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Advances in Brief

Inhibition of Granulocyte-Macrophage Colony-stimulating Factor (GM-CSF) Activity by Suramin and Suramin Analogues Is Correlated to Interaction with the GM-CSF Nucleotide-binding Site[1]

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Abstract

Suramin and suramin analogues strongly inhibit both nucleotide interaction with the nucleotide-binding site of granulocyte-macrophage colony-stimulating factor (GM-CSF) and bioactivity of the molecule as assessed by competition photoaffinity labeling and cell proliferation assay, respectively. The half-maximal inhibition of cell proliferation by suramin occurs at 68 ± 2.5 μM; three suramin analogues achieved comparable activity. The degree of competitive inhibition of nucleotide-binding by these compounds and the inhibition of GM-CSF bioactivity are correlated such that the compounds show similar rank-order by both of these methods. The strong interaction of suramin and related compounds with the nucleotide-binding site may mimic nucleotide-mediated inhibition of GM-CSF bioactivity and may be an important mechanism by which suramin acts as a pharmacological anti-growth factor agent.

Introduction

GM-CSF[3] is a cytokine with proliferative, differentiating, and activating effects on hematopoietic cells (1). These effects require binding of GM-CSF to the high affinity-binding heterodimeric receptor complex (2). Whereas cellular events subsequent to receptor occupancy such as tyrosine phosphorylation of cellular proteins are important (3), many events relating to the specificity of GM-CSF-induced gene expression remain unclear. We have previously described a nucleotide-binding site on recombinant murine GM-CSF and localized this site to the NH_2 terminus of the molecule (4). The rh form of GM-CSF contains an identical site, and its preservation across species barriers suggests an important physiological role (5). GM-CSF is not generally included in the family of cytokines described as heparin-binding growth factors. GM-CSF does, however, appear to interact with matrix proteoglycans in a fashion that modulates biological activity, especially within the marrow microenvironment (6). This interaction with proteoglycans and the known inhibitory activity of suramin against the heparin-binding cytokine acidic fibroblast growth factor, which has recently been noted to also interact strongly with nucleotides (7), led us to examine heparin and suramin for possible interactions with GM-CSF. We have discovered that heparin and suramin strongly compete for the binding of azido-photoaffinity nucleotide analogues to GM-CSF. In examining the capacity of suramin and suramin analogues to inhibit nucleotide binding, we have noted that the degree of such competitive inhibition directly correlates with the degree of growth-inhibitory activity seen against a GM-CSF-dependent leukemic cell line.

Materials and Methods

Materials. rhGM-CSF (derived from *Escherichia coli*) was kindly provided by AMGEN, Inc., and was >95% pure by SDS-PAGE and reverse-phase high performance liquid chromatography analysis. Protein molecular weight standards were obtained from Bio-Rad. All other reagents were analytical grade and were obtained from Sigma Chemical Co. or Aldrich Chemical Co. [γ-^{32}P]8N_3ATP was provided by Research Products International (Mt. Prospect, IL). Suramin and heparin were obtained from Mobay Chemical and Sigma, respectively. Suramin analogues were kindly supplied by Dr. Peter Nickel, with chemical structures as published previously (8).

Photolabeling of rhGM-CSF. Samples containing 1.0 μg of rhGM-CSF in 40–60 μl of photolysis buffer (20 mM NaH_2PO_4, pH 4.5) were incubated at 4°C in Eppendorf tubes with photoprobe for 15 s, followed by a 45-s irradiation at 4°C with a hand-held 254-nm UV lamp (intensity, 7200 μW/cm^2). The reaction was quenched by addition of a protein-solubilizing mixture consisting of 10% SDS, 3.6 M urea, 162 mM DTT, pyronin Y (tracking dye), and 20 mM Tris (pH 8.0). For competition studies, rhGM-CSF was first incubated for 60 s at 4°C with the competitor (heparin, suramin, or suramin analogue), followed by incubation with photoprobe for 15 s at 4°C and photolysis for 45 s at 4°C.

SDS-PAGE. Solubilized protein samples were subjected to electrophoresis in a 10% polyacrylamide separating gel with a 4% stacking gel according to the method of Laemmli (9). Gels were stained with Coomassie brilliant blue R, destained overnight, and dried on a slab gel dryer. Gels were subjected to autoradiography, and ^{32}P incorporation was quantified by excision of the labeled bands and liquid scintillation counting utilizing a Packard Minaxi or Tri-Carb scintillation counter (counting efficiency, 99% for ^{32}P).

Bioassay and Growth-inhibitory Activity of Suramin Analogues. Suramin and suramin analogues were added to GM-CSF-dependent Mo7E cells (10), which had been seeded (1 × 10^4/well) into 96-well microtiter plates with rhGM-CSF. Concentrations of test compounds and rhGM-CSF are as noted in the figure legends. The assay mixture in a final volume of 200 μl/well was then incubated for 72 h at 37°C in a 5% CO_2 humidified incubator and pulsed with [^{3}H]thymidine (1 μCi/well) for the final 4 h. The contents of the wells were harvested onto glass fiber filters, and the amount of [^{3}H]thymidine incorporated into DNA was determined.

Results

Characterization of Competitive Inhibition of Nucleotide Binding. Competition for the nucleotide-binding site of rhGM-CSF was ascertained by the addition of increasing concentrations of suramin, suramin analogues, or heparin to the reaction mixture before azidonucleotide photoprobe activation by UV light. When heparin was examined, strong inhibition of nucleotide binding was noted; half-maximal inhibition occurred at 0.125 μg/50 μl, and virtually complete inhibition occurred by 0.50–1.0 μg/50 μl (data not shown). This led to the examination of suramin, which was noted to potently inhibit the photoinsertion of the azido analogue of ATP (Fig. 1). The half-

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[3] The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; rhGM-CSF, recombinant human GM-CSF; N_3ATP, azidoATP; N_3Ap_4A, azidoadenosine tetraphosphate adenosine; $Ap_{3-5}A$, adenosine tri-pentaphosphate adenosine.

maximal inhibition by suramin was achieved at a concentration of 2.5 μM. This inhibition was 100-fold more potent than the natural nucleotide ATP in this assay.

Examination of a variety of suramin analogues revealed similar strong competitive inhibition (Fig. 1), with the exception of 2 compounds, NF 023 and NF 103. The half-maximal inhibition seen with NF 023 was approximately 5 μM, whereas NF 103 concentrations of 16 μM were necessary for half-maximal inhibition of photoinsertion (Table 1).

Effect of Suramin and Suramin Analogues on GM-CSF-dependent Leukemic Cell Growth. The addition of suramin to a GM-CSF-dependent human leukemia line, Mo7E, inhibited growth in a dose-dependent fashion (Fig. 2). Half-maximal inhibition of growth was achieved at approximately 70 μM; complete inhibition of growth was achieved at 200 μM suramin concentration. These results were similar when GM-CSF was present at maximal stimulatory (plateau) concentrations (0.1 ng/ml) or at lower (linear portion of rhGM-CSF growth-concentration curve; 0.01 ng/ml) concentrations (data not shown). Examination of suramin analogues in the GM-CSF-dependent growth of Mo7E cells revealed reduction in inhibitory potency with NF 023 and NF 103 (Fig. 2). These results mirrored those noted in the nucleotide-binding competition assay (Fig. 1).

At suramin concentrations of 100 and 500 μM, increasing concentrations of GM-CSF could partially overcome suramin inhibition.

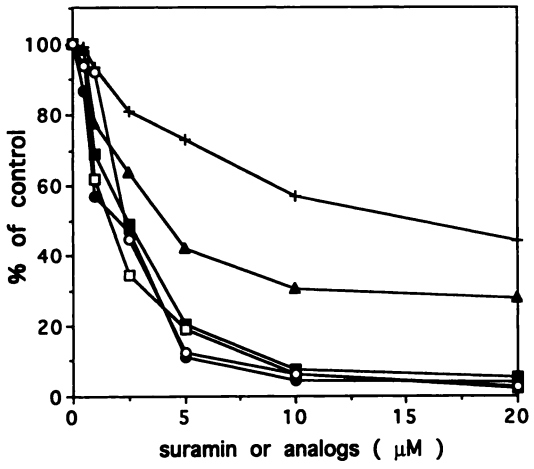

Fig. 1. Protection of [γ'^{32}P]8N_3ATP photoincorporation into rhGM-CSF. rhGM-CSF (1.0 μg) was incubated in photolysis buffer with the indicated increasing concentrations of suramin (■) or suramin analogues NF 032 (●), NF 110 (□), NF 023 (▲), NF 103 (+), and NF 201 (○) for 60 s at 4°C, and then with 25 μM of photoprobe for 15 s and photolyzed. SDS-PAGE analysis and determination of ^{32}P incorporation was as per text; results are expressed as a percentage of the control incorporation of photoprobe. The resulting curve for suramin is a representative of 5 experiments. Suramin analogue results are representative of 2 or 3 experiments, depending on the amounts of compound available to our laboratory.

Table 1 *Inhibitory potency of suramin and suramin analogues*

Competition curves for nucleotide photoprobe incorporation into rhGM-CSF by suramin and suramin analogues (Fig. 1) gave half-maximal inhibitions of photoprobe incorporation as compared to control. Similarly, cell proliferation in the presence of suramin and suramin analogues (Fig. 2) gave half-maximal inhibition of [^{3}H]thymidine uptake. Results are expressed as micromolar concentrations ± 1 SEM.

Compound	Half-maximal inhibition of nucleotide binding (μM)	Half-maximal inhibition of cell proliferation (μM)
Suramin	2.5 ± 0.05	68 ± 2.5
NF110	1.5 ± 0.07	52 ± 3.5
NF032	2.0 ± 0.2	76 ± 1.4
NF201	2.3 ± 0.14	76 ± 1.0
NF023	5.0 ± 0.35	102.5 ± 3.5
NF103	16.0 ± 0.35	141.0 ± 1.4

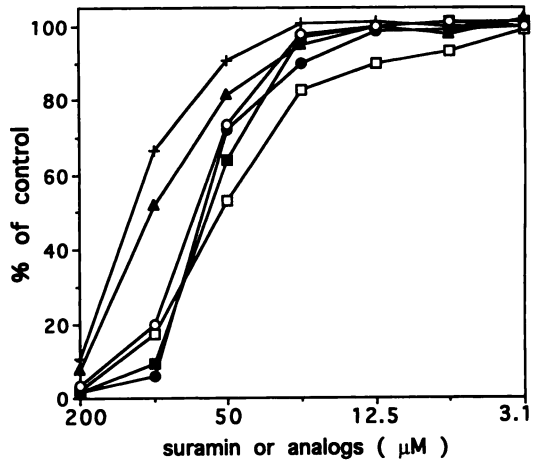

Fig. 2. Comparison of suramin and suramin analogue activity against rhGM-CSF stimulation of Mo7E cell line. M07E cells were seeded at 10^4 cells/well in 200 μl final volume with 0.1 ng/ml rhGM-CSF and the indicated decreasing concentrations of suramin (■) or suramin analogues NF 032 (●), NF 110 (□), NF 023 (▲), NF 103 (+), and NF 201 (○). Cell proliferation was measured by [^{3}H]thymidine uptake as per text, with standard 1:2 dilutions. Cell proliferation that occurred in the presence of suramin or analogue was expressed as a percentage of cell proliferation in control cultures. *Points*, mean of three determinations. The resulting curve for suramin is a representative of 5 experiments. Suramin analogue results are representative of 2 or 3 experiments, depending on the amounts of compound available to our laboratory.

Half-maximal stimulation occurred at GM-CSF concentration of 0.6 ng/ml with 100 μM suramin exposure *versus* 0.035 ng/ml for control; half-maximal stimulation at 500 μM suramin was not attained with GM-CSF concentrations up to 100 ng/ml. Plateau levels of control cell growth could not be reached at either suramin concentration, even with GM-CSF concentrations of 100 ng/ml, fully 3 logs higher than the concentration that resulted in plateau growth of control cultures.

Discussion

Cytokines such as GM-CSF can have growth promoting, differentiating, and cell-activating activities, all of which are mediated by specific ligand-receptor interaction. This interaction triggers certain metabolic events at or near the cell membrane-cytoplasmic area and leads to ligand-receptor internalization (11). This internalization would expose the complex to intracellular pools of nucleotides, which may be of physiological significance due to the presence of a nucleotide-binding site in the NH_2 terminus portion of the molecule (4, 5). The precise mechanism of cytokine signal transduction, specifically the late events related to specific gene transcription, remain unclear. The reports of certain cytokines and their receptors being localized after internalization within the nucleus raises interesting possibilities with regard to conveying specificity of signal transduction (12).

In our previous report of nucleotide interaction with murine and human GM-CSF, we showed the irreversible photolinkage of N_3ATP or N_3Ap_4A into the binding site inhibited GM-CSF bioactivity (4, 5). We have also shown bioactivity modulation with free nucleotide (13). Similar to these effects, albeit with greater potency, we describe in this report suramin interaction, presumably at the nucleotide-binding site, and potent inhibition of growth of a leukemic cell line, which is dependent on GM-CSF for proliferation. The suramin concentrations required for this leukemic cell inhibition are well within the range of concentrations achieved clinically with this agent (14). The plasma concentration goal for therapeutic trials of suramin is reported as 150–300 μg/ml, corresponding to 105–210 μM; greater than 90% inhibition of leukemic cell proliferation occurs with 100 μM suramin concentration, and virtually no proliferation is seen at 200 μM suramin. Autocrine, paracrine, and internal autocrine regulation by GM-

CSF has been described in acute myeloblastic leukemia cells (15, 16). Blockade of such growth factor loops by an anti-growth factor agent such as suramin or analogue, with activity at both the cell surface and intracellular compartments, could be effective in leukemia therapy (17, 18).

The capacity of suramin to strongly compete for the nucleotide-binding site is noteworthy in several respects. First, suramin contains 6 sulfate groups, responsible presumably for its promiscuous activity with polyanion-binding sites on a number of cytokines. The nucleotide binding to GM-CSF is partially dependent on the nucleotide phosphates; progressively less binding is noted as the number of phosphate groups is reduced (4). Weak interaction with tripolyphosphate and PP_i was noted also. Second, the suramin molecule contains planar ring structures, as do the nucleotide molecules we have studied. Finally, the nucleotides with the highest avidity for interaction with GM-CSF, $Ap_{3-5}A$, have, like suramin, a "mirror image" conformation. These similarities in structure may be responsible for the activity of these compounds in down-modulating GM-CSF bioactivity. Our preliminary work suggests that this nucleotide-binding site, or a portion of it, overlaps with a GM-CSF receptor-binding epitope because the occupancy of the binding site interferes with GM-CSF binding to its receptor (13). The location of the nucleotide-binding site in the NH_2 terminus of the molecule supports these data because the NH2 terminus portion of GM-CSF has been reported recently as the site of interaction with the β chain of the GM-CSF heterodimeric receptor complex (19).

The comparison of suramin with five analogues in both the competitive nucleotide-binding assay and the cell proliferation bioassay has yielded three compounds with nearly equivalent activity, one of which is slightly more active than suramin in both the competition of nucleotide binding and the cell proliferation assays (NF 110). The discovery of an analogue with equal bioactivity but less toxicity might allow for dose escalation and greater clinical efficacy. Work with suramin analogues suggests that anti-growth factor activity and neurotoxicity (a major dose-limiting toxicity) can be disassociated with modifications to the suramin molecule (20). The competitive nucleotide-binding assay and bioassay yielded virtually identical results regarding rank-ordering of the activity of the compounds. The differences in suramin and analogue concentrations necessary for half-maximal inhibition in these respective assays is due to the presence of serum proteins, especially albumin, in the cell proliferation assay. Suramin, and presumably analogue, are highly protein bound (17), thus modulating the effective drug concentration in biological systems. NF 023 and NF 103 have similar structures; the latter has methyl groups in the 2 positions of the core aromatic groups. They both differ from suramin primarily in the loss of the aromatic groups, although the analogue NF 110 has loss of both the secondary phenyl rings and also the entire aminonaphthalene trisulfonic acid group. These data are clearly insufficient to arrive at conclusions of structure requirements for maximal interaction of these analogues and other compounds with the putative nucleotide-binding domain of GM-CSF.

The correlation between suramin analogue ability to act as a competitive inhibitor of nucleotide binding and the degree of growth inhibition of the GM-CSF-dependent cell line offers interesting possibilities. The competition binding assay may be useful in rapid screening of a variety of compounds for potential anti-growth factor activity. Correlation of the activities of the compounds, coupled to greater elucidation of the nucleotide-binding site itself, may allow for rational drug design and discovery of less toxic compounds with better anticancer or antiviral activities.

References

1. Metcalfe, D. The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. Blood, *67:* 257–267, 1986.
2. Nicola, N. A., and Metcalfe, D. Subunit promiscuity among hemopoietic growth factor receptors. Cell, *67:* 1–4, 1991.
3. DiPersio, J. F., Golde, D. W., and Gasson, J. D. GM-CSF: receptor structure and transmembrane signaling. Int. J. Cell Cloning, *8:* 63–75, 1990.
4. Doukas, M. A., Chavan, A. J., Gass, C., Campbell, S., and Haley, B. Identification and characterization of a nucleotide binding site on recombinant murine granulocyte/macrophage-colony stimulating factor. Bioconjugate Chem., *3:* 484–492, 1992.
5. Chavan, A. J., Gass, C., Haley, B. E., Boone, T., and Doukas, M. A. Identification of N-terminus peptide of hGM-CSF as the site of nucleotide interaction. Biochem. Biophys. Res. Commun., *208:* 390–396, 1995.
6. Roberts, R., Gallagher, J., Spooncer, E., Allen, T. D., Bloomfield, F., and Dexter, T. M. Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. Nature (Lond.), *332:* 376–378, 1988.
7. Chavan, A. J., Haley, B. E., Volkin, D. B., Marfia, K. E., Verticelli, A. M., Bruner, M. W., Draper, J. P., Burke, C. J., and Middaugh, C. R. Interaction of nucleotides with acidic fibroblast growth factor (FGF-1). Biochemistry, *33:* 7193–7202, 1994.
8. Jentsch, K. D., Hunsmann, G., Hartmann, H., and Nickel, P. Inhibition of human immunodeficiency virus type I reverse transcriptase by suramin-related compounds. J. Gen. Virol., *68:* 2183–2192, 1987.
9. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage 4. Nature (Lond.), *227:* 680–685, 1970.
10. Avanzi, G. C., Lista, P., Giovanazzo, B., Miniero, R., Saglio, G., Benetton, G., Coda, R., Cattoretti, G., and Pegoraro, L. Selective growth response to IL-3 of a human leukaemic cell line with megakaryoblastic features. Br. J. Haematol., *69:* 359–366, 1988.
11. Gasson, J. C. Molecular physiology of granulocyte-macrophage colony-stimulating factor. Blood, *77:* 1131–1145, 1991.
12. Weitzmann, M. N., and Savage, N. Nuclear internalisation and DNA binding activities of interleukin-1, interleukin-1 receptor and interleukin-1/receptor complexes. Biochem. Biophys. Research Commun., *187:* 1166–1171, 1992.
13. Doukas, M. A., Chavan, A., Jacobson, S., Keller, J., Gass, C., Boone, I., and Haley, B. Modification of the nucleotide binding site of granulocyte/macrophage-colony stimulating factor (GM-CSF) decreases bioactivity and receptor interaction (Abstract). Exp. Hematol., *20:* 758, 1992.
14. Cooper, M. R., Lieberman, R., LaRocca, R. V., Grent, P. R., Weinberger, M. S., Headley, D. J., Kohler, D. R., Goldspiel, B. R., Peck, C. C., and Myers, C. E. Adaptive control with feedback strategies for suramin dosing. Clin. Pharmacol. & Ther., *52:* 11–23, 1992.
15. Rogers, S. Y., Bradbury, D., Kozlowski, R., and Russell N. H. Evidence for internal autocrine regulation of growth in acute myeloblastic leukemia cells. Exp. Hematol., *22:* 593–598, 1994.
16. Bradbury, D., Rogers, S., Reilly, I. A. G., Kozlowski, R., and Russell, N. H. Role of autocrine and paracrine production of grandulocyte-macrophage colony-stimulating factor and interleukin-1β in the autonomous growth of acute myeloblastic leukemia cells: studies using purified CD-34 positive cells. Leukemia, *6:* 562–566, 1992.
17. Stein, C. Suramin: a novel antineoplastic agent with multiple potential mechanisms of action. Cancer Res., *53:* 2239–2248, 1993.
18. Huang, S. S., and Huang, J. S. Rapid turnover of the platelet derived growth factor receptor in sis-transformed cells and reversal by suramin. J. Biol. Chem., *263:* 12608–12618, 1988.
19. Hercus, T. R., Cambareri, B., Dottore, M., Woodcock, J., Bagley, C. J., Vadas, M. A., Shannon, M. F., and Lopez, A. F. Identification of residues in the first and fourth helices of human GM-CSF involved in biologicalal activity and in binding to the α- and β-chains of its receptor. Blood, *83:* 3500–3508, 1994.
20. Baghdiguian, S., Nickel, P., and Fantini, J. Double screening of suramin derivatives on human colon cancer cells and on neural cells provides new therapeutic agents with reduced toxicity. Cancer Lett., *60:* 213–219, 1991.