Reversal of TNP-470-Induced Endothelial Cell Growth Arrest by Guanine and Guanine Nucleosides

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ABSTRACT

The mechanism of action of TNP-470 [O-(chloroacetyl-carbamoyl) fumagillol], which potently and selectively inhibits the proliferation of endothelial cells, is incompletely understood. Previous studies have established its binding protein and the most distal effector of its growth arrest activity as methionine aminopeptidase 2 (MetAP-2) and p21^{WAF1/CIP1}, respectively. However, the mechanistic steps between these two effectors have not been identified. We have found that addition of exogenous guanine and guanine-containing nucleosides to culture medium will completely reverse the cytostatic effect of TNP-470 on both cultured bovine aortic and mouse pulmonary endothelial cells. Western blotting showed that supplementation with exogenous guanosine reverses the induction of p21^{WAF1/CIP1} by TNP-470. This “rescue” by guanine/guanosine was abolished when the guanine salvage pathway of nucleotide biosynthesis was inhibited with Immucillin

H, suggesting that TNP-470 might reduce de novo guanine synthesis in endothelial cells. However, an analysis of inosine 5'-monophosphate dehydrogenase, the rate-limiting enzyme in de novo guanine synthesis and target of the antiangiogenic drug mycophenolic acid, showed no TNP-470-induced changes. Curiously, quantitation of cellular nucleotides confirmed that GTP levels were not reduced after TNP-470 treatment. Addition of guanosine at the start of G₁ phase causes a doubling in GTP levels that persists to the G₁/S phase transition, where commitment to TNP-470 growth arrest occurs. Thus, guanine rescue involves an augmentation of cellular GTP beyond physiological levels rather than a restoration of a drug-induced GTP deficit. Determining the mechanism whereby this causes restoration of endothelial cell proliferation is an ongoing investigation.

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing ones, plays an essential role in normal tissue growth and development by supplying cells with the oxygen and nutrients needed to sustain their metabolism. However, for this same reason, angiogenesis also plays a central role in tumor progression: developing tumors can secrete a number of proangiogenic factors to create their own internal vasculature to sustain proliferation of cancer cells (Hahnfeldt et al., 1999). In addition to providing the cancer cells with nutrients and oxygen, the developing vasculature facilitates metastasis by providing tumor cells with access to the general circulation. Aside from cancer, angiogenesis also contributes to the pathophysiology of rheumatoid arthritis (Szek-

anecz et al., 2005), macular degeneration (Nowak, 2006), and other pathological conditions (Pandya et al., 2006). Clinical researchers and basic scientists alike believe that developing therapies that prevent angiogenesis without affecting already existing vasculature (i.e., cytostatic agents rather than cytotoxic agents) will be of great value in the treatment of such conditions.

Although endogenous inhibitors of angiogenesis such as endostatin (Sim, 1998) and endorepellin (Bix et al., 2004) have been identified and characterized, small molecules are superior drug candidates because they are less susceptible to degradation and often have an improved volume of distribution compared with proteins. The serendipitous discovery of the small molecule fumagillin from a fungal contamination of cultured endothelial cells (Ingber et al., 1990) provided researchers with a potent and selective inhibitor of endothelial cell proliferation. Fumagillin at subnanomolar concentrations causes G₁/S phase growth arrest of cultured endothelial cells by activation of p53, and in turn, p21^{WAF1/CIP1} (p21) (Yeh et al., 2000; Zhang et al., 2000). This growth arrest is

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ABBREVIATIONS: TNP-470, O-(chloroacetyl-carbamoyl) fumagillol; BAE, bovine aortic endothelial; MPE, murine pulmonary endothelial; MPA, mycophenolic acid; MetAP-2, methionine aminopeptidase 2; IMPDH, inosine 5'-monophosphate dehydrogenase; pl, isoelectric point; ddATP, dideoxyadenosine triphosphate; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid; RR-M1, M1 subunit of ribonuclease reductase.

reversible and remarkably selective for endothelial cells at such low concentrations. In vivo, fumagillin, and its synthetic analog TNP-470 [O-(chloroacetyl-carbamoyl) fumagillol], retard tumor growth and prolong survival time in studies in mice (Takechi et al., 1994; Gervaz et al., 2000). In corneal micropocket assay, TNP-470 reduced capillary formation in response to proangiogenic growth factor (Yeh et al., 2000).

We and others have shown that fumagillin and TNP-470 bind to and inhibit the metalloprotease methionine aminopeptidase 2 (MetAP-2) (Griffith et al., 1997; Sin et al., 1997), and a later report has confirmed this enzyme as the therapeutically relevant protein target for inhibition of angiogenesis (Yeh et al., 2006). TNP-470 inactivates MetAP-2 by forming a covalent bond with the catalytic His²³¹ in the enzyme active site (Liu et al., 1998a). The specificity of TNP-470 for MetAP-2 over MetAP-1 largely depends on the amino acid located at position 362: Ala362 in MetAP-2 permits access of TNP-470 and its analogs to the ligand binding site, whereas the bulkier Thr residue found at the analogous position in MetAP-1 is prohibitive (Brdlik and Crews, 2004).

The complete mechanism whereby MetAP-2 inhibition should lead to endothelial cell growth arrest has been and continues to be an area of interest. Little is known concerning the intermediate signaling effectors that transduce the cytostatic effect of TNP-470 and lie between MetAP-2 and p53/p21. We have shown that TNP-470 inhibits noncanonical wnt signaling (Zhang et al., 2006) and probably interferes with early, membrane-proximal events in that pathway, although how this causes activation of p53 is yet unknown. One possible cause of p53 activation of growth arrest is depletion of one or more nucleotides within the cell (Taylor et al., 1999; Khan et al., 2000; Bronder and Moran, 2003). Supplying cells with exogenous nucleosides, which become incorporated into purine or pyrimidine salvage mechanisms, restores the correct nucleotide balance and can reverse growth arrest. For example, the immunosuppressant and antiangiogenic drug mycophenolic acid (MPA) arrests growth in cells by inhibition of de novo guanine synthesis, and addition of exogenous guanine to these cells restores proliferation (Chong et al., 2006). Here, we investigated whether supplementation with exogenous nucleosides could attenuate the cytostatic effect of TNP-470 on endothelial cells. We show that the addition of exogenous guanine nucleosides noncompetitively reverses the cytostatic effect of TNP-470 on endothelial cells by blocking p21 induction. It is noteworthy that despite similarities in their activities on endothelial cells TNP-470 affects GTP synthesis differently than mycophenolic acid.

Materials and Methods

Reagents. Tissue culture reagents, including fetal bovine serum (FBS), were obtained from Invitrogen (Carlsbad, CA). Polyclonal antibody to inosine 5'-monophosphate dehydrogenase 1 (IMDPH1) was obtained from Abcam, Inc. (Cambridge, MA), and polyclonal antibodies to p21^{WAF1/CIP1} and cyclin H were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody to the M1 subunit of ribonuclease reductase (RR-M1) was purchased from Cell Signaling Technology (Danvers, MA), and monoclonal antibody to α -tubulin was from Sigma-Aldrich (St. Louis, MO). [³H]thymidine was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA), and [2,8-³H]-hypoxanthine (33.4 Ci/mmol) was purchased from Moravsek Biochemicals (Brea, CA). Immucillin H was a generous gift from Dr. Vern Schramm (Albert Einstein Med-

ical College, New York, NY). Immobilized pH gradient strips were obtained from Bio-Rad Laboratories (Hercules, CA), and protein molecular weight markers for SDS-polyacrylamide gel electrophoresis were from GE Healthcare Bio-Sciences (Little Chalfont, Buckinghamshire, UK). All other reagents were obtained from Sigma-Aldrich unless otherwise specified.

Cell Culture. Primary bovine aortic endothelial (BAE) and murine pulmonary endothelial (MPE) cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Bovine aortic endothelial cells were isolated and cultured as follows: under aseptic conditions, freshly procured calf aorta was cut open lengthwise with sterile surgical scissors, and the intimal surface was gently scraped with sterile razors to collect the endothelial cells. The collected material was pelleted by spinning at 800 rpm for 3 min and then resuspended in 5 ml of 0.25% trypsin·EDTA for 2 min at room temperature to disaggregate the sheets of endothelial cells. The cells were then plated out onto a gelatin-coated dish and grown in a specialized formulation of Dulbecco's modified Eagle's medium (DMEM) containing exclusively D-valine in place of L-valine, further supplemented with 10% FBS, 50 units/ml penicillin, 50 μ g/ml streptomycin, and 1% MEM nonessential amino acids. BAE cells were switched to the more customary L-valine-containing medium upon the first passage. MPE cells were isolated from mouse lung tissue by immunomagnetic selection and cultured as described previously (Yeh et al., 2000).

[³H]Thymidine Incorporation. BAE or MPE cells were seeded into 96-well plates in growth medium at a density of 4000 cells/well. After several hours (overnight) to allow for cell attachment, the growth medium was removed and replaced with serum-free medium. Cells were maintained in serum-free conditions for 48 h to arrest growth and permit cell cycle synchronization in G₀. Cell proliferation was then re-established with fresh medium containing serum and the compound of interest at the specified concentration. After 12 h, each well of cells received another 20 μ l of medium containing 2 μ Ci of [³H]thymidine (70–90 Ci/mmol from PerkinElmer Life and Analytical Sciences). After another 4 h, the cells were harvested from the wells and passed through glass fiber filters by using a Ska-Tron cell harvester (Molecular Devices, Sunnyvale, CA). The filters were washed and then transferred to vials, scintillant was added, and the amount of radioactivity incorporated into the cells in the filters was quantified by scintillation counting. The resulting data were analyzed with Prism software (GraphPad Software Inc., San Diego, CA).

Fluorescence-Activated Cell Sorting for Cell Cycle Analysis. BAE cells were growth-arrested and synchronized by serum deprivation, and then stimulated to re-enter the cell cycle by restoration of 10% FBS to medium. At the time intervals indicated after serum restoration, cells were detached by trypsinization, rinsed in ice-cold PBS, and then resuspended in a propidium iodide-labeling buffer (PBS supplemented with 50 μ g/ml propidium iodide, 0.2% Triton X-100, and 10 μ g/ml RNase A) for 12 h at 4°C in the dark to stain cellular DNA. Using a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA), the DNA content per cell was recorded and the percentages of cells in G₀/G₁, S, and G₂/M phases of the cell cycle were determined by using CellQuest 2.1 acquisition software (BD Biosciences).

Western Blotting. For one-dimensional blotting, MPE cells were drug-treated as described above and then rinsed once in ice-cold PBS. For whole-cell lysates, cells were homogenized in lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholic acid, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml pepstatin A, 10 μ g/ml leupeptin, and 0.3 trypsin inhibitor units/ml aprotinin) and clarified by centrifugation at 14,000 rpm at 4°C for 10 min. For cytoplasmic/nuclear fractions, we used the method described by Schreiber et al. (1989). Sample proteins were resolved by SDS-gel electrophoresis and transferred to nitrocellulose for probing with the antibody indicated.

Two-Dimensional Blotting. MPE cells were drug-treated, rinsed once with ice-cold 250 mM sucrose, and then harvested in isoelectric focusing buffer (8 M urea, 4% CHAPS, and 2 mM tributylphosphine). Samples were dounced several times and clarified by centrifugation at 14,000 rpm at 4°C for 10 min. Sample proteins were resolved first by isoelectric focusing on immobilized pH gradients strips for 20,000 V·h followed by SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose. After membrane block in 5% nonfat milk, samples were probed for IMPDH1 by using rabbit polyclonal antibody and developed by using chemiluminescence.

Reporter Assay for p53 Induction. MPE cells stably expressing the p53-driven luciferase reporter construct pG13Py-luc (gift of Bert Vogelstein, Johns Hopkins University, Baltimore, MD) were plated into 12-well plates and drug-treated as indicated. Luciferase activity was measured by using the Luciferase Assay System from Promega (Madison, WI) according to the manufacturer's instructions. Luciferase activity was measured on a Wallac Victor² automated plate reader (PerkinElmer Wallac), and results were normalized to protein concentrations determined by using the bicinchoninic acid method.

Immunofluorescence. MPE cells were grown on glass coverslips in six-well plates for 24 h, followed by the indicated overnight drug treatment. They were then washed with PBS and fixed in PBS supplemented with 3.7% paraformaldehyde for 10 min. After permeabilization with 0.25% Triton X-100 in PBS, the cells were blocked with 1% BSA for 1 h. The cells were then stained with anti-IMPDH1 antibody at 1:200 for 60 min and then incubated with the secondary antibody (Alexa Fluor 594-conjugated goat anti-rabbit) at 1:500 for another hour. Coverslips were washed three times with PBS every time after antibody incubations. Vectashield mounting medium (Vector Laboratories, Burlingame, CA) was applied to protect fluorophore activity, after which the cells were visualized by confocal microscopy.

IMPDH Activity Assay. IMPDH activity in intact BAE cells was determined by measuring the amount of ³H released from the C-2 position of [2,8-³H]inosine 5'-monophosphate that was formed in the cells during incubation with [2,8-³H]hypoxanthine. In brief, 10⁶ BAE cells were plated into 60-mm plates and then drug-treated as indicated. The cells were then rinsed twice in serum-free DMEM and incubated in serum-free DMEM with continued drug application along with 25 μ Ci/dish of [2,8-³H]hypoxanthine (33.4 Ci/mmol). At 0, 30, 60, 120, 150, and 180 min, 80- μ l aliquots of medium were removed from each dish, combined with 500 μ l of ice-cold activated charcoal suspension (100 mg/ml in 5% TCA), mixed, and centrifuged for 10 min at 1300g. For each sample, 100 μ l of the supernatant was analyzed for radioactivity by scintillation counting. The amount of spontaneous liberation of ³H was measured in growth medium in the absence of cells and found to be <0.75% of the total amount of

[2,8-³H]hypoxanthine added; this background was subtracted from all values to arrive at the net amount of ³H released by the activity of IMPDH.

Quantitation of Cellular Nucleotides by HPLC. Endothelial cells were drug-treated as described, rinsed twice in ice-cold PBS containing 20 μ M dipyrindamole, and harvested from the dishes in ice-cold 70% methanol containing 5 mM sodium fluoride and 12.5 mM β -glycerophosphate. After incubation on ice for 15 min, samples were centrifuged at 14,000 rpm for 10 min to pellet the protein, and the supernatant was recovered and evaporated to dryness. After addition of 100 μ l of 15% TCA and vigorous vortexing to remove any remaining sample protein, the TCA in each sample was removed by two half-volume extractions using triethylamine:1,1,2-trichloroethane (both from Aldrich Chemical Co., Milwaukee, WI) in a ratio of 45:55, and the resultant aqueous layer was analyzed for nucleotide content by injection of 50 μ l onto the HPLC (Shimadzu, Braintree, MA). Before injection, each sample was spiked with a known amount of the synthetic nucleotide ddATP as an internal standard. The nucleotides were separated from each other by using a Partisil SAX column (Whatman, Clifton, NJ) and a gradient of H₂O to 300 mM potassium phosphate buffer at a flow rate of 1.5 ml/min. The size of the peaks was determined with a diode array detector with a channel set to 270 nm, and the identities of the four NTP peaks were confirmed by measurement of their UV absorbance spectra.

Results

In the first series of experiments (Fig. 1), the ability of individual nucleosides to reverse the cytostatic effect of TNP-470 on cultured BAE cells was tested. Alone ("unsupplemented"), TNP-470 was potently cytostatic against BAE cells, exhibiting an IC₅₀ value of 0.37 ± 0.12 nM. Initially, all of the nucleosides were tested by supplementing the culture medium at both 1 mM and 100 μ M. At the higher concentration of 1 mM, all of the nucleosides inhibited BAE cell proliferation even in the absence of TNP-470 (data not presented); however, at the lower concentration of 100 μ M, the nucleosides had differing effects on BAE cell growth. The pyrimidine nucleosides (cytidine, thymidine, and uridine) all were still growth suppressive even at 100 μ M (Fig. 1A). In contrast, adenosine at 100 μ M was mitogenic and enhanced BAE cell proliferation by approximately 50% in the absence of TNP-470. However, despite this mito-

Fig. 1. Effects of small molecules on TNP-470-induced growth arrest of endothelial cells. Data points represent the mean $\pm$ S.E. of three to four independent experiments. A, effects of exogenous nucleosides on TNP-470-induced growth arrest in BAE cells. B, guanine and guanine-containing nucleosides rescue BAE cells from TNP-470-induced growth arrest.

Fig. 2. Guanosine rescue of TNP-470-induced growth arrest requires time for metabolic processing. A, exogenous guanosine is less effective at rescuing against TNP-470 when added to BAE cells in late G₁ phase compared with the start of G₁ phase. B, Western blot after serum restoration to cells for the proliferating cell protein marker, RR-M1 (top) and α -tubulin loading control (bottom). C, flow cytometric analysis of endothelial cell progression through G₁ phase into S phase. The amount of cellular DNA was measured by propidium iodide labeling. D, guanosine rescue of BAE cells requires the activity of the guanine salvage pathway. E, guanosine rescue of MPE cells requires the activity of the guanine salvage pathway.

genic activity, adenosine was unable to diminish the cytostatic effect of TNP-470: BAE cells were equally inhibited by TNP-470 in the absence or presence of adenosine. However, supplementation of BAE cells with exogenous guanosine at 100 μ M completely "rescued" the BAE cells from TNP-470-induced growth arrest (Fig. 1A), even at the highest concentration of TNP-470 used (100 nM). Concentrations of guanosine in the medium ≤ 20 μ M provided no reversal of TNP-470, whereas concentrations between 20 and 80 μ M were able to only partially reverse TNP-470 growth arrest. In the absence of TNP-470, guanosine at 100 μ M was not mitogenic or growth suppressive on BAE cells. This stands in contrast to both adenosine and all of the pyrimidine nucleosides. Thus, the ability of guanosine to effectively rescue endothelial cells against TNP-470 is specific for that nucleoside.

The ability of exogenous deoxyguanosine and the purine base guanine itself to rescue BAE cells from TNP-470-induced growth arrest was subsequently tested (Fig. 1B), and they were found to have similar rescuing activity as guanosine. Deoxyguanosine at 100 μ M was nearly as effective at rescuing BAE cell proliferation as guanosine, with only a small cytostatic effect of TNP-470 observed at its highest concentration. Guanine at 100 μ M was able to reverse 50% of the cytostatic TNP-470 effect without changing the IC_{50} value of the antiangiogenic drug; however, guanine at 200 μ M completely rescued BAE cells from TNP-470. Such dose-dependent reduction in TNP-470 efficacy by guanine with no change in TNP-470 potency may best be described pharmacologically as a noncompetitive reversal.

It is interesting to note that the addition of guanosine, etc., to BAE cells was most effective at countering the TNP-470 cytostatic effect when added to cells arrested in G_0 phase and/or re-entering the cell cycle at G_1 phase (Fig. 2A). The ability of guanosine to rescue BAE cell proliferation from TNP-470 was markedly reduced as supplementation of growth medium with the nucleoside occurred at progressively later time points in G_1 phase. Cell cycle progression was charted by both expression of the M1 subunit of ribonuclease reductase, RR-M1, which is absent in arrested cells (Mann et al., 1988) but reacquired in cycling cells (Fig. 2B), and quantitation of cellular DNA content using propidium iodide staining and flow cytometry (Fig. 2C). RR-M1 was undetectable in endothelial cells that had been serum-starved for 48 h, which indicated a state of quiescence had been achieved. Upon restoration of FBS to the growth medium, RR-M1 levels slowly increased and were readily de-

tectable by 8 h. Quantitation of cellular DNA in Fig. 2C shows that 8 h corresponds to the earliest indication of the cells entering S phase (up to 18.9% from 12.8% at 4.5 h); and at 12 h, the percentage of cells in S phase has further doubled to nearly 40%. Because the addition of guanosine at these late time points in G_1 phase has no rescuing effect against TNP-470, it suggested that time was required for the metabolic conversion of guanosine (and deoxyguanosine and guanine) into another form to rescue endothelial cell proliferation. One possibility involves the guanine salvage pathway, which can scavenge extracellular guanine and guanine nucleosides for conversion into GTP to supplement the de novo GTP synthesis pathway.

Immucillin H is a small-molecule inhibitor of purine nucleoside phosphorylase (Kicska et al., 2002), a key enzyme in the guanine salvage pathway. Incubation of BAE cells with exogenous guanosine and Immucillin H completely blocked the guanosine rescue effect and restored TNP-470 growth arrest (Fig. 2D). The ability of guanosine to rescue endothelial cells from the cytostatic effect of TNP-470 via the guanine salvage pathway is not a species-specific phenomenon, because MPE cells exhibit the same behavior (Fig. 2E). Thus, the antagonizing effect of guanine and guanine nucleosides on TNP-470 growth arrest is not restricted to endothelial cells from a particular species or specific anatomical origin (e.g., arteriolar versus venous endothelial cells).

TNP-470-dependent endothelial cell growth arrest directly results from the induction of the cyclin inhibitor, p21^{WAF1/CIP1}. As shown in Fig. 3A, TNP-470 causes the up-regulation of p21^{WAF1/CIP1} in both the nuclear and cytoplasmic compartments of the endothelial cell [p21^{WAF1/CIP1} appears as a doublet when detected using this antibody, as we have shown previously (Yeh et al., 2000)]. It is generally believed that the nuclear-localized p21^{WAF1/CIP1} results in cell cycle arrest; although there have been reports of cytoplasmic-localized p21^{WAF1/CIP1} possessing its own biological activities, the extent to which they may be relevant to cell cycle arrest by TNP-470 is unknown. Cotreatment of endothelial cells with 100 μ M guanosine in addition to TNP-470 markedly decreases the p21^{WAF1/CIP1} induction caused by the latter. Curiously, although exogenous guanosine completely reduces cytoplasmic levels of p21^{WAF1/CIP1} back to the basal levels seen in vehicle-treated cells, a small amount of elevated nuclear p21^{WAF1/CIP1} remains. However, because proliferation is restored in guanosine-treated endothelial cells, this "residual" nuclear p21^{WAF1/CIP1} appears to be of nominal importance. The decrease in p21^{WAF1/CIP1} levels caused by guanosine could be

Fig. 3. Guanosine reverses the effects of TNP-470 on downstream mediators of growth arrest. A, exogenous guanosine reverses TNP-470 induced up-regulation of cytoplasmic- and nuclear-localized p21^{WAF1/CIP1} in endothelial cells. Representative Western blot results for p21^{WAF1/CIP1} (top), the nuclear marker cyclin H (middle), and the cytoplasmic marker α -tubulin (bottom) are shown. B, exogenous guanosine blocks TNP-470 activation of the tumor-suppressor transcription factor p53. Results represent the mean $\pm$ standard error of five independent experimental replications.

caused by an upstream effect (inhibition of p21 transcription) or a downstream effect (promotion of p21 degradation). It is known that p21^{WAF1/CIP1} up-regulation depends on TNP-470 activation of the tumor-suppressing transcription factor p53. Therefore, to see whether guanosine is decreasing p21^{WAF1/CIP1} through an upstream effect, the ability of the nucleoside to reverse TNP-470-triggered p53 activation was tested. Endothelial cells stably expressing a p53-driven firefly luciferase plasmid, pG13Py-luc, were treated with TNP-470 in the absence or presence of exogenous guanosine (Fig. 3B). TNP-470 caused a 3.3-fold increase over basal levels of luciferase activity; however, when these cells were treated with both TNP-470 and 100 μ M guanosine, the p53 response was significantly reduced by ~48%. Although reversal of TNP-470-triggered p53 induction by guanosine is incomplete and may explain the residual p21 in Fig. 3A, it seems nevertheless sufficient to permit the resumption of cell cycling. Furthermore, this result shows that guanosine rescues growth by acting at or upstream of p53, an area of the mechanism of TNP-470 that still needs elucidation.

Huang et al., 2005 have shown that the immunosuppressant drug MPA, which inhibits de novo guanine synthesis and thereby depletes cellular stores of GTP (Senda et al., 1995), can also trigger endothelial cell growth arrest. As with TNP-470, the growth inhibition of endothelial cells by mycophenolic acid is cytostatic, p21-dependent, and reversible by cotreatment with exogenous guanosine (Chong et al., 2006). Mycophenolic acid blocks cell proliferation by inhibiting the enzyme IMPDH1 (Chong et al., 2006), which is the rate-limiting step in de novo guanine synthesis. Curiously, reduction of IMPDH activity, like p21 induction, has been shown to be a necessary consequence of p53 activation for growth arrest (Liu et al., 1998b). Because TNP-470 shows marked similarities to MPA in terms of its cytostatic activity and could potentially affect the processing of several proteins via its inhibition of MetAP-2, the effects of TNP-470 on IMPDH1 in endothelial cells were directly tested. The abundance, post-translational modification, subcellular localization, and overall catalytic activity were compared in the absence or presence of TNP-470. Western blotting of MPE cells showed that TNP-470 does not change the cellular levels of IMPDH1 (Fig. 4A). Indeed, two-dimensional Western blots of TNP-470-, MPA-, and vehicle-treated MPE cells showed no significant changes in isoform distribution (Fig. 4B), with multiple isoforms of IMPDH1 detected: the majority of IMPDH1 immunoreactivity ran with pI of 6.1, with significant amounts of the enzyme running at pI of 6.0 and 6.2. There was also a poorly resolved streak of immunoreactivity running from pI 5.1 to approximately 5.5. The nature of the differences between these isoforms of IMPDH1 was not determined; however, it is clear that they are unaltered by treatment with TNP-470 or MPA.

Assays of IMPDH activity revealed that, whereas mycophenolic acid strongly inhibited the enzyme (Fig. 4C), TNP-470 had no effect. Likewise, TNP-470 had no effect on the subcellular distribution of IMPDH1 (Fig. 4D, center) com-

pared with control cells (Fig. 4D, left), whereas MPA induced the previously reported (Ji et al., 2006) formation of large aggregates of the inactivated enzyme (Fig. 4D, right). Thus, in contrast to the effects of mycophenolic acid, IMPDH1 seems to be completely unaffected by TNP-470 despite the observed similarities in their effect on endothelial cells. Finally, treatment of endothelial cells with both drugs at once (Fig. 3E) revealed no changes in IC₅₀ values for either drug in the absence or presence of a submaximal concentration of the other inhibitor (100 nM for MPA and 0.5 nM for TNP-470). More specifically, TNP-470 had an IC₅₀ value of 0.43 ± 0.08 nM in the absence of MPA and 0.60 ± 0.21 nM in its presence; similarly, mycophenolic acid had an IC₅₀ value of 106 ± 17 nM in the absence of TNP-470 and 116 ± 33 nM in its presence, both values being consistent with previously published estimates of MPA potency against endothelial cells (Huang et al., 2005; Chong et al., 2006). However, in either case where both drugs were applied to cells, the maximum proliferation was decreased to approximately half that observed in the single drug dose responses. The additive cytostatic effect of these inhibitors is consistent with their each having a different mechanism of action.

Although IMPDH is a key enzyme in de novo guanine synthesis, there are other enzymes (e.g., GMP synthetase), which, if inhibited by the presence of TNP-470, would similarly lead to reduction of cellular GTP levels. Alternatively, TNP-470 treatment could cause the stabilization of an enzyme that accelerates GTP breakdown. Therefore, to definitively test whether TNP-470 treatment leads to decreases in cellular GTP levels in endothelial cells, extracts of drug-treated cells were subjected to HPLC analysis to quantitate nucleotide levels. Whereas treatment with 1 μ M mycophenolic acid causes the predicted selective decrease in cellular GTP (Fig. 5B) compared with control cells (Fig. 5A), treatment with 10 nM TNP470 does not change GTP levels (Fig. 5C). Therefore, surprisingly, inhibition of MetAP-2 in endothelial cells by TNP-470 clearly does not lead to suppression of de novo guanine synthesis.

Nevertheless, the fact that Immucillin H blocks the ability of guanosine to rescue against TNP-470 strongly implies that guanosine first must be converted to guanine nucleotide before it can restore angiogenesis. Measurements of nucleotide levels in endothelial cells after 12 h of guanosine treatment (Table 1) confirm that GTP levels double in the presence of 100 μ M guanosine. Levels of ATP and CTP do not change, whereas UTP levels fall slightly, consistent with the known reciprocal regulation of UTP and GTP (Sokoloski and Sartorelli, 1985).

Discussion

Adenosine and guanosine both have been reported as mitogenic to various kinds of cells (Yalowitz and Jayaram, 2000; Dubey et al., 2002), and indeed, the pharmacological growth pattern we observed in Fig. 1A supports the notion that adenosine is mitogenic to endothelial cells. However, despite

Fig. 4. Lack of effect of TNP-470 on inosine 5'-monophosphate dehydrogenase. A, TNP-470 does not alter the abundance of IMPDH1 in endothelial cells. B, TNP-470 does not alter isoelectric points of IMPDH1 isoforms in endothelial cells. Top, vehicle-treated cells; middle, 10 nM TNP-470-treated cells; bottom, 1 μ M MPA-treated cells. C, TNP-470 does not alter IMPDH catalytic activity. Results presented are the mean $\pm$ S.E. of three separate experiments. D, TNP-470 does not alter subcellular localization of IMPDH1. Left, vehicle-treated cells; center, 10 nM TNP-470-treated cells; right, 1 μ M MPA-treated cells. E, MPA and TNP-470 have an additive cytostatic effect on endothelial cells. Results are the mean $\pm$ S.E. of three independent experiments.

Fig. 5. Quantitation of cellular nucleotides in vehicle-treated cells (A), 1 μ M mycophenolic acid-treated cells (B), and 10 nM TNP-470 treated cells (C). Representative chromatographs are shown. ddATP was used as an internal standard to verify proper sample injection into the HPLC.

TABLE 1
Cellular levels of NTPs after supplementation with exogenous guanosine for 12 h
Values represent quantitation by area under the curve (arbitrary units). Mean $\pm$ standard error.

	Control	TNP-470	Guanosine	TNP-470 and Guanosine
UTP	2883 $\pm$ 317	2943 $\pm$ 35	1772 $\pm$ 110	1913 $\pm$ 225
CTP	664 $\pm$ 93	693 $\pm$ 39	461 $\pm$ 16	547 $\pm$ 85
ATP	13,998 $\pm$ 1994	14,908 $\pm$ 704	14,110 $\pm$ 1973	14,450 $\pm$ 1280
GTP	2539 $\pm$ 508	2721 $\pm$ 545	5512 $\pm$ 567	5372 $\pm$ 431

its ability to enhance endothelial cell growth, adenosine was unable to overcome the cytostatic effect of the antiangiogenic drug TNP-470. Guanosine, however, showed a different pharmacological pattern when added to BAE cells in that it 1) completely reversed the growth inhibition even at maximum concentrations of TNP-470, and 2) restored proliferation to the same levels as seen in untreated BAE cells. This pattern is consistent with guanosine or its metabolites acting along the same signaling pathway as TNP-470, but at a point downstream of MetAP-2 inhibition and upstream of distal effectors where commitment to cell division is determined. In fact, the point of action of guanosine "rescue" seems to occur at or upstream of p53 activation. The potentially wide-ranging effects of MetAP-2 inhibition have made elucidating the mechanism of TNP-470 in between these two effectors a challenging task.

That the ability of exogenous guanosine to rescue endothelial cells (both bovine and murine) from TNP-470 is progressively diminished when the nucleoside is added at progressively later time points into G₁ phase shows that early events, including some metabolic processing, are necessary for guanine/guanine nucleotides to rescue. This metabolic processing is probably the guanine salvage pathway: in addition to being able to synthesize purines bases like guanine de novo from building block compounds such as glycine, aspartic acid bicarbonate, etc., most cells have the ability to scavenge free purine bases and/or purine-containing ribonucleosides and deoxyribonucleosides to maintain critical cellular levels of these essential metabolites. The enzymes comprising this scavenging mechanism are collectively referred to as the "guanine salvage pathway." Thus, conversion of exogenous guanine, guanosine, and deoxyguanosine into GMP inside the endothelial cells is essential for rescue from TNP-470. In support of the notion that exogenous guanosine is acted on by the salvage pathway to accomplish TNP-470 rescue, the salvage pathway inhibitor Immucillin H also blocked guanosine rescue and reinstated the cytostatic effect of TNP-470.

The observations that exogenous guanosine rescues endothelial cells from TNP-470-induced growth arrest, and that Immucillin H can counter this rescuing effect, led us to speculate that TNP-470 may be inhibiting the ability of the endothelial cells to synthesize their own endogenous guanine/guanine nucleotides. There are a number of drugs that inhibit cell growth by disrupting de novo purine or pyrimidine base synthesis. For example, the immunosuppressants leflunomide (Cherwinski et al., 1995) and mycophenolic acid (Senda et al., 1995) block immune cell proliferation by inhibiting de novo uridine and de novo guanine synthesis, respectively. Mycophenolic acid has been shown to inhibit endothelial cell growth as well (Huang et al., 2005). It accomplishes this by inhibiting the enzyme IMPDH, which catalyzes the first committed step in de novo guanine synthesis. Chong et al. (2006) have also shown that inhibition of the enzyme subtype IMPDH1 causes growth arrest by mycophenolic acid in endothelial cells, whereas inhibition of IMPDH2 is of nominal consequence to endothelial cells and rather more important for growth arrest of lymphocytes. Because of the similarities (cytostatic growth arrest, p53 induction, and exogenous guanosine rescue of drug effect) between TNP-470 and mycophenolic acid, it was tempting to speculate that the

former may also alter IMPDH1 activity in some way to disrupt de novo guanine synthesis. However, thorough examination of endothelial cell IMPDH1 failed to show any changes in subcellular localization, enzyme abundance and isoform distribution, or overall catalytic activity after TNP-470 treatment. Meanwhile, mycophenolic acid-treated endothelial cells showed the expected loss of IMPDH activity and formation of annular aggregates of the protein that have been reported previously (Ji et al., 2006). Thus, the mechanism whereby TNP-470 may disrupt de novo guanine synthesis in endothelial cells is different from mycophenolic acid in that the effect of the former is IMPDH1-independent. The differences in their respective mechanisms are also somewhat underscored by their different receptor proteins.

The proximal and distal mediators of the cytostatic effect of TNP-470 have been firmly established as MetAP-2 (Griffith et al., 1997; Sin et al., 1997) and p21^{WAF1/CIP1} (Yeh et al., 2000; Zhang et al., 2000), respectively. The task now remains to identify those signaling proteins that connect the inhibition of MetAP-2 by TNP-470 with the cessation of cell cycling triggered by p53. We recently discovered that TNP-470 inhibits noncanonical wnt signaling (Zhang et al., 2006), a pathway that has since been shown to be important for angiogenesis (Cirone et al., 2008) and is affected by the behavior of G proteins. In this study, we have shown that the resultant growth inhibition from TNP-470 treatment of endothelial cells is completely reversed by the addition of exogenous guanine and guanine-nucleosides acting via the guanine salvage pathway. Whereas such guanine reversal of growth arrest is commonly observed with known antiangiogenics that act by suppressing de novo guanine synthesis, TNP-470 itself causes no decreases in cellular GTP levels. Thus, despite many similarities in their activity, TNP-470 and mycophenolic acid act differently to produce endothelial cell growth arrest. It is possible that TNP-470, through inhibition of MetAP-2, may decrease a small, unique compartment of the guanine nucleotide pool instead of guanine nucleotide synthesis, leading to the eventual induction of p21, and that the measurement of total cellular GTP may not reflect such a localized GTP decrease. Alternatively, the boost in cellular GTP levels after guanine supplementation may affect other metabolic activities, such as mitogenic G protein signaling, which themselves could have a role in TNP-470 growth arrest. The noncompetitive manner in which exogenous guanosine rescues against TNP-470 suggests that it acts downstream in the effector pathway from MetAP-2. There are reports in the literature of small G proteins being inhibited by TNP-470 (Satchi-Fainaro et al., 2005; Nahari et al., 2007). There are also numerous reports (Habas et al., 2001; Ahumada et al., 2002; Katanaev et al., 2005) describing the importance of G proteins in noncanonical wnt signaling, a signaling pathway that is important to angiogenesis and also inhibited by TNP-470. It is tempting to speculate that the addition of exogenous guanosine to TNP-470-arrested endothelial cells and the resultant increase in cellular GTP levels can alter the behavior of downstream G proteins whose upstream effectors have been "silenced" by the drug, thus restoring the proliferating phenotype.

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