodologies to identify small molecule inhibitors of ITK, a kinase in the same family of Tec but unique to T cells. ITK inhibitors have the potential to target T cell malignancies, which we have demonstrated for T cell acute lymphoblastic leukemia [7]. Here we have characterized a novel kinase inhibitor, CTA191, with selectivity for ITK over ETK and BTK. Molecular modeling studies demonstrated that this inhibitor binds strongly to ITK's ATP binding groove. Using different CTCL lines, we demonstrate CTA191's ability to target and kill malignant T cells. Importantly, ITK inhibition had little effect on primary T cells demonstrating their natural resistance to the inhibitor. Molecular characterization of CTA191's mechanism revealed that it induces apoptosis in part by upregulating BCL-2L11 and by inducing activation of caspase-3/7. Data reveals that both the intrinsic and extrinsic apoptosis pathways are engaged by CTA191 (Supplementary Fig. 1). These experiments highlight the importance of pursuing targeted kinase inhibition to treat CTCL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was supported by a grant from the NIH (# 1DP2OD008752-01) awarded to EM and NIH grants (# CA165263 and # CA150197) to HJK and (# CA160132) to KSL and a research grant from C-Tag Bioscience Inc to RL. This work was also supported by the Drs. Martin and Dorothy Spatz Charitable Foundation, as well as a grant from the National Cancer Institute (NCI P30CA093373). EM was also supported by career awards from the Burroughs Wellcome Fund and the Howard Hughes Medical Institute.

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Fig. 1.

ITK inhibitor synthesis, binding and specificity. (A) The T cell signaling cascade demonstrating that ITK inhibition may disrupt activation of downstream transcription factors (NFAT, AP1, NF-κB). (B) Synthesis of CTA191. i) L-valine methyl ester hydrochloride (1 eq.), DIEA (2.2 eq.), DMF, 45 min; ii) 1-(3-aminopropyl)piperidine (1 eq.), DIEA (1 eq.), DMF, 2 h; iii) Pd/C (10%, 200 mg/mmol), ammonium formate (20 eq.), ethanol, reflux, 3 h; iv) 4-(4-Pyridyl) benzaldehyde (1 eq.), DMF, overnight (C) Low energy binding mode predicted from computational docking of CTA191 to ITK. The model indicates that CTA191 interacts with residues near the ATP-binding region. CTA191 also displays good shape complementarity in this binding mode. (D) Purified kinases (20 nM, ITK, ETK, and BTK) and peptide substrate were incubated with [γ -³³P]ATP in a kinase reaction increasing concentrations of CTA191. As a measure of functional kinase activity, the resulting products were then analyzed by TLC assay demonstrating specificity for ITK over ETK and BTK.

Fig. 2.

CTA191 targets malignant T cells (A) CTA191 inhibits phosphorylation of ITK. Phosphorylation of ITK and downstream kinases (Erk and IκB) were quantified with Luminex (Bio-Plex Phosphoprotein detection reagents, BioRad). CTA191 significantly inhibited the phosphorylation of ITK and the downstream kinase IκB in both cell lines at 1 min ($p < 0.05$ Student's t -test). CTA191 also inhibited the phosphorylation of downstream Erk at 15 min in the HTB176 but not the CRL2105 CTCL cell line. (B) CTA191 inhibits growth of CTCL. B34, CTA056, and CTA191 were assayed for their ability to inhibit CRL2105 and HTB176 cells, left and right graphs respectively using the WST1 reagent (added for last 3–4 h of a 48-h culture). CTA191 was found to be superior in its ability to inhibit CRL2105 and HTB176 ($p < 0.00001$, ANOVA, $n = 4$). (C) CTA191 kills malignant T cells in a dose-dependent fashion. The HTB176 cell line was incubated in parallel with ITK inhibitors B34, CTA056 and CTA191 and dead cells were detected using the flow cytometry-based “live dead” assay (PI measures dead cells and Calcein AM measures live cells) ($n = 2$). At 2000 nM, all cells incubated with CTA191 were dead. (D) CTA191 has minimal cytotoxic effect against primary T cells. Compared to HTB176, primary T cells were highly resistant to CTA191-induced killing ($n = 2$). (E) CTCL lines upregulate anti-apoptotic and pro-apoptotic genes in response to CTA191. HTB176 and CRL2105 were incubated with CTA191 for six hours and then RNA was extracted using RNeasy plus mini kit (Qiagen, CA) ($n = 3$). Apoptosis regulating genes induced by CTA191 included BCL2, BCL2L11, FAS, and CFLAR ($p < 0.05$, nonparametric t -test). (F) CTA191 induces caspase-3/7 activation. Green fluorescence detected at 6 and 8 h following CTA191 incubation demonstrates that CTA191 induces caspase-3/7 activation in CTCL cell lines ($n = 2$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)