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Selective cytotoxicity of azatyrosinamides against *ras*-transformed NIH 3T3 cells

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Abstract—This study aims to develop novel azatyrosinamide compounds structurally modified from *ras*-specific antioncogenic azatyrosine. Analogues **4**–**15** were prepared and their inhibition on the growth of wild-type and *ras*-transformed NIH 3T3 cell lines was compared. Compound **12** was found to be the most active with IC_{50} 16.5 ± 2.2 μM which is 458-fold more potent than that of azatyrosine. The selective toxicity, defined as $IC_{50\ \text{wild-type}}/IC_{50\ ras\text{-transformed}}$ for this compound was 138.5.

Small GTPases of the Ras superfamily are crucial components of signal transduction pathways leading from cell-surface receptors to the control of cell proliferation, differentiation, or death. The mammalian genome contains three *ras* genes that encode highly related proteins of 21 kDa termed H-Ras, N-Ras, and K-Ras with its two variants, K-Ras4A and K-Ras4B, generated from two alternative fourth exons. The three *ras* genes are concurrently expressed in most mouse and human tissues.[1] Uncontrolled Ras-signaling has been implicated in the development of human cancer.[2] Signaling transmitted through Ras results in activation of several downstream effectors, with the Raf/ERK1/ERK2 and the phosphatidylinositol 3-kinase/AKT pathways playing a crucial role in modulation of the *ras* target genes which in turn regulate cell functions.[3,4] Azatyrosine (L-β-(5-hydroxy-2-pyridyl)alanine), an antibiotic from *Streptomyces chibanensis*,[5] was first reported by Shindo-Okada et al. to suppress the growth of NIH 3T3 cells transformed by c-Ha-ras, c-Ki-ras, N-ras, or c-raf oncogene while having no effect on the growth of wild-type cells.[6] Those azatyrosine-treated transformed cells that survived reverted to an apparently normal phenotype and the normal appearance and growth characteristics of the cells persisted for months after removal of the compound.[7,8] Some of the revertant clone demonstrated complete loss of tumorigenicity in nude mice.[6] Studies also proved that this compound is involved in the regulation of other oncogenic cell growth.[9–12] The high reversion efficiency toward oncogenic transformed cells combined with low toxicity to normal cells suggested azatyrosine to be a new lead for developing anticancer agents.

However, the potential of azatyrosine in cancer treatment is limited due to its potency. Relatively high concentrations (1–2 mM)[10] are necessary for the activity. We believe that this is partially due to the low intracellular availability of azatyrosine. The intracellular availability is limited not only by the number and the turnover rate of the tyrosine amino acid transporter, but also by the fact that azatyrosine must compete with tyrosine for access to the transporter. Structural modification of azatyrosine such that it is no longer dependent upon the membrane-associated amino acid transporter therefore becomes a rational approach to circumvent this limitation. This report describes the synthesis of a series of ester and amide derivatives of azatyrosine. The in vitro inhibitory activities of the analogues were determined. Selective toxicity in terms of inhibition on

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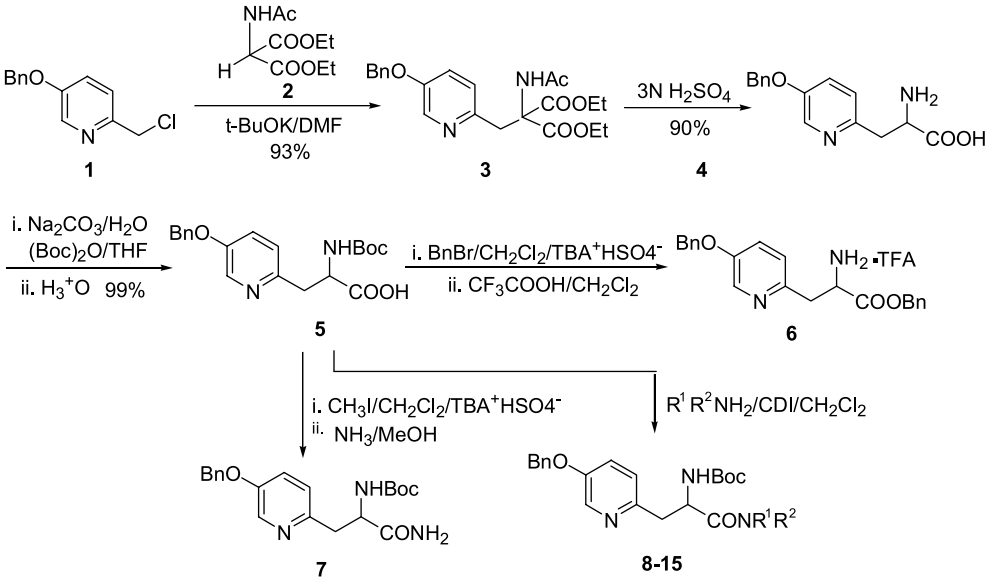
the growth of wild-type and *ras*-transformed NIH 3T3 cell lines was also determined.

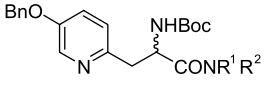
Analogues **4**–**15** of azatyrosine were prepared via the route depicted in Scheme 1. Condensation of **2** with **1**[13] afforded a malonate derivative (**3**), which upon hydrolysis with aqueous 3-N sulfuric acid afforded the benzyl derivative of azatyrosine **4**. Protection of the amino group of **4** with Boc gave analogue **5**. Benzylation of **5** followed by the removal of Boc group gave the ester analogue **6**. Amide analogues **7**–**15** were obtained by condensation of **5** with a variety of amines.

The synthesized compounds[14] were screened on wild-type and *ras*-transformed NIH 3T3 cells for the comparison of their cytotoxicity.[15] Screening was carried out in 96-well plate and the cells, with a density of 1500–2500 cells/well, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C under an atmosphere of 7.5% CO_2 in air. Growth curves for each cell line were established. The DMSO solution of each compound was incubated with the cell for 48 h. The Hansen MTT assay method was modified for estimating the cell number.[16] In brief, the absorption of generated formazan blue at $\lambda_{570\ nm}$ was recorded with ELISA reader. The number of surviving cells was determined from a calibration curve derived by correlating the absorption to the cell number determined from a hemacytometer. At least three experiments were carried out for each compound and IC_{50} was calculated using



Scheme 1.

Table 1. Growth inhibition of azatyrosine and its analogues against wild-type and *ras*-transformed NIH 3T3 cell lines



Compound	R^1	R^2	IC_{50} (μM)[a]		
			Wild-type	*ras*-Transformed	SI[b]
Azatyrosine	—	—	10793.0 ± 471.8	7554.8 ± 417.5	1.42
4	—	—	—	1000	—
5	—	—	—	1000–2000	—
6	—	—	—	100	—
7	H	H	1716.8 ± 10.4	73.1 ± 41.8	23.5
8	*n*-Propyl	H	989.7 ± 707.3	34.8 ± 0.8	28.4
9	Allyl	H	213.7 ± 111.5	74.2 ± 15.2	2.9
10	Propagyl	H	410.8 ± 247.3	45.8 ± 1.65	9.0
11	Cyclopropyl	H	1926.1 ± 898.7	104.5 ± 83.8	18.4
12	Cyclohexyl	H	2284.8 ± 1763	16.5 ± 2.2	138.5
13	$-(CH_2)_5\tilde{N}$		74.8 ± 18.7	42.0 ± 3.9	1.8
14	$-(CH_2)_4\tilde{N}$		148.1 ± 12.5	59.3 ± 17.8	2.5
15	$-(CH_2)_2-S-(CH_2)_2-$		154.4 ± 3.9	50.3 ± 1.04	3.1

[a] Data presented are means ± SD of 3–5 experiments.
[b] SI denotes $IC_{50\ wild\text{-}type}/IC_{50\ ras\text{-}transformed}$.

sigmoidal regression. SI, the selectivity of growth inhibition of each test compound, was defined as the ratio of IC_{50} on wild-type to IC_{50} on *ras*-transformed cell lines.

The inhibitory activity of compounds **4**–**15** is summarized in Table 1. The acid analogues **4** and **5** were slightly more active than azatyrosine on inhibiting the growth of *ras*-transformed cells. The ester analogues **6** showed an IC_{50} about 75-fold lower than that of azatyrosine. Comparison of the IC_{50} between azatyrosine and its amide analogue **7** indicated that high lipophilicity might increase the intracellular bioavailability for exhibiting the inhibitory activity. Thus, we further synthesized a series of amide analogues **8**–**15**. As indicated in Table 1, all the amide analogues showed higher activity than azatyrosine on both cell lines. Compound **12** exhibited the highest activity among all the amide analogues tested with IC_{50} 16.5 ± 2.2 μM, a concentration 458-fold lower than that for azatyrosine. The SI of compound **12** was 138.5, indicating its high selective toxicity on *ras*-transformed cells. Although cyclic tertiary amides (**13**–**15**) showed good activity, their SI's were unfortunately low.

In conclusion, most of the compounds exhibited an inhibitory effect on *ras*-transformed NIH 3T3 cells with activities more potent than that of azatyrosine. Compound **12** was the most active with the highest selective toxicity on *ras*-transformed cells. Whether the activity came from the compound per se or from azatyrosine, the potential metabolite, need to be further investigated.

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14. All compounds were characterized by ^{1}H NMR, mass and C, H, N elemental analysis. Spectral data of representative compounds. **8**: mp 117.0–117.5 °C; ^{1}H NMR ($CDCl_3$) δ, ppm: 8.23 (pyridine-α-H, 1H, d, $J = 2.6$ Hz), 7.37 (Ph, 5H, m), 7.17 (pyridine-γ-H, 1H, dd, $J = 2.6$, 8.6 Hz), 7.10 (pyridine-β-H, 1H, d, $J = 8.6$ Hz), 6.73 (NH, 1H, br), 6.19 (–C*H*–CO, 1H, d, $J = 6.8$ Hz), 5.06 (Ph–CH_2–, 2H, s), 4.46 (NH, 1H, br), 3.11 (–CH_2CHCONHCH_2–, 4H, m), 1.41 (*t*-Bu, 9H, s), 1.36 (–NHCH$_2$CH_2CH$_3$, 2H, hexalet, $J = 7.2$ Hz), 0.77 (–NHCH$_2$CH$_2$CH_3, 3H, t, $J = 7.2$ Hz); EI-MS (70 eV), 339 (100%), 248, 225, 91; Anal. Calcd for $C_{23}H_{31}N_3O_4$: C, 66.81; H, 7.56; N, 10.16. Found: C, 66.43; H, 7.26; N 10.20.
15. NIH 3T3 wild-type and NIH 3T3 val^{12}-*ras* cells were kindly supplied by Professor W. P. Wang of the Immunology Department of National Taiwan University Medical College and Dr. M. Campa of Duke University, respectively.
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