Antitumor Agents

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Pharmacophore Reassignment for Induction of the Immunosurveillance Cytokine TRAIL**

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Abstract: *Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is an immunosurveillance cytokine that kills cancer cells but demonstrates little toxicity against normal cells. While investigating the TRAIL-inducing imidazolinopyrimidinone TIC10, a misassignment of its active structure was uncovered. Syntheses of the two isomers, corresponding to the published and reassigned structures, are reported. The ability of each to induce TRAIL expression in macrophages was investigated and it was found that only the compound corresponding to the reassigned structure shows the originally reported activity; the compound corresponding to the published structure is inactive. Importantly, this structural reassignment has furnished a previously unknown antitumor pharmacophore.*

Cancer immunosurveillance relies on various effector functions of the immune system that can modify both induced and spontaneous carcinogenesis.[1,2] TRAIL is an immunosurveillance cytokine that plays a critical role in this process through its ability to selectively induce apoptosis in cancer cells over normal cells.[2–6] The TRAIL gene is expressed in a variety of tissues and cells,[3] including dendritic cells, natural killer (NK) cells, and monocytes/macrophages.[7] Its expression is controlled by several transcriptional regulators, such as the transcription factors NF-κB and p53.[8] The reduction of TRAIL expression by neutralizing antibodies and the ablation of TRAIL expression in mice lacking the *TRAIL* gene results in the development of carcinogen-induced fibrosarcomas, sarcomas, and lymphomas; especially in p53-deficient mice.[9,10] These data are also consistent with observations that a change in TRAIL expression in immune cells is associated with TRAIL resistance in cancer cells.[11] The effectors of TRAIL production in immune cells are thus of clinical relevance[7] and could also be used to achieve a model system for studying the complex immunosurveillance signaling system.

A recent report highlighted imidazolinopyrimidinone **1** (Figure 1), obtained from the NCI, which was able to induce TRAIL expression in a variety of cell lines.[12] This compound, designated TIC10 (TRAIL-inducing compound 10), was demonstrated to be thermally stable and orally bioavailable, thus surmounting liabilities inherent in recombinant TRAIL therapies. It was also able to induce tumor regression in several xenograph models with no observed toxicity at four times the effective dose.

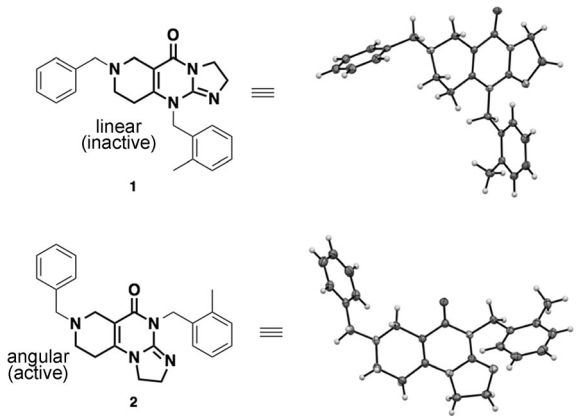

Figure 1. Structures of the imidazolinopyrimidinones synthesized in this study. The tricyclic core of each structure is highlighted in bold. The compound with an angular structure (**2**) and not that with a linear structure (**1**) induces TRAIL expression.

It is important to note that the effective dose of TIC10 (25 mg kg^{-1}) was as a stand-alone therapy and thus suffers from the same resistance mechanisms observed in other TRAIL therapies.[13] We have reported how quorum-sensing signaling molecules (acylhomoserine lactones) can overcome tumor-cell TRAIL resistance through the inhibition of NF-κB.[14] In terms of antitumor therapy, the combination of these two treatments could address the fundamental shortcomings of TRAIL as a therapeutic.[15] As a logical starting point, **1** was required; yet despite a wealth of biological data, no chemical synthesis was reported.

TIC10 (**1**) was prepared in four steps from 4-chloronicotinic acid (Scheme 1). Briefly, acylation of an activated carboxylic acid followed by a double displacement reaction, subsequent hydrogenation, and reductive amination afforded **1** in 52% overall yield. This structure was confirmed by mass spectrometric, NMR spectroscopic, and X-ray crystallographic analyses (Figure 1).

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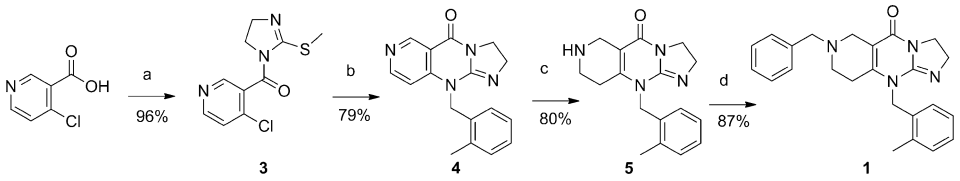


Scheme 1. Synthesis of the reported structure of TIC10 (**1**). Reagents and conditions: a) $SOCl_2$, 90 °C, 1 h, then 2-methylthio-2-imidazoline hydroiodide, Et_3N, CH_2Cl_2, 0 °C to RT, 19 h, 96%; b) 2-methylbenzylamine, K_3PO_4, *N*,*N*-dimethylacetamide, reflux, 1 h, 79%; c) 45 psi H_2(g), PtO_2, MeOH/TFA, RT, 5 h, 80%; d) PhCHO, $Na(OAc)_3BH$, AcOH, CH_2Cl_2, RT, 4 h, 87%.

The biological activity of **1** was measured by RT-PCR analysis of *TRAIL* mRNA expression in the murine macrophage cell line RAW 264.7. Surprisingly, no change in *TRAIL* mRNA expression over controls was observed, even at doses as high as 10 μM (Figure 2a) or with prolonged exposure (Figure 2b). In order to confirm the disparity between our results when using **1** and those reported by Allen et al.,[12] a sample of TIC10 (**2a**) was acquired from the NCI.[16]

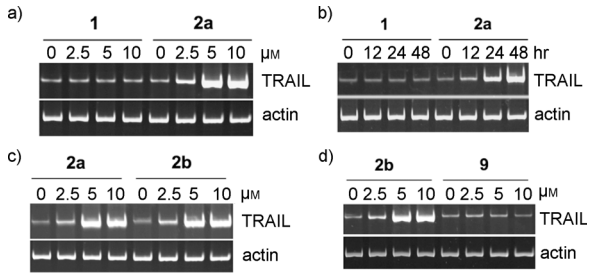


Figure 2. Angular fusion of the tricyclic core is required for imidazolinopyrimidinone-mediated induction of *TRAIL*. a) Induction of *TRAIL* mRNA in RAW cells treated for 48 h with the indicated dose of linear (**1**) or angular (**2a**) TIC10. b) Induction of *TRAIL* mRNA following treatment with **1** or **2a** (5 μM) for the indicated times. c) Dose-dependent response to repository (**2a**) or synthesized (**2b**) angular imidazolinopyrimidinone after 48 h exposure. d) Dose-dependent response to angular (**2b**) or commercial (**9**) imidazolinopyrimidinone.

In sharp contrast to our results with **1**, this material was able to significantly induce *TRAIL* mRNA expression over controls at doses as low as 2.5 μM (Figure 2a) and within 12 h of exposure (Figure 2b). The induction appeared linear over 48 h at 5 μM (Figure 2b).

Because of the stark discrepancy in the biological activities of these materials, an in-depth structural analysis of **2a** was initiated. The material obtained from the repository was examined through heteronuclear multiple-bond correlation (HMBC) NMR spectroscopy, which, unexpectedly, revealed no correlation between the methylene protons of the imidazoline ring and the carbonyl carbon atom. Meanwhile, significant correlation was observed between the benzylic protons of the 2-methylbenzyl moiety and the carbonyl carbon atom. This data is more consistent with a constitutional isomer of TIC10 possessing an angular (rather than linear) tricyclic core (**2**). This critical structural difference between **1** and **2** might also explain why the imidazoline protons in the ^{1}H NMR spectrum for **2** appear as a congested multiplet ($\delta \approx 3.9$ ppm), whereas as imidazoline protons in the ^{1}H NMR spectrum for **1** are cleanly separated into two triplets ($\delta \approx 4.1$ and 3.8 ppm) owing to a marked difference in shielding by the conjugated system (Figure 3). In addition to the equivocality of the NMR data, the structure of the repository TIC10 (**2**) was ultimately confirmed by X-ray crystallographic analysis (Figure 1).

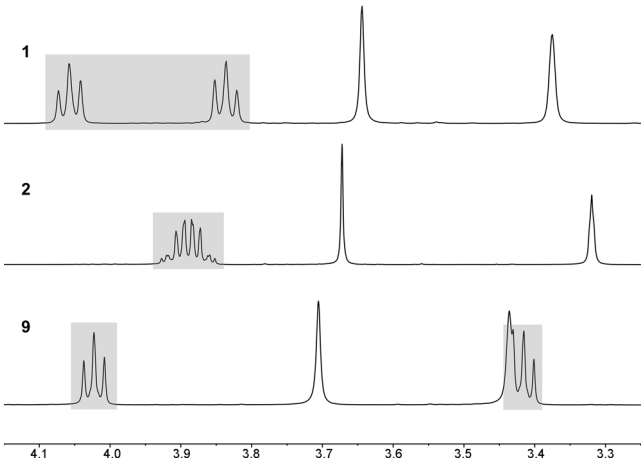
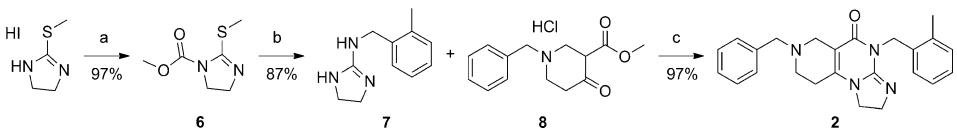


Figure 3. ^{1}H NMR overlay of **1**, **2**, and **9**. The imidazoline ring protons have distinct patterns corresponding to the linear or angular core structure.

Our new target, angular imidazolinopyrimidinone **2**, was prepared in three steps in 82% yield (Scheme 2). Guanidine **7** was obtained in two steps from commercially available 2-methylthio-2-imidazoline hydroiodide.[17] Subsequently, a mixture of **7** and 1-benzyl-4-oxopiperidine-3-carboxylate hydrochloride (**8**) in refluxing methanol and sodium methoxide afforded **2** almost exclusively; a trace amount of **1** was detected by ^{1}H NMR following work-up of this reaction but it was removed by subsequent purification. We rationalize this result by considering that the imidazoline ring nitrogen atoms of **7** possess both statistical and steric advantages over the benzylic nitrogen atom of **7**. Initial attack by nitrogen at the



Scheme 2. Synthesis of the actual structure of TIC10 (**2**). Reagents and conditions: a) methyl chloroformate, Et_3N, CH_2Cl_2, 0 °C to RT, 44 h, 97%; b) 2-methylbenzylamine, MeOH/AcOH, reflux, 45 h, 87%; c) NaOMe, MeOH, reflux, 18 h, 97%.

ketone carbonyl of **8** affords an aminocarbinol intermediate, which undergoes intramolecular cyclocondensation and dehydration to provide **2**. Its structure was confirmed by mass spectrometric and NMR spectroscopic analyses. Our synthetic product **2b** was able to induce *TRAIL* mRNA expression with similar potency to the material obtained through the repository (**2a**; Figure 2c).

During the preparation of this manuscript, linear imidazolinopyrimidinone (TIC10; **1**) purportedly became commercially available.[18] This commercial material (**9**) also proved unable to induce *TRAIL* expression in RAW cells (Figure 2d). X-ray crystallographic analysis revealed this compound to be a third constitutional isomer (Figure 4). It has the same linear tricyclic core as **1**, but the 2-methylbenzyl substituent is attached to the adjacent (imidazoline ring) nitrogen atom.

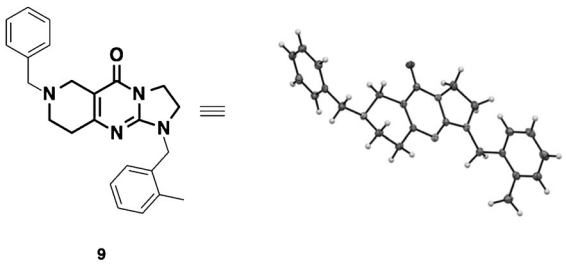


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Figure 4. The structure of a commercially available imidazolinopyrimidinone. The tricyclic core is highlighted in bold. This compound proved unable to induce *TRAIL* expression.

In view of these discrepancies, a brief discussion of the heterocyclic chemistry of **1**, **2**, and **9** is warranted. With regard to the cyclocondensation step, depending on the reaction conditions used to form the carbinol, it may be possible to obtain either product isomer (**1** or **2**). Indeed, it has been reported that the K_2CO_3-mediated reaction of a β-keto ester with a 2-amino-2-oxazoline (a type of unsymmetrical 1,1-diamine) affords a mixture of linear and angular products.[19] Empirical and theoretical evidence supported the notion that *"the endocyclic nitrogen atom is the most nucleophilic and attacks the most electrophilic carbon of the biselectrophile. A ring closure between the exocyclic nitrogen atom and the second electrophilic center concludes the bicyclic heterocycle synthesis."*[19] This is consistent with our own observations of the synthesis of **2**. One may speculate that the original synthesis of **1** unexpectedly produced **2** instead (Figure 5, path a). In the absence of rigorous structure elucidation through correlation NMR spectroscopy and/or X-ray crystallographic analysis, misassignment is possible. Only with full spectral data (and X-ray crystal structures) in hand can the discrepancies really be appreciated. Also, whereas there is a plethora of literature precedent for linear fused pyrimidinones akin to **1**, there is a paucity of literature precedent for angular fused pyrimidinones akin to **2**. Finally, one may speculate that compound **9** may have arisen through attempted N-alkylation of a linear fused precursor en route

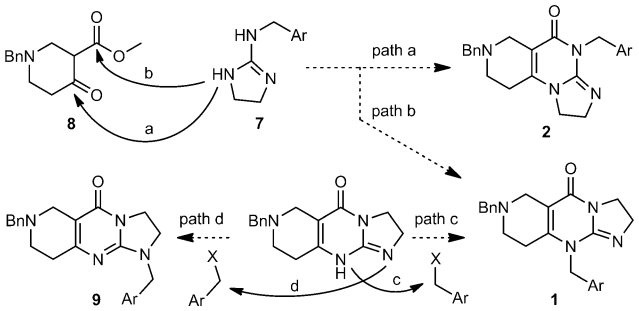

Figure 5. A plausible rationale for undesired condensation and alkylation outcomes during imidazolinopyrimidinone synthesis.

to **1** (the originally reported TRAIL-inducing compound), where N-alkylation of the wrong nitrogen atom[20] afforded **9** (Figure 5, path d) instead of **1**.

The report by Allen et al.[12] extolled the clinical significance of TIC10, a fact further borne out by its recent commercial availability and that it is featured in an investigational new drug (IND) application.[21] However, our findings imply that the angular fusion of the tricyclic core (as in **2** rather than **1**) is a required feature of this pharmacophore for *TRAIL* induction. Given the structural misassignment explained herein, this drug development pursuit must thus be questioned.

With a concise synthesis for the active material (**2**) and irrefutably structural confirmation through X-ray crystallographic analysis, further evaluation of this angular scaffold with respect to *TRAIL* induction can be undertaken. It will now also be possible to examine with confidence the structure–activity relationships (SAR) of this scaffold, explore its mechanism of action, proceed with subsequent optimization, and initiate studies into possible combination therapies. Finally, this angular imidazolinopyrimidinone has not been previously disclosed and thus represents a promising new pharmacophore for further exploration as a therapeutic.

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