Azatyrosinamides: Novel RAS-related Anticancer Agents

CHUN-LI WANG[1], ON LEE[2], CHIU-FEN HUANG[3], ERIC I-CHIAN LI[3], CHE-MING TENG[4], SHIOW-LIN PAN[5], JANG-FENG LIAN[1,6], FENG-SHOU CHANG[1,6], JING-PING LIOU[1] and HUI-PO WANG[1,6]

[1]*School of Pharmacy, College of Pharmacy, and* [6]*Center of Excellence for Cancer Research, Taipei Medical University, Taipei, Taiwan, R.O.C.;*
[2]*Biomedical Technology and Device Research Laboratories, Industrial Technology Research Institute, Hsinchu City, Taiwan, R.O.C.;*
[3]*Graduate Institute of Pharmacology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, R.O.C.;*
[4]*Institute of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan. R.O.C.;*
[5]*Institute of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Zhunan Town, Miaoli County, Taiwan, R.O.C.*

Abstract. *Background: We previously reported on the design and synthesis of novel azatyrosinamide derivatives selective for ras-transformed NIH3T3 cells and with improved toxicity over azatyrosine. This study was aimed at investigating the mechanism of action and the antitumour activity of these compounds in ras-transformed cells. Materials and Methods: Nine azatyrosinamides were previously screened for anticancer activity in both wild-type and ras-transformed NIH3T3 cells; the most active compounds were further tested in vitro and in vivo. Results: HPW98-1 and HPW98-2 induced formation of apoptotic bodies in ras-transformed NIH3T3 cells in vitro and inhibited anchorage-independent growth. Excess tyrosine reduced the cytotoxic effect of azatyrosine, but not of HPW98-1 and HPW98-2. HPW98-1 reduced vascular endothelial growth factor-mediated angiogenesis in a Matrigel plug assay and attenuated growth of a ras-transformed NIH3T3 xenograft and a human SW620 xenograft. Conclusion: Our results support the continued study of HPW98-1 for its potential use in the treatment of RAS-related cancers.*

The RAS superfamily is involved in various receptor-kinase signalling cascades that regulate target gene expression and in turn modulate cell proliferation, differentiation, and apoptosis (1-3). Uncontrolled RAS signalling is closely related to tumorogenesis (4-7), and may even cause cellular resistance to radiotherapy or chemotherapy (1, 6). Strategies targeting RAS activation (11), RAS post-translational modification (12-14), downstream effectors (15-20), or RAS–RAF protein interaction (21, 22) have yielded mixed results.

Selective inhibition of cancer cells remains the major obstacle in cancer chemotherapy. Therefore, most efforts in anticancer agent development are focused on enhancing the selectivity to key components along the RAS signalling pathway (10). Azatyrosine [l-beta-(5-hydroxy-2-pyridyl)-alanine] has been shown to exhibit selective cytotoxicity towards *ras*-transformed NIH3T3 rat fibroblast cells (23) by incorporating into cellular proteins to replace tyrosine, resulting in labile phosphorylation signals that interrupt tumour signalling pathways (24, 25). Because azatyrosine demonstrated poor intracellular bioavailability, it was used as a template to design azatyrosinamides which had improved cell penetration and significant cytotoxicity selectively towards *ras*-transformed NIH3T3 cells (26). To investigate their therapeutic potential, we evaluated their anticancer activity in *in vitro* and *in vivo ras*-transformed cancer models and investigated the underlying mechanism(s). Due to the fact that RAS superfamily members are key regulators of the vascular endothelial growth factor-induced angiogenesis (27), the antiangiogenesis activity of one compound was also investigated.

Correspondence to: Hui-po Wang, Ph.D., Professor of Taipei Medical University College of Pharmacy. 250 Wu-Hsing St., Taipei, Taiwan 110, R.O.C. Tel: +886 227361661 ext. 6100, Fax: +886 227390671, e-mail: hpw@tmu.edu.tw

Key Words: Azatyrosinamides, *ras*, antitumour agent, NIH3T3, SW620 human colon xenograft, angiogenesis.

Materials and Methods

Chemicals. Azatyrosine and azatyrosinamides compounds HPW98-1 to 9 (Figure 1) were synthesized as previously described (26). All chemicals used were of analytical grade and obtained from Sigma Chemicals (St. Louis, MO, USA), unless otherwise indicated.

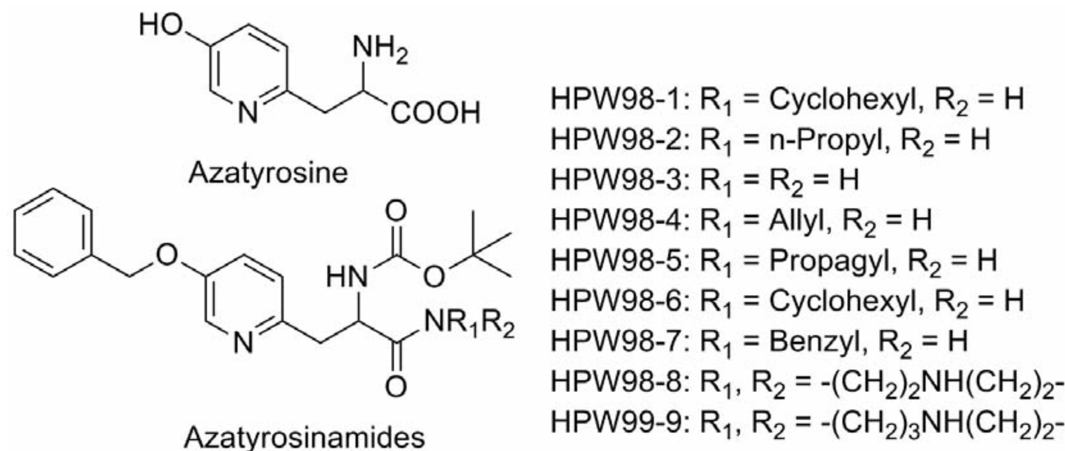

Figure 1. *Structures of azatyrosine and azatyrosinamides.*

Cell culture. NIH3T3 wild-type and NIH3T3 Val^{12}-*ras* cells were kindly supplied by Professor W.P. Wang of the Immunology Department, National Taiwan University, Taipei, R.O.C. and Dr. M. Campa of Duke University, Durham, NC, U.S.A., respectively. SW620 human colorectal adenocarcinoma [(ATCC) CCL-227] was obtained from the Institute of Food Technology, Taiwan, R.O.C. The cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM; Hyclone Inc., Logan, UT, USA), Dulbecco's modified Eagle's medium (DMEM; Biofluids Co., Rockville, MD, USA), or Leibovitz's L-15 medium (Gibco Co., Grand Island, NY, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Biological Industries, Haemek, Israel), in accordance with the ATCC guidelines. The cells were cultured in a humidified atmosphere of 5% CO_2 at 37˚C.

Cell growth inhibition. Cytotoxicity was determined by performing a colourimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay of treated cells (28, 29). The control wells contained medium plus cells (total absorbance) or medium-alone (background absorbance). Formazan absorbance was measured at 590 nm with a Dynatech MR5000 microplate reader (Dynatech Laboratories, Chantilly, VA, USA); cell death was calculated as the percentage reduction of formazan absorption.

Nuclear morphology observations. Hoechst 33258 dye was used to investigate changes in nuclear morphology that may relate to signalling pathways involved in apoptosis (29). Briefly, 1×10^5 cells treated with or without the test compound for 48 h were plated on glass coverslips and fixed with 2% paraformaldehyde (PFA) solution [comprising of 2 ml of PFA and 98 ml of phosphate-buffered saline (PBS)], kept at 37˚C for 20 min. PFA was then removed and the cells were washed thrice with PBS, and submerged in cold, 100% methanol for 10 min. The cells were washed with PBS three more times and then incubated in 1 ml of Hoechst 33258 solution [8 μg/ml in water containing 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ)] in the dark for 1 h. Cells were examined by fluorescence light microscopy with a source of wavelength 340–380 nm and emission wavelength of 465 nm.

Anchorage-independent growth assay. Effect of compounds on anchorage-independent growth of cancer cells was assessed by performing the soft agar colony formation assay (30-31). Cells (2×10^3) were mixed with 1.5 ml of 0.35% agarose gel [containing 5% agarose in 0.1 ml of DMEM (with 7.5% calf serum) and 0.65 ml of test material (diluted by DMEM from stock solution to 0.75 ml)] and plated on a 0.6% agarose gel base in a 6-well plate (1.5 ml/well). The plate was incubated at 37˚C for 14 days, and the colonies (≥50 cells) were then counted.

Animal husbandry. Athymic nude mice and BALB/c mice with severe combined immune deficiency (SCID) (6–8 weeks old) were purchased from National Taiwan University Animal Center (Taipei, Taiwan, R.O.C.) and acclimatized for one week in a housing room under a 12-h light/dark cycle and maintained at 23±1˚C and 39-43% relative humidity; water and food were provided *ad libitum*. All procedures involving the use of animals were in compliance with the guidelines for the use of experimental animals and approved by the Institutional Animal Care and Use Committee.

Matrigel plug angiogenesis assay. Matrigel plug assay was performed as described previously (32-33). Athymic nude mice were injected with 500 μl of Matrigel® containing vascular endothelial growth factor (VEGF; 150 ng/ml, *s.c.*) or basic fibroblast growth factor (bFGF; 150 ng/ml, *s.c.*). In the VEGF model, HPW98-1 was administered by oral gavage at doses of 30, 100, or 300 mg/kg/day. In the bFGF model, mice were given doses of 100 or 300 mg/kg/day. After a 6-day treatment, the mice were euthanized by pentobarbital overdose. The plugs were carefully clipped; effects on angiogenesis were measured using a haemoglobin detection assay kit. Briefly, the plug was gently homogenized and 20 μl of whole blood were added to 5 ml of Drabkin's solution for 15 min at room temperature; absorbance was measured at 530-550 nm. Haemoglobin concentration was calculated based on haemoglobin standards.

In vivo tumour suppression study. In vivo antitumour efficacy was evaluated in mice bearing tumour xenografts. NIH3T3-*ras*/7-4 or SW620 tumour cells (5×10^6) were injected subcutaneously into the

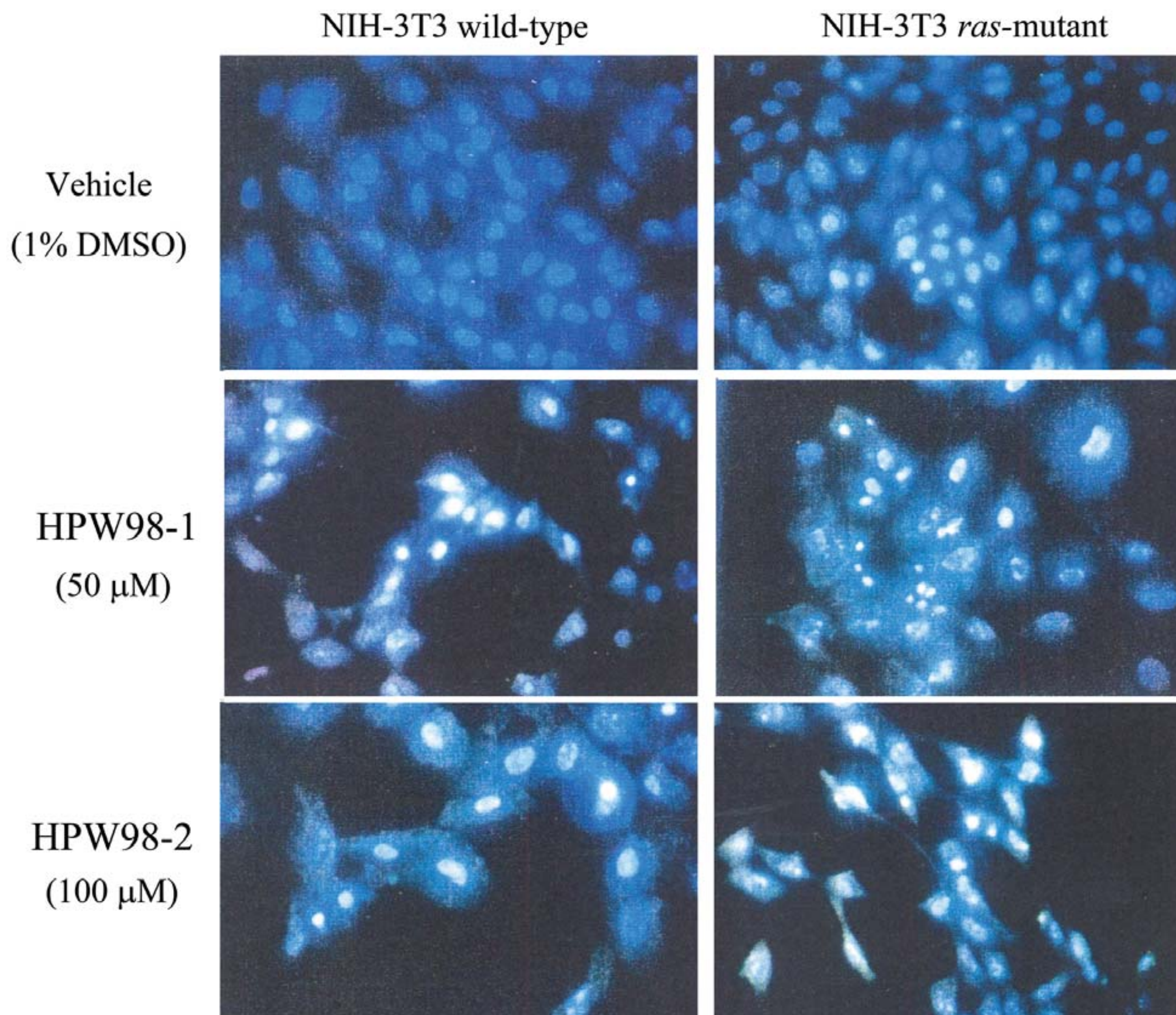

Figure 2. *Nuclear morphology of wild-type and ras-transformed NIH3T3 cells (×200). Azatyrosinamides induced apoptotic body formation in wild-type and ras-transformed NIH3T3 cells. A bright spot under the fluorescent microscope revealed condensed DNA inside nucleus (vehicle). Compared to control, the multiple bright small dots inside single cells represent the formation of apoptotic bodies in response to treatment with HPW98-1 and HPW98-2.*

right flank of BALB/c SCID or athymic nude mice, respectively (6-8 weeks old, n=5). For the NIH3T3 xenograft model, HPW98-1 (30, 60, or 120 mg/kg/day; *i.p.*) or vehicle [10% hydroxylpropyl-β-cyclodextrin$_{(aq)}$] was administered 24 h after tumour cell injection for up to 20 days after inoculation. For the SW620 xenograft model, HPW98-1 (25 mg/kg/day; *i.p.*) or vehicle [10% hydroxylpropyl-β-cyclodextrin$_{(aq)}$] was administered, starting at day 3 or 10 after tumour cell inoculation and continued up to day 60 after inoculation. Tumour volume, represented as width (mm)^2 × depth (mm), was monitored every other day.

Statistical analysis. All data are expressed as the mean±SD from at least three independent experiments. Statistical significance was determined by two-way analysis of variance (ANOVA) by using Microsoft Excel and significance was set at $p<0.05$.

Results

Azatyrosinamides induced apoptotic body formation in wild-type and ras-transformed NIH3T3 cells. Wild-type and *ras*-transformed NIH3T3 cells treated with HPW98-1 and HPW98-2 were stained with Hoechst 33258 and examined by fluorescence microscopy. Images revealed that both treatments induced the formation of condensed incomplete nuclear fragments representing the formation of apoptotic bodies (Figure 2).

Azatyrosinamides exhibited a different mechanism of action from that of azatyrosine. Azatyrosine and HPW98-1, HPW98-2

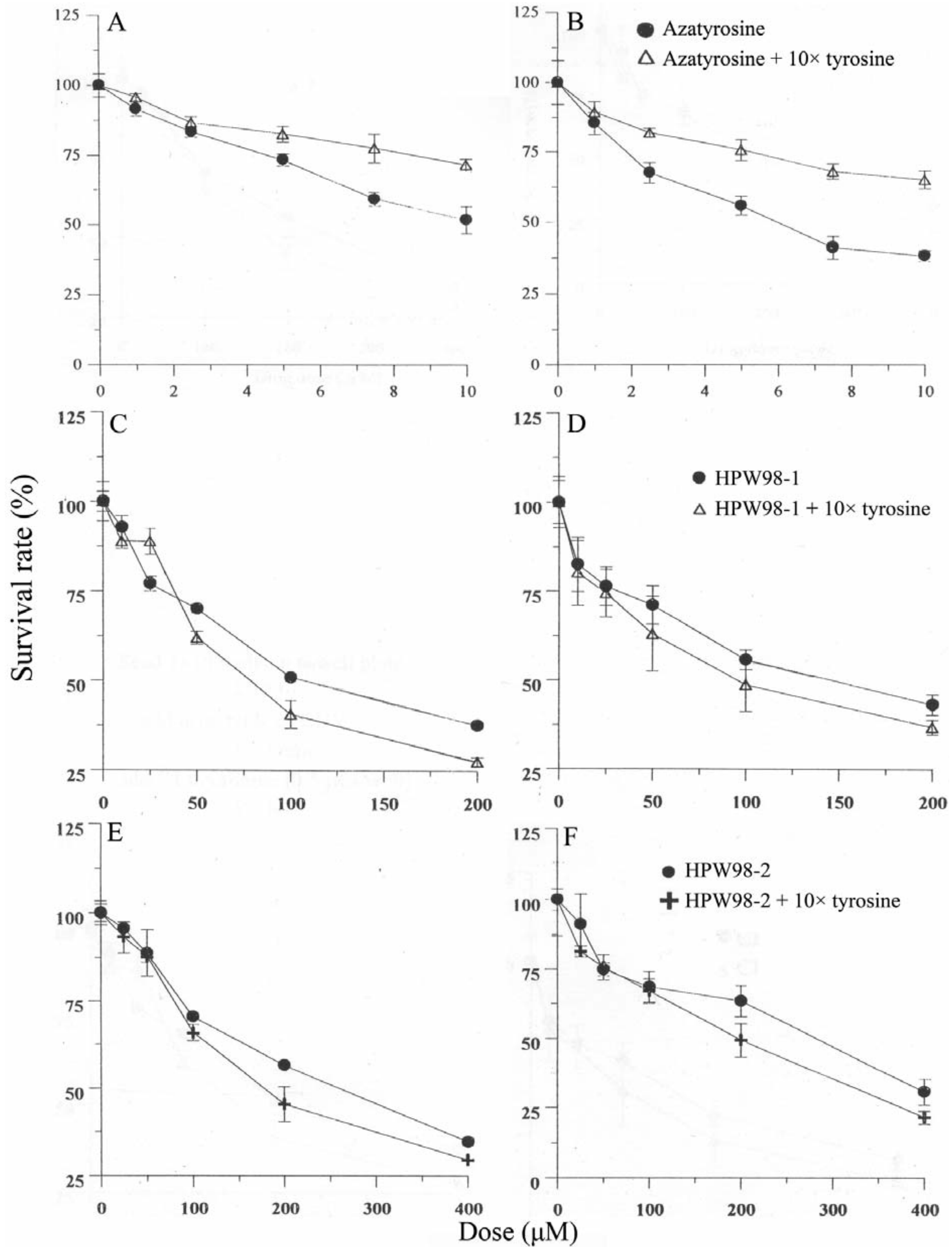

Figure 3. *Growth inhibition of wild-type (A, C, E) and ras-transformed (B, D, F) NIH3T3 cells by azatyrosine (A-B), HPW98-1 (C-D), and HPW98-2 (E-F). Azatyrosine, but not azatyrosinamides, induced growth inhibition that was prevented by excess tyrosine supplementation (n=3, mean±S.D.). Tyrosine was added at 10× concentration of test compounds.*

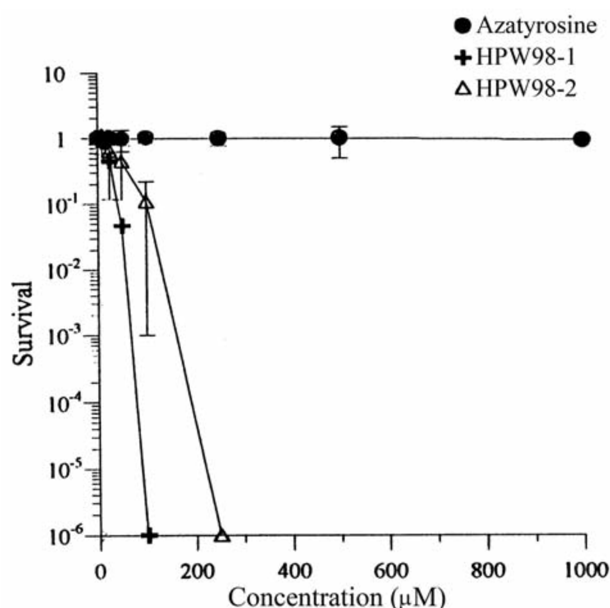

Figure 4. *Effect of HPW98-1 and HPW98-2 on anchorage-independent growth of ras-transformed NIH3T3 cells using the soft agar assay. These azatyrosinamides exhibited dose-dependent inhibition of ras-dependent colony survival (n=3, mean±S.D.).*

reduced survival of both wild-type and *ras*-transformed NIH3T3 cells in a dose-dependent manner (Figure 3). Excess tyrosine attenuated the effect of azatyrosine, (Figure 3A and B), but not of HPW98-1 (Figure 3C and D) and HPW98-2 (Figure 3E and F) in both wild-type and *ras*-transformed NIH3T3 cells, suggesting that the mechanism underlying growth inhibition by azatyrosinamides is different from that by azatyrosine.

Azatyrosinamides reduced ras-dependent survival colony formation. Colony formation in a soft agar assay is a characteristic of the malignant transformation caused by ras mutations (34-35). Colony formation of *ras*-transformed NIH3T3 cells was resistant to azatyrosine at concentrations of up to 1 mM (Figure 4). Conversely, the azatyrosinamide HPW98-1 (IC_{50} 23 μM) and HPW98-2 (IC_{50} 30 μM) strongly inhibited colony formation. HPW98-1 totally inhibited *ras*-dependent colony formation at a concentration of <100 μM.

Azatyrosinamides suppressed solid tumour growth in vivo. HPW98-1 suppressed tumour growth in BALB/C SCID mice bearing *ras*-transformed NIH3T3 xenografts in a dose-dependent manner. Compared to controls, HPW98-1 administered at doses of 30, 60, and 120 mg/kg/day; *i.p.*, respectively, reduced tumour size to 68%, 14%, and 1%, 20 days after treatment (Figure 5A). HPW98-1 (25 mg/kg/day; *i.p.*) also reduced tumour volume in athymic nude mice bearing *ras*-transformed SW620 colon tumour xenografts; tumour volume was 19% and 28% relative to controls at day 60 when treatment was started at day 3 and 10, respectively (Figure 5B).

Azatyrosinamides inhibited VEGF-induced angiogenesis in vivo. VEGF and bFGF are two major RAS-regulated contributors to tumour angiogenesis. We therefore investigated the antiangiogenesis activity of HPW98-1 in mice, which exhibited VEGF- or bFGF-induced angiogenesis by using the Matrigel plug angiogenesis assay. Haemoglobin levels in Matrigel plugs in VEGF-treated mice decreased to 19%, 19%, and 13% after treatment with HPW98-1 at doses of 30, 100, and 300 mg/kg/day; *p.o.*, respectively (Figure 6). No antiangiogenic effect was found in the bFGF-induced model. These data suggest that similarly to the clinically used kinase inhibitors, HPW98-1 might disrupt the VEGF receptor (VEGFR) signalling pathway.

Discussion

We previously found that azatyrosine suppressed the growth of *ras*-transformed NIH3T3 cells, although with an IC_{50} that exceeded 1 mM because of poor intracellular bioavailability. To improve bioavailability and thereby increase potency, we designed and synthesized nine azatyrosinamide derivatives (26). Compared to azatyrosine, azatyrosinamides exhibited a 70- to 700-fold increase in cytotoxicity and a 2- to 138-fold increase in selectivity towards *ras*-transformed NIH3T3 cells. Apoptotic bodies formed in *ras*-transformed NIH3T3 cells after treatment with HPW98-1 and HPW98-2, suggesting that a *ras*-dependent apoptosis pathway may contribute to growth inhibition in these cells. HPW98-1, the most potent and selective azatyrosinamide derivative, suppressed growth of *ras*-transformed NIH3T3 and *ras*-transformed SW620 xenografts *in vivo*, at least partly by disrupting signalling downstream of VEGFR activation. In addition to inhibiting tumour growth, HPW98-1 also exerted an anti-angiogenic effect.

In both the MTT and soft agar assays, we found that the sensitivity of *ras*-transformed NIH3T3 cells to the cytotoxic effect of HPW98-1 and HPW98-2 was significantly greater than to that of azatyrosine. In contrast to azatyrosine, which showed no inhibitory effect on colony formation at concentrations up to 1 mM, both HPW98-1 and HPW98-2 inhibited colony formation with an IC_{50} of 30 and 23 μM, respectively. Although long-term incubation with azatyrosine resulted in growth inhibition of cancer cells *in vitro*, the high concentrations required restrict its development for therapeutic use.

Our data suggest that the mechanisms by which the azatyrosinamide derivatives exert their effects differs from that of azatyrosine. Azatyrosine, a tyrosine analogue with a nitrogen atom replacing the carbon-2 atom of the phenol group, competes with and replaces endogenous tyrosine during

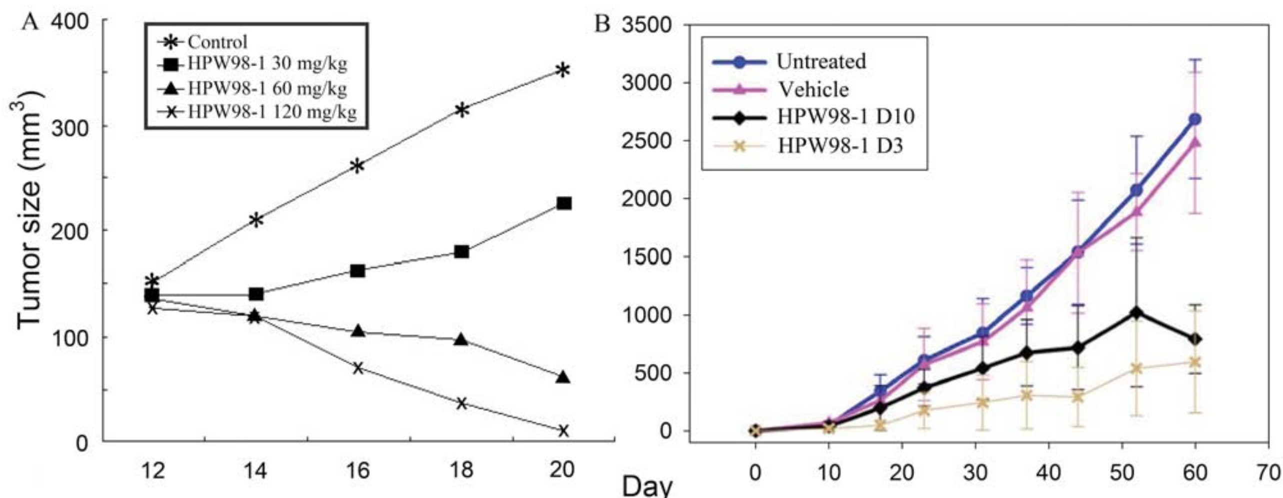

Figure 5. *Effect of HPW98-1 on solid tumour growth in vivo. A: Tumour suppression effect of HPW98-1 (30, 60, and 120 mg/kg/day, i.p.) in BALB/C mice bearing a ras-mutated NIH3T3 xenograft (n=5). Control group was treated with 10% hydroxylpropyl-β-cyclodextrin$_{(aq)}$. B: Tumour suppression in SCID mice bearing a SW620 human colon xenograft. Mice were either untreated, or treated with vehicle or HPW98-1 (25 mg/kg/day, i.p.) starting at day 10 (D10) or day 3 (D3) after tumour cell inoculation (mean±S.D., n=8-10).*

translation (24). Purro *et al*. (25) proposed that azatyrosine incorporation into the C-terminus of alpha-tubulin might inhibit cancer cell proliferation and thereby convert *ras*-, *raf*-, or *cErbB-2*-transformed NIH3T3 cells from a malignant to a normal phenotype. This incorporation is competitively inhibited in the presence of excess tyrosine. Restoration of cell viability by excess tyrosine is a unique property of azatyrosine-mediated growth inhibition. In our study, however, excess tyrosine did not affect growth suppression caused by azatyrosinamide derivatives, whereas it did reduce the cytotoxic effect exerted by azatyrosine, consistent with the notion that the inhibitory mechanisms are different.

Azatyrosinamide derivatives exerted an antiangiogenic effect downstream of select growth factor pathways. In particular, *ras*-dependent, VEGF-induced angiogenesis was markedly inhibited by HPW98-1 at a dose of 30 mg/kg in the Matrigel plug assay, whereas bFGF-induced angiogenesis was unaffected. The reason for this is not entirely clear. It was shown that expression of the mutant ras oncogenes is associated with the up-regulation of VEGF expression (36). Thus, the use of azatyrosinamide derivatives may provide an additional therapeutic approach for treating VEGF-associated cancers. The successful development of multiple drugs targeting the VEGF pathway is a testimony to the effectiveness of this therapeutic approach (37-39).

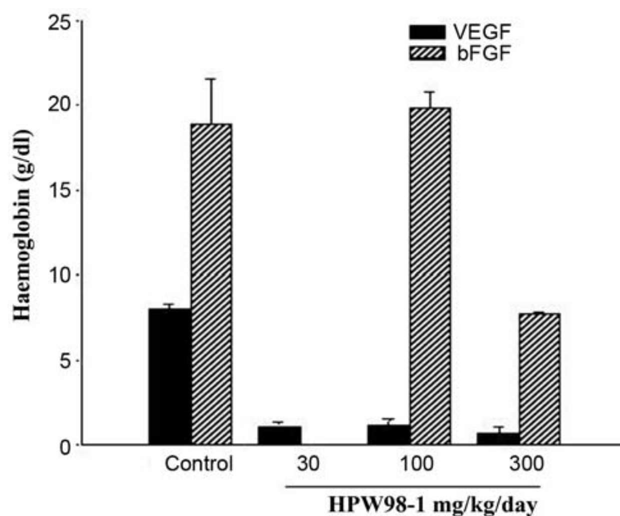

Figure 6. *Matrigel plug angiogenesis assay demonstrating the effect of HPW98-1 on growth factor-induced angiogenesis in vivo in athymic nude mice. Antiangiogenesis activity of HPW98-1 on vascular endothelial growth factors (VEGF; 150 ng/ml, s.c.) and basic fibroblast growth factors (bFGF; 150 ng/ml, s.c.) induced blood plug formation was measured as haemoglobin concentration (mean±S.D., n=3).*

Conclusion

In conclusion, azatyrosinamides exhibited selective anticancer activity towards *ras*-transformed NIH3T3 cells. VEGF-associated antiangiogenesis may contribute to the tumour suppressive effect of HPW98-1. The profound tumour suppressive effect of this compound on *ras*-transformed NIH3T3 xenograft and SW620 xenograft *in vivo* warrants further investigation of this in human cancer.

Acknowledgements

This study was supported by the Ministry of Economics, Republic of China (100-EC-17-A-20-S1-196 and 101-EC-17-A-08-S1-215). We are grateful to Dr. Chin-Ho Li of the Institute of Toxicology, College of Medicine, National Taiwan University, for his comments and help in revising this manuscript.

References

1 McCubrey JA, Steelman LS, Abrams SL, Bertrand FE, Ludwig DE, Bäsecke J, Libra M, Stivala F, Milella M, Tafuri A, Lunghi P, Bonati A and Martelli AM: Targeting survival cascades induced by activation of RAS/RAF/MEK/ERK, PI3K/PTEN/AKT/mTOR, and JAK/STAT pathways for effective leukemia therapy. Leukemia *22*: 708-722, 2008.

2 Reuther GW and Der CJ: The Ras branch of small GTPase: Ras family members don't fall far from the tree. Curr Opin Cell Biol *12*: 157-165, 2000.

3 Shaw RJ and Cantley LC: RAS, PI(3)K and mTOR signalling controls tumour cell growth. Nature *441*: 424-430, 2006.

4 Ayllon V and Rebollo A: RAS-induced cellular events (Review) Mol Membr Biol *17*: 65-73, 2000.

5 Barbacid M: RAS genes. Annu Rev Biochem *56*: 779-827, 1987.

6 McKenna WG, Muschel RJ, Gupta AK, Hahn SM and Bernhard EJ: RAS signal transduction pathway and its role in radiation sensitivity. Oncogene *22*: 5866-5875, 2003.

7 Roberts PJ and Der CJ: Targeting the RAF-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene *26*: 3291-3310, 2007.

8 Karnoub AE and Weinberg RA: Ras oncogenes: Split personalities. Nat Rev Mol Cell Biol *9*: 517-531, 2008.

9 Gysin S, Salt M, Young A and McCormick F: Therapeutic strategies for targeting Ras proteins. Genes Cancer *2*: 359-372, 2011.

10 Wong KK: Recent development anti-cancer agents targeting RAS/RAF/MEK/ERK pathway. Recent Pat Anticancer Drug Discov *4*: 28-35, 2009.

11 Taveras AG, Remiszewski SW, Doll RJ, Cesarz D, Huang EC, Kirshmeier P, Pramanik BN, Snow ME, Wang Y-S, del Rosario JD, Vibulbhana B, Bauera BB, Browna JE, Carra D, Catinoa J, Evansa CA, Girijavallabhana V, Heimarka L, Jamesa L, Liberlesa S, Nasha C, Perkinsa L, Seniora MM, Tsarbopoulosa A, Gangulya AK, Austb R, Brownb E, Delisleb D, Fuhrmanb S, Hendricksonb T, Kissingerb C, Loveb R, Sissonb W, Villafrancab E and Webberb SE: Ras oncoprotein inhibitors: The discovery of potent, ras nucleotide exchange inhibitors and the structural determination of a drug–protein complex. Bioorg Med Chem *5*: 125-133, 1997.

12 Basso AD, Kirschmeier P and Bishop WR: Lipid posttranslational modifications: Farnesyl transferase inhibitors. J Lipid Res *47*: 15-31, 2006.

13 Njorge FG, Taveras AG, Kelly J, Remiszewski S, Mallams AK, Wolin R, Afonso A, Cooper AB, Rane DF, Liu Y-T, Wong J, Vibulbhan B, Pinto P, Deskus J, Alvarez CS, del Rosario J, Connolly M, Wang J, Desai J, Rossman RR, Bishop WR, Patton R, Wang L, Kirschmeier P, Bryant MS, Nomeir AA, Lin C-C, Liu M, McPhail AT, Doll RJ, Girijavallabhan VM and Ganguly AK: (+)-4-[2-[4-(8-Chloro-3, 10-dibromo-6, 11-dihydro-5H-benzo[5, 6]cyclohepta[1, 2-b]-pyridin-11(R)-yl)-1-piperidinyl]-2-oxo-ethyl]-1-piperidinecarboxamide e (SCH-66336): A very potent farnesyl protein transferase inhibitor as a novel antitumor agent. J Med Chem *41*: 4890-4902, 1998.

14 End DW, Smets G, Todd AV, Applegate TL, Fuery CJ, Angibaud P, Venet M, Sanz G, Poignet H, Skrzat S, Devine A, Wouters W and Bowden C: Characterization of the antitumor effects of the selective farnesyl protein transferase inhibitor R115777 *in vivo* and *in vitro*. Cancer Res *61*: 131-137, 2001.

15 Friday BB and Adjei AA: Advances in targeting the RAS/RAF/MEK/ERK mitogen-activated protein kinase cascade with MEK inhibitors for cancer therapy. Clin Cancer Res *14*: 342-346, 2008.

16 Hennessy BT, Smith DL, Ram PT, Lu Y and Mills GB: Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov *4*: 988-1004, 2005.

17 Thompson N and Lyons J: Recent progress in targeting the RAF/MEK/ERK pathway with inhibitors in cancer drug discovery. Curr Opin Pharmacol *5*: 350-356, 2005.

18 Sebolt-Leopold JS, Dudley DT, Herrera R, Becelaere KV, Wiland A, Gowna RC, Tecle H, Barrett SD, Bridges A, Przybranowski S, Leopold WR and Saltiel AR: Blockade of the MAP kinase pathway suppress growth of colon tumours *in vivo*. Nat Med *5*: 810-816, 1999.

19 Yeh TC, Marsh V, Bernat BA, Ballard J, Colwell H, Evans RJ, Parry J, Smith D, Brandhuber BJ, Gross S, Marlow A, Hurley B, Lyssikatos J, Lee PA, Winkler JD, Koch K and Wallace E: Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase kinase 1/2 inhibitor. Clin Cancer Res *13*: 1576-1583, 2007.

20 Barrett SD, Bridges AJ, Dudley DT, Saltiel AR, Fergus JH, Flamme CM, Delaney AM, Kaufman M, LePage S, Leopold WR, Przybranowski SA, Sebolt-Leopold J, Becelaere KV, Doherty AM, Kennedy RM, Marston D, Howard WA Jr, Smith Y, Warmus JS and Tecle A: The discovery of the benzhydroxamate MEK inhibitors CI-1040 and PD 0325901. Bioorg Med Chem Lett *18*: 6501-6504, 2008.

21 Wilhelm S, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, Schwartz B, Simantov R and Kelley S: Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. Nat Rev Drug Discov *5*: 835-844, 2006.

22 Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, Bremer R, Gillette S, Kong J, Haass NK, Sproesser K, Li L, Smalley KSM, Fong D, Zhu Y-L, Marimuthu A, Nguyen H, Lam B, Liu J, Cheung I, Rice J, Suzuki Y, Luu C, Settachatgul C, Shellooe R, Cantwell J, Kim S-H, Schlessinger J, Zhang KYJ, West BL, Powell B, Habets G, Zhang C, Ibrahim PN, Hirth P, Artis DR, Herlyn M and Bollag G: Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. Proc Natl Acad Sci USA *105*: 3041-3046, 2008.

23 Shindo-Okada N, Makabe O, Nagallara H and Nishimura S: Permanent conversion of mouse and human cells transformed by activated ras or raf genes to apparently normal cells by treatment with the antibiotic azatyrosine. Mol Carcinog *2*: 159-167, 1989.

24 Monden Y, Hamano-Takaku F, Shindo-Okada N and Nishimura S: Azatyrosine–mechanism of action for conversion of transformed phenotype to normal. Ann NY Acad Sci *886*: 109-121, 1999.

25 Purro SA, Bisig CG, Contin MA, Barra HS and Arce CA: Post-translational incorporation of the antiproliferative agent azatyrosine into the C-terminus of alpha-tubulin. Biochem J *375*: 121-129, 2003.

26 Wang HP, Hwang TL, Lee O, Tseng YJ, Shu CY and Lee SJ: Selective cytotoxicity of azatyrosinamides against *ras*-transformed NIH 3T3 cells. Bioorg Med Chem Lett *15*: 4272-4274, 2005.
27 Rak J, Yu JL, Klement G and Kerbel RS: Oncogenes and angiogenesis: Signaling three-dimensional tumor growth. J Investig Dermatol Symp Proc *5*: 24-33, 2000.
28 Carmichael J, DeGraff WG and Gazdar AF: Evaluation of tetrazolium-based semiautomated colorimetric assay: Assessment of radiosensitivity. Cancer Res *47*: 943-946, 1987.
29 Kepp O, Galluzzi L, Lipinski M, Yuan J and Kroemer G: Cell death assays for drug discovery. Nat Rev Drug Discov *10*: 221-237, 2011.
30 Hamburger AW and Salmon SE: Primary bioassay of human tumour stem cells. Science *197*: 461-463, 1977.
31 Selever J, Gu G, Lewis MT, Beyer A, Herynk MH, Covington KR, Tsimelzon A, Dontu G, Provost P, Di Pietro A, Boumendjel A, Albain K, Miele L, Weiss H, Barone I, Ando S and Fuqua SA: Dicer-mediated up-regulation of BCRP confers tamoxifen resistance in human breast cancer cells. Clin Cancer Res *17*: 6510-6521, 2011.
32 Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, Pauly RR, Grant DS and Martin GR: A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. Lab Invest *67*: 519-528, 1992.
33 Brown JL, Cao ZA, Pinzon-Ortiz M, Kendrew J, Reimer C, Wen S, Zhou JQ, Tabrizi M, Emery S, McDermott B, Pablo L, McCoon P, Bedian V and Blakey DC: A human monoclonal anti-ANG2 antibody leads to broad antitumor activity in combination with VEGF inhibitors and chemotherapy agents in preclinical models. Mol Cancer Ther *9*: 145-156, 2010.
34 Chang TY, Tsai WJ, Chou CK, Chow NH, Leu TH and Liu HS: Identifying the factors and signal pathways necessary for anchorage-independent growth of *Ha-ras* oncogene-transformed NIH/3T3 cells. Life Sci *73*: 1265-1274, 2003.
35 Hahn WC, Counter CM, Lundberg AS, Reijersbergen RL, Brooks MW and Weinberg RA: Creation of human tumour cells with defined genetic elements. Nature *400*: 464-468, 1999.
36 Rak J, Mitsubishi Y, Bayko L, Filmus J, Shirasawa S, Sasazuki T and Kerbel RS: Mutant ras oncogene upragulate VEGF/VPF expression: Implications for induction and inhibition of angiogenesis. Cancer Res *55*: 4575-4580, 1995.
37 Bukowski RM, Yasothan U and Kirkpatrick P: Pazopanib. Nat Rev Drug Discov *9*: 17-18, 2010.
38 Langmuir PB and Yver A: Vandetanib for the treatment of thyroid cancer. Clin Pharmacol Ther *91*: 71-80, 2012.
39 Kerbel RS: Tumour angiogenesis. N Engl J Med *358*: 2039-2049, 2008.

Received November 26, 2012
Revised December 26, 2012
Accepted January 3, 2013