A small-molecule inhibitor of the ribonucleolytic activity of human angiogenin that possesses antitumor activity

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The results of previous preclinical and clinical studies have identified angiogenin (ANG) as a potentially important target for anticancer therapy. Here we report the design and implementation of a high-throughput screening assay to identify small molecules that bind to the ribonucleolytic active site of ANG, which is critically involved in the induction of angiogenesis by this protein. Screening of 18,310 compounds from the National Cancer Institute (NCI) Diversity Set and ChemBridge DIVERSet yielded 15 hits that inhibit the enzymatic activity of ANG with K_i values <100 μM. One of these, NCI compound 65828 [8-amino-5-(4′-hydroxybiphenyl-4-ylazo)naphthalene-2-sulfonate; $K_i = 81$ μM], was selected for more detailed studies. Minor changes in ANG or ligand structure markedly reduced potency, demonstrating that inhibition reflects active-site rather than nonspecific binding; these observations are consistent with a computationally generated model of the ANG·65828 complex. Local treatment with modest doses of 65828 significantly delayed the formation of s.c. tumors from two distinct human cancer cell types in athymic mice. ANG is the likely target involved because (*i*) a 65828 analogue with much lower potency against the enzymatic activity of ANG failed to exert any antitumor effect, (*ii*) tumors from 65828-treated mice had fewer interior blood vessels than those from control mice, and (*iii*) 65828 appears to have no direct effect on the tumor cells. Our findings provide considerable support for the targeting of the enzymatic active site of ANG as a strategy for developing new anticancer drugs.

Angiogenin (ANG), a 14.1-kDa monomeric protein, was originally isolated as a human tumor-derived angiogenesis factor (1), and has since been identified as a potentially important target for anticancer therapy. Monoclonal antibodies and an antisense oligonucleotide directed against ANG are highly effective at inhibiting the establishment of s.c. human colon, prostate, breast, lung, and fibroblast tumors in athymic mice (2–4). These antagonists also protect mice from regional iliac lymph node metastases after implantation of human prostate cancer cells directly in the prostate gland (3, 5). Strikingly, the metastasis experiments with antisense reveal a strict correlation between the extent of reduction in ANG expression in the primary tumors and the degree of protection achieved (3). Another ANG antagonist, an 11-amino acid peptide, dramatically reduces liver metastases in mice injected with human colorectal carcinoma cells (6).

An association between ANG and cancer has been observed in more than 20 clinical studies to date. ANG mRNA and/or protein was elevated in colorectal (7, 8), gastric (7), pancreatic (9), breast (10, 11), prostate (5), melanoma (12), and urothelial (13) cancer lesions compared with the corresponding nonneoplastic tissues. In some cases, high tissue ANG was shown to correlate with cancer progression or poor prognosis (8, 9, 14). ANG levels in serum were significantly increased in patients with colorectal (15), gastric (16), pancreatic (9), ovarian (17), renal cell (18), and urothelial (13) cancer and with melanoma (19). Moreover, ANG was shown to be up-regulated by hypoxia in cultured melanoma cells but not in normal melanocytes, and the degree of induction correlated with the metastatic potential of the cell line used (12).

As ANG antagonists for clinical use, low molecular weight compounds would offer tremendous advantages over the agents (proteins, oligonucleotides, and peptides) tested thus far in mice. One attractive strategy for developing such inhibitors is to exploit a unique feature of ANG: it possesses a ribonucleolytic activity that is essential for angiogenicity (20–22). In general, targeting of enzymatic active sites has proved to be particularly successful for drug development, especially when, as in the case of ANG, the three-dimensional structure is known (23) and "rational" approaches can be incorporated. ANG is a member of the pancreatic RNase superfamily, with 33% sequence identity to bovine pancreatic RNase A (24). Although it contains analogues of the catalytic triad and the major substrate-binding residues of RNase A, it exhibits only weak ribonucleolytic activity toward standard RNase substrates (25). Its natural substrate has not been determined, but may reside in the nucleolus of vascular endothelial cells, where ANG accumulates after binding to the cell surface (26).

Previous efforts to identify small active-site-directed inhibitors of ANG have focused on nucleotides (27, 28). Although some of the compounds tested had mid-to-upper nanomolar K_i values with RNase A, K_i values with ANG were no better than $\approx$500 μM under physiological conditions. This unfavorable starting point, together with obstacles to the crystallographic study of ANG·nucleotide complexes that have been encountered (29), make the development of nucleotide-based ANG inhibitors a daunting prospect. As an alternative, we have now devised a high-throughput screening (HTS) assay for ANG and used it to conduct a wider search for low molecular weight inhibitors. This approach has yielded two potential leads that bind an order of magnitude more tightly than any of the nucleotides; one of these is here demonstrated to have antitumor activity in mice at modest doses. Thus small molecules directed at the active site of ANG may constitute a previously unrecognized class of anticancer agents.

Materials and Methods

Materials. Human ANG and its R5A (Arg-5 → Ala) variant were prepared as described (30). Oligonucleotides were from Integrated DNA Technologies (Coralville, IA). Chemical libraries were from the National Cancer Institute (NCI; Bethesda, MD) and ChemBridge Corporation (San Diego), as were all compounds denoted with prefixes N and C, respectively. Tyger Scientific (Princeton, NJ) resynthesized compound N-65828 and provided the analytical information cited below except for the

Abbreviations: ANG, angiogenin; HTS, high-throughput screening; NCI, National Cancer Institute; 6-FAM, 6-carboxyfluorescein.

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MS data, which were collected by the authors with a Micromass Platform LCZ instrument and electrospray ionization. $[M - H]^-$ was 418.0 Da vs. 418.1 Da (calc). Sodium 6-amino-5-(2-benzenesulfonylphenylazo)-4-hydroxynaphthalene-2-sulfonate (catalog no. S321443) and DMSO (Hybri-Max grade) were purchased from Sigma–Aldrich. 5′-Phosphothymidine 3′-pyrophosphate ($P'\rightarrow5'$) adenosine 3′-phosphate was from earlier studies (27). Rabbit reticulocyte lysate *in vitro* translation system, luciferase assay system, and luciferase mRNA were from Promega; the translation and assay systems were used according to the manufacturer's recommendations. Precautions for minimizing RNase contamination were described previously (31).

Kinetic Analyses. *Fluorimetric assays.* A modification of an earlier method (32) was used with the fluorogenic substrate 6-FAM–$(mA)_2rC(mA)_2$–Dabcyl, where 6-FAM is the fluorophore 6-carboxyfluorescein, mA is 2′-*O*-methylriboadenosine, rC is ribocytidine, and Dabcyl is the quencher 4-(4-dimethylaminophenylazo)benzoic acid. The fluorescence increase accompanying substrate cleavage at 37°C was monitored with a Jobin-Yvon-Spex (Longjumeau, France) FluoroMax-2 fluorimeter (λ_{ex} = 495 nm; λ_{em} = 525 nm). Assay mixtures contained 20 nM substrate, 400 nM ANG, and 10 μg/ml BSA in buffer A (20 mM Hepes–NaOH/100 mM NaCl, pH 7.0). Values of k_{cat}/K_m were determined from initial velocities as described (33).

HPLC assays. Reaction mixtures (30 μl) containing ANG (5 μM) and substrate [20 μM $(dA)_5rC(dA)_2$, a concentration expected to be $\ll K_m$ (33, 34)] in buffer A were incubated for 2 hr at 37°C and then injected onto a Mono Q HR5/5 (Amersham Pharmacia) column. Products and substrate were separated with a 9-min linear gradient from 0.19 M to 0.28 M NaCl in 10 mM Tris·HCl, pH 8.0, at ambient temperature at a flow rate of 1.5 ml/min. Peak areas at 254 nm for substrate and the product $(dA)_5rC$ cyclic 2′,3′-phosphate were used to calculate k_{cat}/K_m values (35). Test compounds were assayed initially at a concentration of 25 μM; those that inhibited by >10% were investigated further. K_i values were calculated from the dependence of k_{cat}/K_m on free inhibitor concentration, [I] (31); typically, at least four concentrations of inhibitor (10–100 μM) were used. R5A-ANG was assayed in 0.2 M Mes, pH 5.9, because activity in buffer A is too low for accuracy; R5A data are compared with those for ANG under the same conditions.

HTS. HTS was performed in black polypropylene 384-well plates (Nalge Nunc). Twenty microliters of 800 nM ANG in 40 μg/ml BSA were delivered into each well with a Multidrop liquid dispenser (Labsystems, Chicago). Test compounds (100 nl) were then added with a solid pin array device (V & P Scientific, San Diego); stock solutions were 10 mM (NCI) or 5 mg/ml (ChemBridge) in DMSO. The reaction was initiated by addition of substrate (20 μl of 50 nM 6-FAM–$(mA)_2rC(mA)_2$–Dabcyl) in 2× buffer A with the Multidrop dispenser. The plates were film-sealed and incubated for 1.5–2 hr at 37°C in the dark, and fluorescence was then measured with a Wallac 1420 Victor^2 multilabel counter (excitation: 485 nm; emission: 535 nm) (Perkin–Elmer Life Sciences). The median value for each plate (≈1,500 counts per second) was normalized to fluorescence intensity of 1.0; this value was similar to that measured when no library compound was added.

Luciferase mRNA Cleavage Assay. Pretranslation assay mixtures contained 50 ng of luciferase mRNA, 50 μM test compound, 60 nM ANG, and buffer A in a total volume of 5 μl. After 15 min of incubation at 30°C, 45 μl of translation mixture was added. After another 90 min at 30°C, the amount of luciferase produced was determined by transferring 5 μl of sample to wells containing 95 μl of luciferase substrate on a 384-well plate. Luminescence was measured with the Wallac counter described above.

Modeling of ANG·Inhibitor Complex Structures. Ligand docking to the ANG (PDB ID code 1B1I) active site was carried out in AUTODOCK 3.0 (36) as described for RNase A (31). Docking was constrained to a 22.5-Å cubic grid whose origin corresponds to the position of the phosphorus atom in the superimposed coordinates of free ANG and the ANG·phosphate complex (PDB ID code 1H52) (29).

Antitumor Activity in Mice. Effects of test compounds on the establishment of tumors from PC-3 and HT-29 cells were determined as described previously (2, 3). Cells (1×10^4 and 1.25×10^5 for PC-3 and HT-29, respectively) were injected s.c. in the shoulder of 6- to 7-week old athymic mice on day 0; in the protocols used, these numbers of cells have been shown to be sufficient to produce tumors in all mice given PC-3 cells (3, 5) and >97% of mice injected with HT-29 cells (2). Treatment or control solutions (100 μl) were administered locally s.c. six times per week until day 35; the first dose was given 5–10 min before injection of the cells. Test compounds were 400, 80, and 16 μg/ml in PBS containing 4%, 0.8%, and 0.16% DMSO (from 10 mg/ml stock solutions in DMSO), respectively. Control groups were injected with PBS containing the same concentration of DMSO. *P* values for statistical significance were from Mantel–Cox tests performed on Kaplan–Meier survivor functions; results for treatment groups were compared with those for appropriate DMSO control groups. There were no detectable differences in the survivor functions for control groups given PBS alone and those injected with PBS containing up to 4% DMSO.

Histology. Tissue sections from tumors (weight range: 18–89 mg; average: 47 mg) were examined for blood vessel content by factor VIII staining (2).

Results

HTS Development. Until recently, no assays for the ribonucleolytic activity of ANG were available that could be adapted for use in HTS. Because activity toward common small RNase substrates such as dinucleotides is extremely low (25), kinetic measurements typically required ≈10 μM ANG, and it was necessary to monitor the reaction by HPLC. Assays with polynucleotide substrates (37) used somewhat lower enzyme concentrations, but would be problematic to implement on microtiter plates. In 1999, Kelemen *et al.* (32) reported an assay for RNase A and ANG that appeared to have sufficient sensitivity and other characteristics compatible with HTS. The substrates are small oligonucleotides containing a single ANG-cleavable bond, a fluorophore at the 5′ end, and a quencher at the 3′ end. Cleavage relieves the internal quenching and produces a substantial increase in fluorescence.

For HTS, we opted to use the substrate 6-FAM–$(mA)_2rC(mA)_2$–Dabcyl and to conduct assays at pH 7 rather than the less physiological, but more kinetically optimal, pH value of ≈6 used in previous studies (28, 32). Initial rate assays in cuvettes yielded a k_{cat}/K_m value of $34 \pm 1\ M^{-1}\ s^{-1}$. On this basis, an ANG concentration of 400 nM, sufficient to cleave ≈5% of the substrate in 1 hr, was selected for HTS. Testing in microtiter plates showed that the fluorescence increase during this reaction could be quantitated with good precision by using 20 nM substrate; the fluorescence reading after 2 hr of incubation was 5-fold higher than the background from buffer and uncleaved substrate.

An important consideration that must be taken into account for the HTS assay is the extent to which the ANG preparation used is free of adventitious RNases. As with all other substrates reported, RNase A cleaves 6-FAM–$(mA)_2rC(mA)_2$–Dabcyl several orders of magnitude more rapidly than does ANG [$k_{cat}/K_m = (3.1 \pm 0.1) \times 10^7\ M^{-1}\ s^{-1}$]. Therefore, the presence of less than 0.0001% of this common laboratory contaminant in an ANG

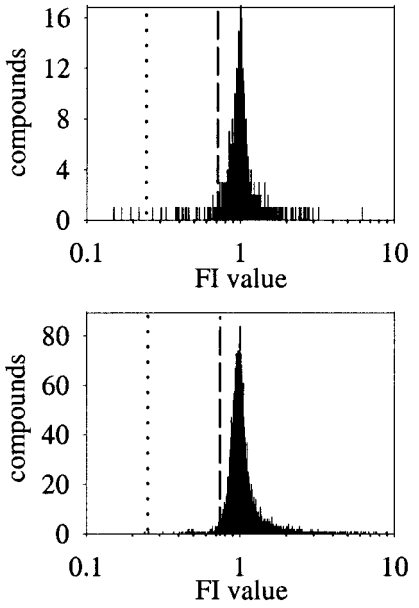

Fig. 1. Frequency of fluorescence readings from HTS of NCI (*Upper*) and ChemBridge (*Lower*) libraries. Readings are normalized to the median value for each plate (fluorescence intensity FI ≡ 1). The dotted line indicates the background value, and the dashed line shows the cutoff for classification as a hit.

preparation would invalidate any results obtained. We established that our ANG did not contain any significant amount of RNase A by demonstrating that activity was decreased by <10% upon addition of the inhibitor 5′-phosphothymidine 3′-pyrophosphate ($P'\rightarrow5'$) adenosine 3′-phosphate at a concentration of 1 μM, well above the K_i for RNase A but >100-fold below that for ANG (27).

HTS and Follow-Up Assays to Eliminate False Positives. The NCI Diversity Set (1,990 compounds) and ChemBridge DIVERSet E (16,320 compounds) were screened at concentrations of 25 μM or 12.5 μg/ml (20–70 μM), respectively. The results are shown in Fig. 1. Twenty-nine NCI compounds and 173 ChemBridge compounds reduced the fluorescence readings by at least 33% compared with the median value for each plate (minus background) and were classified as hits.

Some of the hits were expected to be false positives because of delivery errors, light scattering, or optical absorbance of test compounds. The final evaluation of inhibitors was to be performed with a rigorous HPLC assay that is not subject to these artifacts. However, this assay is time-consuming and the number of hits obtained from HTS is fairly large. Therefore, we first used a rapid secondary screening method, independent of that used for HTS, as a filter. In this method, luciferase mRNA is incubated with ANG in the presence and absence of putative inhibitor and then added to an *in vitro* translation system; the dilution used (10-fold) is sufficient to prevent any significant further RNA degradation by ANG and minimizes any influence of the test compounds on translation. After translation, the sample is diluted another 20-fold into a luciferase substrate mixture for quantification of protein product by luminescence. ANG concentrations of 30 and 60 nM in the absence of inhibitor commonly result in luminescence reductions of 38% and 70%, respectively, compared with the level measured when ANG is omitted. Sixty nanomolar ANG was used for inhibitor testing, and compounds were designated as hits if they appeared to rescue more than 50% of mRNA (i.e., if the readings were higher than that measured for 30 nM ANG without inhibitor) when used at 50 μM. Twelve compounds from each library satisfied this criterion and were investigated further by HPLC.

Previous HPLC assay methods with dinucleotide substrates (34) were deemed unsuitable for studying the secondary screening hits because (*i*) ANG activity in the pH 7 buffer is too low and (*ii*) test compounds might not separate adequately from substrate or products. These problems were averted by using the substrate $(dA)_5rC(dA)_2$ and a modified anion-exchange HPLC procedure. The k_{cat}/K_m value for this octanucleotide is 27.0 ± 1.1 M^{-1} s^{-1}, severalfold higher than for any dinucleotide. The substrate and its hexanucleotide cleavage product have considerably greater net charge than any of the test compounds and were well resolved in all cases.

Of the 24 compounds assayed by HPLC, 15 had apparent K_i values <100 μM (Fig. 2), and 5 produced no detectable inhibition. The 4 that inhibited most effectively (K_i < 11 μM) all contained copper complexes. These were dismissed from further consideration because development of such hits would be difficult and because ANG is known to be inhibited effectively by free cupric ions (37), which might be present in these samples. N-13778 (K_i = 42 μM) was rejected because it contains antimony. C-181431, C-180582, C-180553, and C-216112 are structurally related. C-181431 has the lowest K_i value of any inhibitor lacking metal ions (41 ± 7 μM), and its structure seems particularly amenable to both rational design and combinatorial approaches for improving affinity, suggesting that this compound may have promise as a potential lead. Similar considerations apply to N-65828 (K_i = 81 ± 7 μM), which has greater aqueous solubility than C-181431 and was more readily available. Therefore, this latter inhibitor was selected for further studies, including testing for antitumor activity *in vivo*.

Interaction of N-65828 with ANG. Before proceeding with animal studies, we considered it critical to establish that inhibition by N-65828 reflects active-site rather than nonspecific binding. The plot of k_{cat}/K_m vs. [I] for N-65828 conformed well to the theoretical shape for simple inhibition ($r^2 = 0.99$), as was the case for all of the inhibitors examined; this observation already suggests that binding is specific. More conclusive evidence was obtained by measuring the effects of minor changes in enzyme and/or ligand structure on K_i. Many active-site variants of ANG and analogues of N-65828 were available for this purpose. The choice of which to use was guided by a model for the complex of ANG with N-65828 generated by AUTODOCK 3.0. In this model (Fig. 3), the azo group sits in the catalytic center with His-13 and His-114 on either side, the substituted naphthalene component forms four hydrogen bonds (two between the sulfonate and the side chain of Arg-5, and two involving the amino group and the main-chain O of Arg-5 and the imidazole of His-8), and the OH of the biphenyl component hydrogen bonds with Asp-41 and Arg-121. In view of the apparent prominence of the interactions involving Arg-5 and the sulfonate group, we examined the effect of replacing Arg-5 with Ala. R5A was not inhibited detectably by 250 μM N-65828, indicating that the K_i value is >1 mM (compared with 58 μM for ANG under the same conditions).

Findings with analogues of N-65828 are also consistent with specific binding at the active site (Fig. 4). The compounds tested retain the 5-phenylazonaphthalene core of N-65828, but differ in the substitutions on the rings. In N-45571, the phenol is replaced by an *N*-acetamide that, according to the model, would not interact with ANG; K_i is increased by ≈4-fold. N-45557, which lacks the 2-sulfonate on the naphthalene ring, and contains a sulfo rather than an amino group at position 8 and a hydroxyl instead of the phenol group, has a K_i value >10-fold higher than for N-65828. S321443 also binds at least 10-fold less tightly than N-65828. This compound retains the 2-sulfo group on the naphthalene ring and has 6-amino and 4-hydroxyl groups, both of which might form hydrogen bonds with ANG according to the

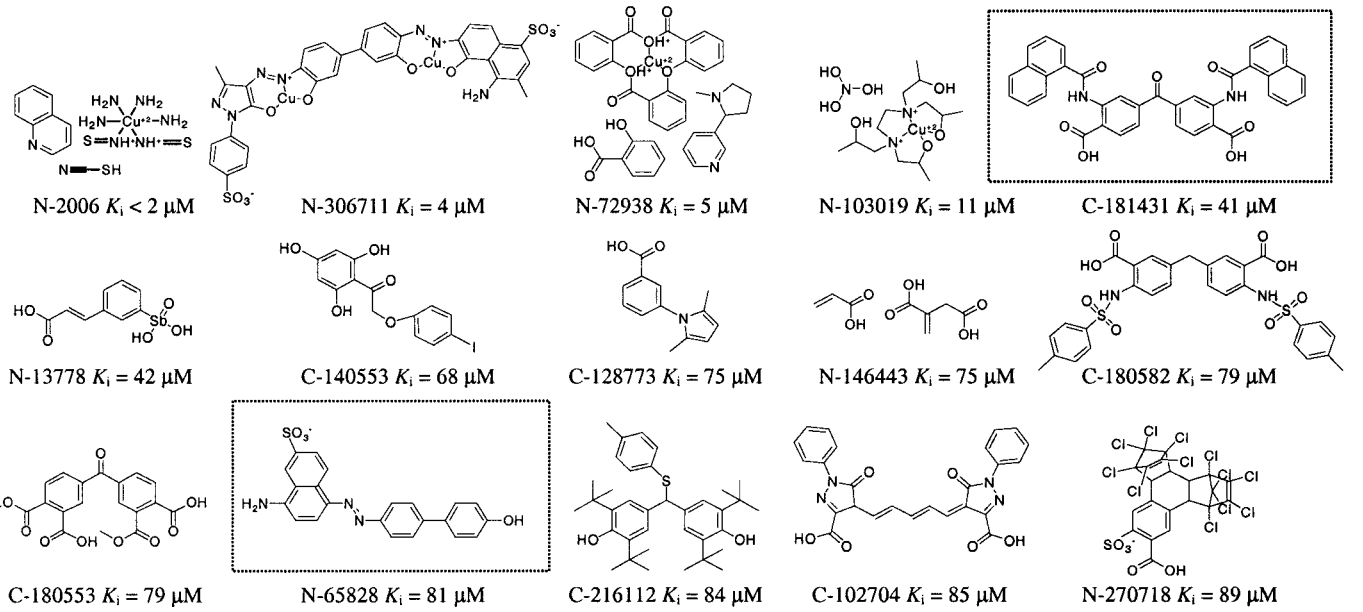

Fig. 2. Chemical structures and K_i values of ANG inhibitors identified by HTS of the NCI and ChemBridge libraries. The two compounds considered to be potential leads are boxed.

AUTODOCK model. However, the benzenesulfonyl group would be expected to clash with His-13, Lys-40, and Gln-117.

Antitumor Activity of N-65828. The efficacy of N-65828 *in vivo* was examined by using s.c. human tumor xenograft models in athymic mice (2, 3) and local administration of the inhibitor. In the initial test, PC-3 prostate cancer cells were used with three doses of inhibitor (40, 8, and 1.6 μg/day, corresponding to ≈1.4, 0.3, and 0.06 mg/kg per day on average) and four mice per group. Mice receiving the higher two doses developed tumors more slowly than those in the corresponding vehicle control groups. This experiment was then repeated with a larger number of mice (Fig. 5 *A* and *B*). Again there was an appreciable delay in the appearance of tumors in the groups receiving 40 μg and 8 μg/day of N-65828 (*P* values for the two combined experiments are <0.0001 and 0.0003, respectively). Two mice were still tumor-free 25 days after all of the mice in the vehicle control groups had tumors and 14 days after treatment had ceased on day 35. We also included groups of mice treated with 40 μg and 8 μg/day of N-45557, one of the N-65828 analogues shown to be ineffective as an inhibitor of ANG's ribonucleolytic activity. The rates of tumor appearance in these mice were very similar to those in the vehicle control groups (Fig. 5 *A* and *B*).

It is well known that some compounds in the NCI libraries are impure or have even been misidentified (38). To ensure that the observed antitumor activity of N-65828 was actually due to the compound listed, additional tests were performed with newly synthesized material whose structure and purity (>95%) were established by NMR, MS, elemental analysis, TLC, and C_{18} HPLC. The K_i for inhibition of the enzymatic activity of ANG was identical to that for the NCI preparation. Direct comparison in mice (n = 8 and n = 12, respectively, with n = 8 for vehicle controls) showed that the resynthesized material is at least as effective as that from NCI (*P* values for doses of 8–40 μg/day vs. vehicle controls are 0.0037–0.0008). The resynthesized inhibitor was also tested for efficacy *in vivo* against a second human

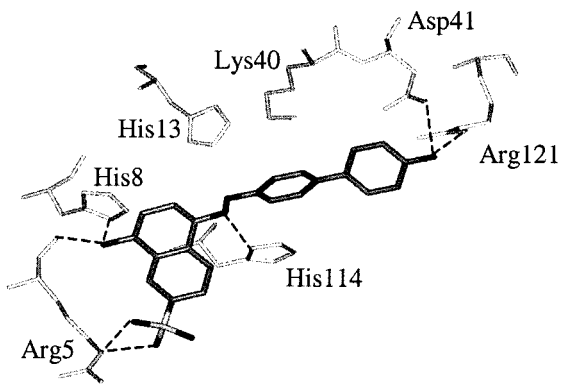

Fig. 3. Structure of the complex of ANG (light) with N-65828 (dark) as predicted by AUTODOCK. Hydrogen bonds are denoted with broken lines. The figure was drawn with INSIGHT II (Accelrys; Burlington, MA).

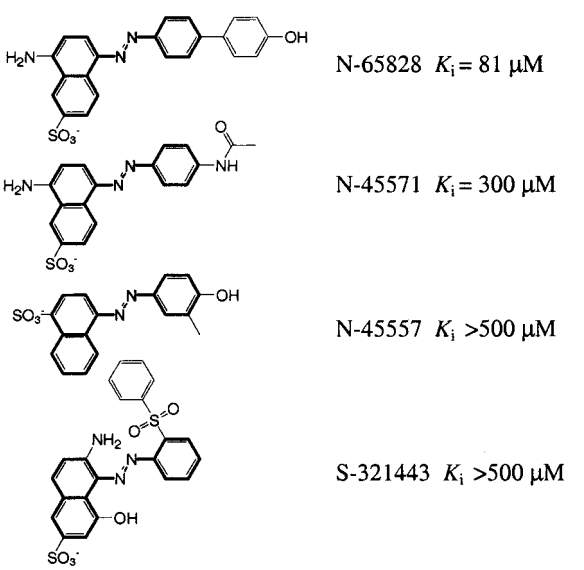

Fig. 4. Chemical structures and K_i values of N-65828 and three analogues predicted to bind less tightly on the basis of the modeled complex.

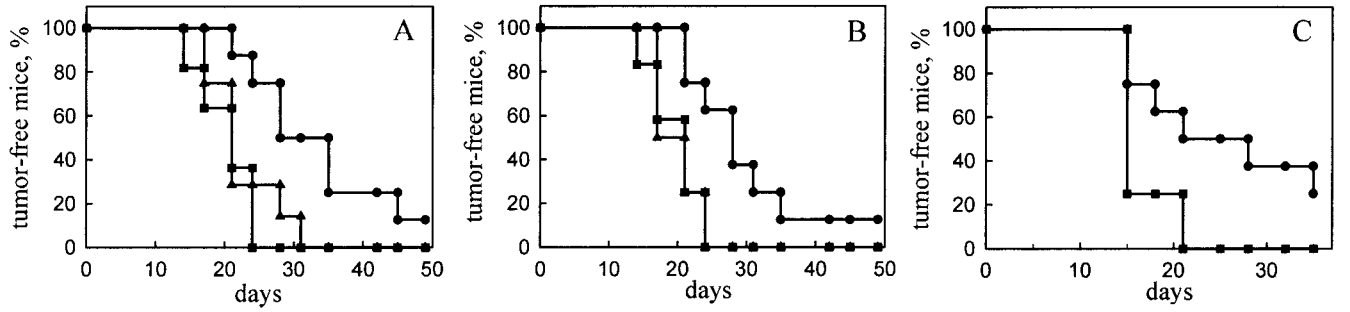

Fig. 5. Prevention of PC-3 (*A* and *B*) and HT-29 (*C*) tumor formation by N-65828. Cells were injected s.c. on day 0, and treatment or control solutions (●, N-65828; ■, vehicle; ▲, N-45557) were administered locally s.c. six times per week starting on the same day. Mice were examined twice per week for palpable tumors. Treatment doses: 40 μg/day (*A* and *C*); 8 μg/day (*B*). $n = 8$ for all groups except the vehicle controls in *A* and *B*, where $n = 12$.

tumor cell line, HT-29 (colon adenocarcinoma) (Fig. 5*C*). The compound again caused a significant delay in the appearance of tumors ($P = 0.02$), and 2 of the 8 mice in the treatment group were still tumor-free at sacrifice (14 days after all animals in the control group had tumors).

The mice in all of the treatment groups gained weight normally and had no apparent liver or kidney abnormalities at necropsy, suggesting that N-65828 is not toxic.

Effects of N-65828 on Tumor Vasculature. The extent and pattern of vascularization in PC-3 tumors from mice treated with N-65828 (40 or 8 μg/day; $n = 7$) were compared with those in weight-matched tumors from mice treated with the corresponding vehicle control solutions ($n = 9$). The average number of interior blood vessels was 53% lower in the treatment groups than in the controls, although the numbers of peripheral vessels were similar. These differences are not statistically significant, but follow the same trend observed previously for prostate tumors in mice treated with anti-ANG antibody or antisense vs. vehicle-treated mice (3, 5). As noted in the earlier reports, the development of interior vessels is thought to reflect true angiogenesis, whereas peripheral vessels might originate by co-option of preexisting vasculature (39).

Discussion

In recent years, inhibition of tumor angiogenesis has become one of the most active avenues for development of new anticancer drugs, and more than 30 anti-angiogenesis agents have entered clinical trials (40). Some of these antagonists have been directed at individual molecules, including several of the many known inducers of angiogenesis and their receptors and proteins that participate in downstream processes such as tissue remodeling. Others have targeted endothelial cells more generally, often without any defined mechanism of inhibition. As of this writing, no resounding successes have been reported in these trials, and it is too early to discern which, if any, of the specific approaches already being tested will yield drugs that are both effective and safe for human therapy.

Considerable evidence from preclinical and clinical studies, summarized above, suggests that ANG plays an important role in the establishment, progression, and metastatic dissemination of numerous types of human cancers. Yet no agent known to interfere, either directly or indirectly, with the actions of ANG has been tested in humans. Indeed, the ANG antagonists that have been amply demonstrated to be active in mice have potentially formidable drawbacks as drugs. The mAbs would probably need to be administered at relatively large doses: the vasculature in an adult cancer patient contains anywhere from $\approx$1 mg of ANG to more than twice this amount (9), requiring from 6 to >12 mg of mAb for neutralization of the protein in this compartment alone. Long-term treatment with high doses of protein can be problematic in terms of both practical considerations and side-effects, although immunogenicity can be minimized by using chimeric (4) or more fully "humanized" versions of the antibody. The other ANG antagonist investigated extensively in mice belongs to a class of compounds, antisense oligonucleotides, whose use in humans remains experimental (41).

The present work now provides a pathway for the development of small-molecule inhibitors of ANG for anticancer therapy, using the ribonucleolytic active site of ANG as the target and HTS as the starting point for lead identification. The viability of this approach depends, in part, on the strength of one of its underlying hypotheses: that the catalytic center of ANG is critically involved in the induction of angiogenesis by this protein. The results of mutational studies have provided considerable support for this view. ANG variants with markedly decreased enzymatic activity invariably have reduced angiogenic activity as well. These include the catalytically inactive derivatives H13A, H13Q, H114A, H114N, and K40Q, as well as others (K40R and T44H) showing less extensive losses of enzymatic activity (20–22). The properties of T44H are particularly noteworthy because a different replacement of Thr-44 (by Asp) has essentially no impact on either activity (22). Crystal structures of the K40Q and H13A variants show no significant changes beyond the site of the substitution, establishing that the loss of angiogenicity is directly attributable to disruption of the catalytic apparatus (23). In addition, parallel increases in catalytic efficiency and angiogenic potency have been observed with some ANG variants (42).

These mutational findings establish that the angiogenic action of ANG requires an intact catalytic site, and thereby suggest that molecules directed at this site would have antitumor activity. However, at least two plausible scenarios can be envisioned in which this would not be the case. First, the antitumor effects of antibodies and antisense DNA might be due not to interference with angiogenesis [although a reduction in tumor vasculature has indeed been observed (2, 3, 5)] but to inhibition of some other activity of ANG, independent of the active site. Tumor cells are known to secrete several additional angiogenesis factors (43), and it is possible that the vascularization induced by these molecules is sufficient to foster growth and metastasis when ANG is inhibited. Moreover, ANG has at least one cancer-promoting activity unrelated to its angiogenic and ribonucleolytic actions: it can serve as an adhesion molecule for tumor cells (44). If this is the property of ANG that is most critical for tumor formation, then active-site-directed inhibitors might not be effective anticancer agents. Second, it is possible that the role of the active site of ANG in the angiogenic mechanism does not involve catalysis, but binding of some activating ligand that potentiates cellular interactions such as receptor binding. In this case, compounds that bind to this site and inhibit ribonucleolytic activity might even enhance the biological actions of the protein.

The results of the study reported here largely allay these theoretical concerns and provide considerable support for targeting of the enzymatic active site of ANG as a strategy for developing new anticancer drugs. N-65828, a compound selected purely on the basis of a screen for ribonucleolytic activity inhibitors, was shown to significantly delay the formation of tumors from two distinct human cancer cell types. Although we cannot exclude the possibility that N-65828 acts on other molecules critical for tumor establishment, three lines of evidence, taken together, point to ANG as the target most likely involved. (*i*) An analogue of this compound that has greatly reduced potency against the enzymatic activity of ANG also fails to exert any protective effect against PC-3 tumors (Fig. 5). (*ii*) Tumors from mice treated with N-65828 have fewer interior (but not peripheral) blood vessels than tumors from the control groups, a trend seen previously for two antagonists that were demonstrably ANG specific (mAb and antisense) (3, 5). (*iii*) Data listed at an NCI web site (http://dtp.nci.nih.gov/docs/cancer/searches/cancer_open_compounds.html) show that N-65828, at concentrations up to 100 μM, does not inhibit the growth in culture of PC-3, HT-29, or any of 57 other human tumor cell lines tested. These data suggest that the antitumor activity of this compound does not derive from a direct effect on tumor cells, and are consistent with inhibition of angiogenesis or some other process involving interactions with the host as the basis for protection.

The utility of ANG inhibitors N-65828 and C-181431 as leads that can ultimately be developed into anticancer agents for human therapy is unclear at this stage. The requisite affinity improvements (probably more than two orders of magnitude) can potentially be achieved by structure-based design and/or combinatorial methods. For optimization of N-65828, the computational model of the ANG complex may already serve as a useful guide; results with N-65828 analogues and ANG variants described above, and other findings in these areas to be reported elsewhere (J.L.J. and R.S., unpublished data), are in complete agreement with this model. These follow-up studies have already yielded several analogues that have 4- to 20-fold greater potency *in vitro*. Crystallographic and NMR experiments are needed to determine actual complex structures. Along with increasing affinity, lead optimization will also likely involve making the compounds more "drug-like" (45, 46)—e.g., replacing the azo group of N-65828 or adding some hydrophilic substituents to C-181431. Finally, we note that application of the HTS method developed here to additional libraries might well yield leads that already have more favorable K_i values and/or physical properties than do the compounds thus far identified.

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