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Discovery of AICAR Tfase inhibitors that disrupt requisite enzyme dimerization

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Abstract—The discovery of a new class of aminoimidazole carboxamide ribonucleotide transformylase (AICAR Tfase) inhibitors through screening peptidomimetic libraries (>40,000 compounds) that act by inhibiting requisite enzyme dimerization is disclosed. In addition to defining key structural features of the lead compounds responsible for the activity, kinetic analysis of the remarkably small inhibitors established that they act as noncompetitive, dissociative inhibitors of AICAR Tfase with the prototypical lead (**A1B3**, Cappsin 1) exhibiting a K_i of 3.1 ± 0.3 μM. Thus, the studies define a unique approach to selectively targeting AICAR Tfase over all other folate-dependent enzymes, and it represents only one of a few enzymes for which inhibition achieved by disrupting requisite enzyme dimerization has emerged from screening unbiased combinatorial libraries.
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Aminoimidazole carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase (ATIC) is a bifunctional enzyme that catalyzes the final two steps in the purine biosynthetic pathway.[1] The aminoimidazole carboxamide ribonucleotide transformylase (AICAR Tfase) activity of the enzyme utilizes N^{10}-formyl tetrahydrofolic acid (10-f-FH_4) to transfer a form- yl group to AICAR installing the final carbon for the formation of the purine nucleus,[2] whereas the cyclohydrolase domain catalyzes the dehydrative ring closure to provide IMP (Fig. 1). Unlike other folate-dependent enzymes, AICAR Tfase has proven challenging to target selectively with competitive substrate or cofactor analogues. Nonetheless, inhibition of ATIC is an attractive target for antineoplastic intervention.[2]

Key to understanding the unique behavior of ATIC is the recently disclosed X-ray structure of the enzyme.[3] The structure revealed that the AICAR Tfase active site is formed by enzyme dimerization and at the dimer interface enlisting residues from two monomers to create the substrate and folate binding pockets, whereas the IMP cyclohydrolase active site is isolated in separate domains of each monomer in the dimer. These structural insights suggested that, in addition to developing competitive inhibitors that target the active sites, compounds that block enzyme dimerization would also inhibit AICAR Tfase activity. Not only would this provide a unique approach to selective inhibition of AICAR Tfase over all other folate-dependent enzymes, a challenge that has not been successfully addressed with active site

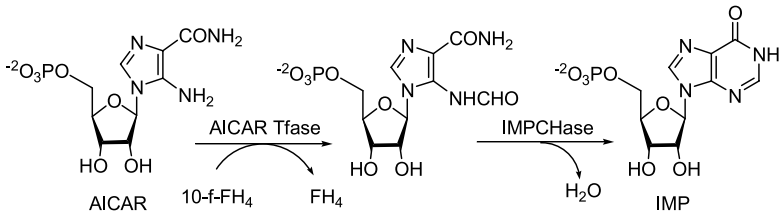

Figure 1. Reactions catalyzed by ATIC.

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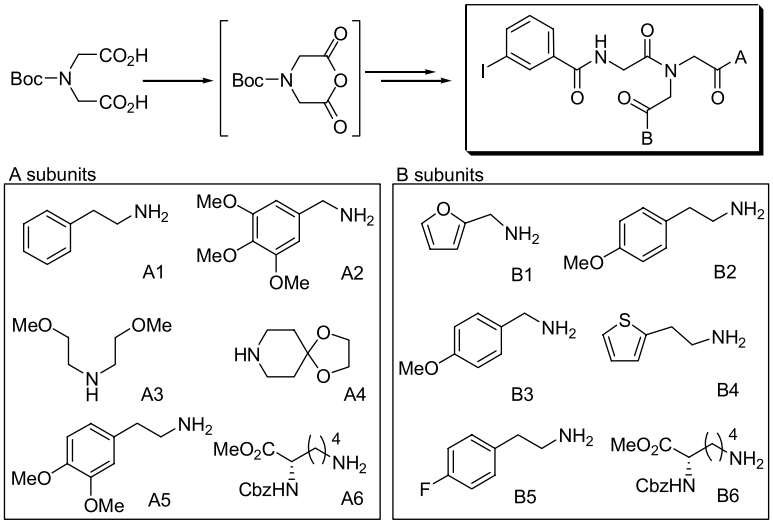

Figure 2. Thirty-six-member mixture exhibiting inhibition of ATIC.

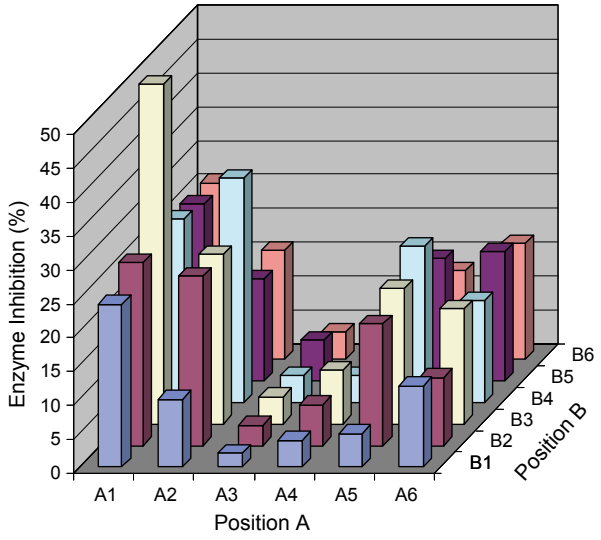

Figure 3. Percent inhibition of AICAR Tfase activity of ATIC by the 36 individual compounds: 50 μM, 50 nM ATIC, 60 μM 10-f-FH_4, 50 μM AICAR @ 5 min.

competitive inhibitors, but the discovery of such dimerization inhibitors would represent a welcome new addition to a still small group of enzymes that have been targeted by such an approach.[4,5] Given previous success in identifying small-molecule modulators of protein–protein interactions from our peptidomimetic libraries,[6] the collection was screened for dissociative inhibitors of ATIC.

The library of more than 40,000 compounds was tested for inhibition of AICAR Tfase activity in an assay measuring consumption of 10-f-FH_4[7] enlisting a relatively high concentration of enzyme (50 nM) to insure only effective inhibitors would be detected.[8] The most potent activity was observed with a mixture of 36 compounds (Fig. 2) which showed inhibition of the enzyme when screened at a total concentration of 50 μM. Each of the 36 compounds was synthesized using the solution-phase protocol enlisted for the preparation of the libraries (isolation of the intermediates and final products by liquid–liquid acid–base extraction[9]) and individually screened in the assay (Fig. 3). The discernable trends for Position A are that an aromatic substituent is required and that phenethylamine (A1) was the most effective substituent. No trend was apparent for Position B and all compounds contained the iodobenzoylglycinamide. The most potent and representative active compounds were selected for further analysis and screened at decreasing concentrations and each exhibited concentration-dependent inhibition (Fig. 4A).

Kinetic analysis of the most potent inhibitor, **A1B3** (Cappsin 1), defined the mechanism of inhibition. A Lineweaver–Burk plot indicated that the compound is a noncompetitive inhibitor of the enzyme (Fig. 5). Parallel lines at various inhibitor concentrations relative to the enzyme alone in a Zhang–Poorman plot establish that Cappsin 1 is a dissociative, rather than allosteric, noncompetitive inhibitor of ATIC (Fig. 6).[10] Notably, the parallel and especially the linear behavior of the inhibition over a nearly 100-fold enzyme concentration

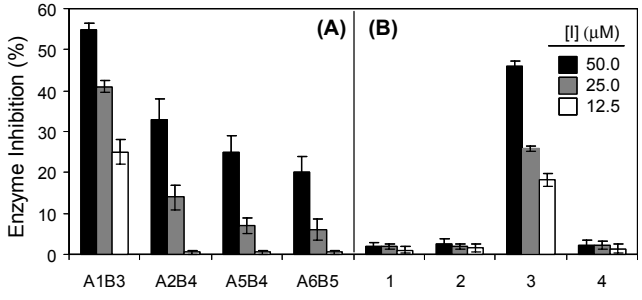

Figure 4. (A) Each of the selected inhibitors AXBX exhibits concentration-dependent inhibition of AICAR Tfase. (B) Compounds **1**, **2**, and **4** lose their inhibitory activity whereas **3** (Cappsin 2) retains activity against AICAR Tfase and contains the essential structural elements of the lead compound (50 nM ATIC, 60 μM 10-f-FH_4, 50 μM AICAR @ 5 min).

conducted at the relatively high enzyme concentrations (5–300 nM vs 1 nM characteristic of most HTS) insure the behavior is not characteristic of a nonspecific aggregate mechanism of inhibition.[8,11] A modified Zhang–Poorman plot established a 1:1 stoichiometry for Cappsin 1 binding to an ATIC monomer (Fig. 7). The K_d of ATIC in the presence of AICAR and 10-f-FH_4 was measured as 3.2 ± 0.5 nM. Using this value measured for the K_d, the K_i of Cappsin 1 was established to be 3.1 ± 0.3 μM.

A structure scan of Cappsin 1 was undertaken to identify its components that confer activity (Fig. 8). Removal of the iodide or the phenethylacetamide substituent, and replacement of the iodobenzoylglycinamide side chain with a Boc group abrogated activity (Fig. 4B). However, deletion of the *p*-methoxybenzylacetamide substituent provided a smaller molecule (**3**, Cappsin 2) that retained nearly all the inhibitory potency of Cappsin 1 (**A1B3**). Thus, the phenethylamine and iodobenzoylglycinamide are the essential components of the lead inhibitor that confer its inhibitory activity. Notably, each of the active constituents of the original mixture bear these structural characteristics

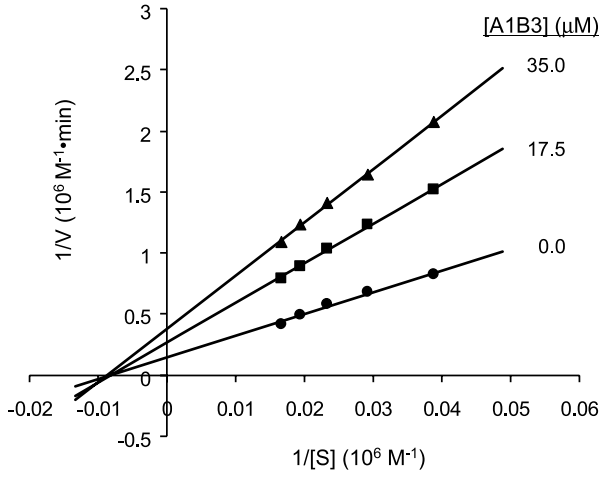

Figure 5. Lineweaver–Burk plot indicates that **A1B3** is a noncompetitive inhibitor of AICAR Tfase.

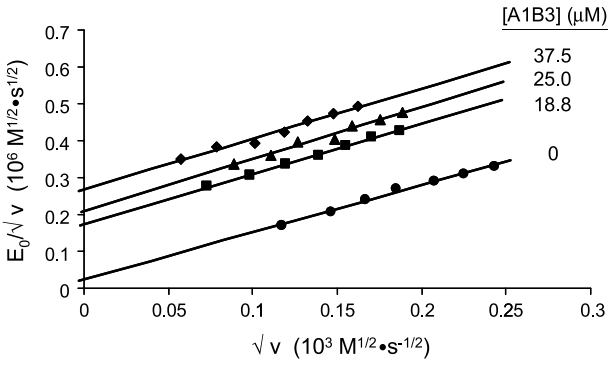

Figure 6. Zhang–Poorman plot for **A1B3** (Cappsin 1) establishes that the molecule is a dissociative inhibitor of ATIC dimerization: 5–300 nM ATIC, 60 μM 10-f-FH_4, 50 μM AICAR.

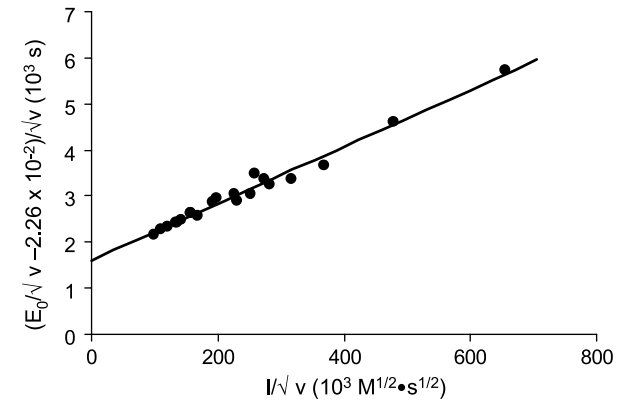

Figure 7. A modified Zhang–Poorman plot indicates a 1:1 binding stoichiometry for **A1B3** to ATIC monomer.

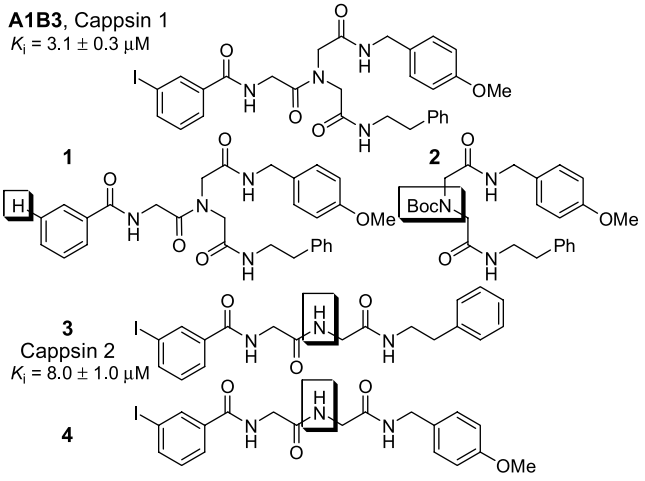

Figure 8. Scan of A1B3.

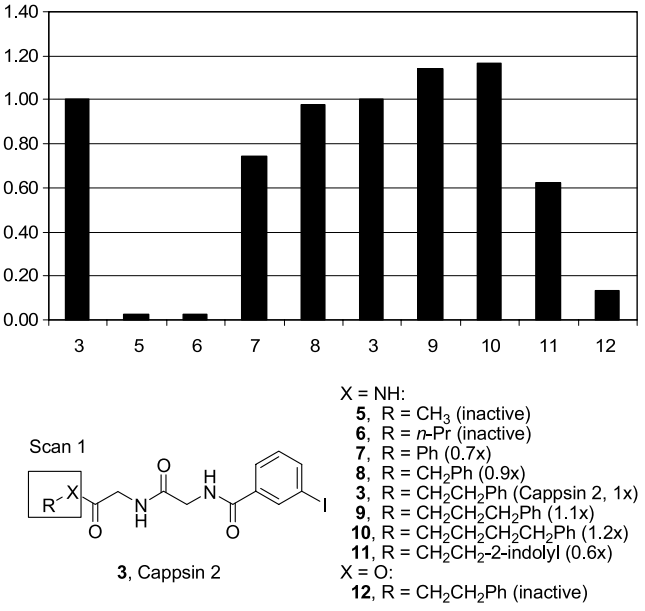

Figure 9. Scan 1: average inhibition at 25 and 50 μM reported relative to Cappsin 2 (45% average inhibition by Cappsin 2; 50 nM ATIC, 60 μM 10-f-FH_4, 50 μM AICAR @ 5 min).

(Fig. 2), and the remarkable distinction between **3** and **4** highlight the selectivity observed with Cappsin 1 and 2 (**A1B3** and **3**). The experimentally established 1:1 binding stoichiometry along with observations that Cappsin 1 and 2 do not form aggregates in buffer under the conditions of the assay[12] insure the inhibitors are not acting by such a general nonspecific mechanism. Consistent with these expectations and the anticipated selectivity for AICAR Tfase inhibition versus other folate-dependent enzymes, Cappsin 1 and Cappsin 2 failed to inhibit glycinamide ribonucleotide transformylase (GAR Tfase), and dihydrofolate reductase (DHFR): 0% inhibition at 50 μM. These observations along with the fact that addition of 0.01% Triton X-100 had little effect on the AICAR Tfase inhibitory potency of Cappsin 1 and 2 confirmed that they are not acting as nonspecific, aggregate enzyme inhibitors.[11]

A small library of compounds was prepared to provide additional insights into the key structural features of Cappsin 2 responsible for its properties. In Scan 1, removal of the phenethyl side chain (**5**) or phenyl group (**6**) from Cappsin 2 eliminated activity; replacement of the phenethyl amide linkage with an ester eliminated activity (**12**); and smooth, progressive increases in potency were observed in the series $(CH_2)_nPh$ (n = 0–4 (**3**, **7**–**10**), 4 ⩾ 3 > 2 > 1 > 0) (Fig. 9).

Although the removal of the *p*-methoxybenzylacetamide of Cappsin 1 to provide Cappsin 2 resulted in only a small drop in potency, its replacement with an *N*-acetic acid (**13**), methyl acetate (**14**), or simple methyl group (**15**) eliminated activity (Fig. 10). Removal of this central linking amide and replacement with an ethyl spacer yielded **17**, the only compound in Scan 2 that retained any appreciable activity. To date, the limited efforts to conformationally restrict this portion of the structure have not resulted in more active inhibitors and typically have abrogated potency.

Finally, an initial series of *m*-iodophenyl substitutions (Scan 3) revealed that a *m*-substituent is optimal ($m > p$ (0.2x) > o (inactive) for I), that a well-defined trend for the halide substituents exists (I > Br > Cl > F > H (inactive)), and that other large, soft hydrophobic substituents may substitute for I (SMe (0.75x), Ph (0.75x); but not NO_2, Me, OMe, inactive) (Fig. 11).

Herein, a new class of AICAR Tfase inhibitors were discovered through screening combinatorial libraries (>40,000 compounds) that act by inhibiting requisite enzyme dimerization. Kinetic analysis of the remarkably small inhibitors established that they act as noncompetitive, dissociative inhibitors of ATIC with the prototyp-

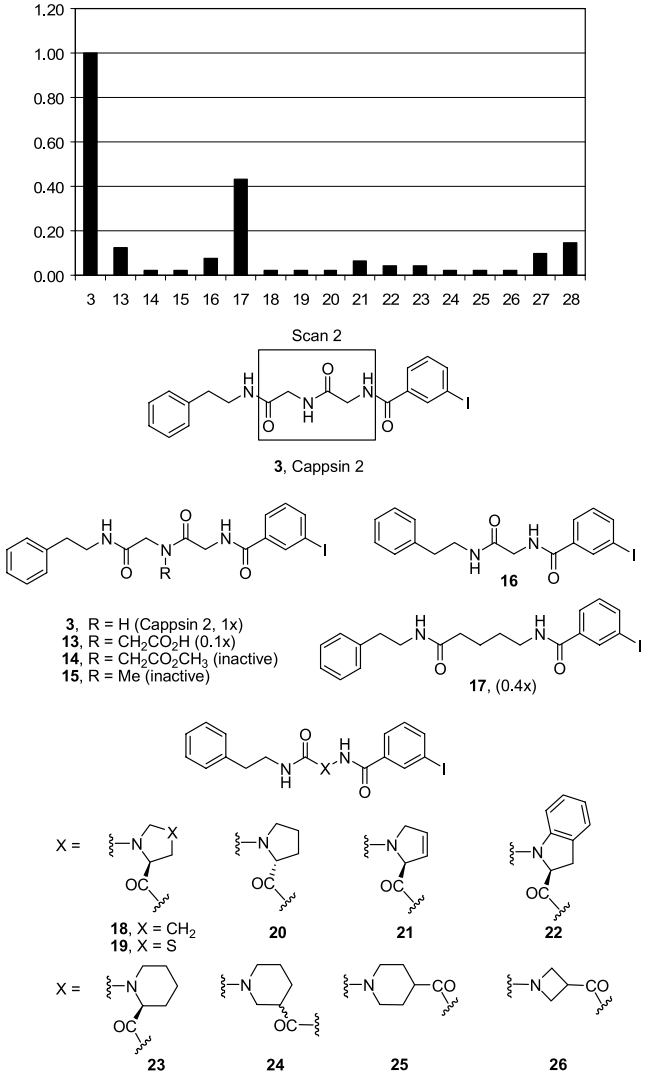

Figure 10. Scan 2: average inhibition at 25 and 50 μM reported relative to Cappsin 2 (45% average inhibition by Cappsin 2; 50 nM ATIC, 60 μM 10-f-FH_4, 50 μM AICAR @ 5 min).

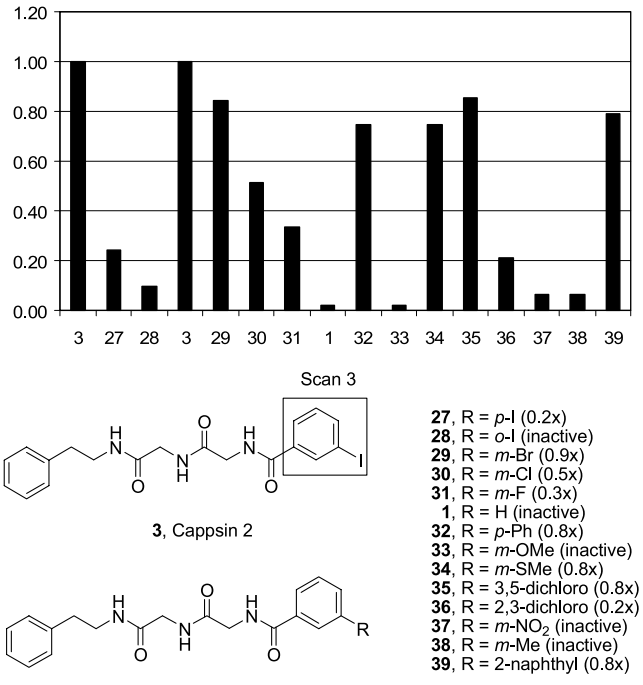

Figure 11. Scan 3: average inhibition at 25 and 50 μM reported relative to Cappsin 2 (45% average inhibition by Cappsin 2; 50 nM ATIC, 60 μM 10-f-FH_4, 50 μM AICAR @ 5 min).

ical lead Cappsin 1 (**A1B3**) exhibiting a K_i of 3.1 ± 0.3 μM. A first level structure scan of this lead defined the essential and surprisingly selective features required for activity and provides the basis on which library elaboration of Cappsin 1 and 2 may be conducted. More fundamentally, the studies define a unique approach to selectively targeting AICAR Tfase over all other folate-dependent enzymes and, to our knowledge, represents only the second enzyme for which inhibition achieved by disrupting requisite enzyme dimerization emerged from screening unbiased combinatorial libraries.

Acknowledgments

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Supplementary data

Experimental details for **A1B3**, **A2B4**, **A5B4**, **A6B5**, and **1**–**4**, and for the enzymatic assays are provided. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.bmcl.2005.03.094.

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12. Light scattering: Buffer alone (8.2 kcps), buffer containing 50 μM Cappsin 2 and 1% DMSO (9.1 kcps). Typical aggregates result in an increase in scattering intensity of at least an order of magnitude, see Refs. 8 and 11.