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AD_____

Award Number: DAMD17-98-1-8467

TITLE: Diphenylureas for Treatment of Prostate Cancer

PRINCIPAL INVESTIGATOR: Delwood C. Collins, Ph.D.

CONTRACTING ORGANIZATION: University of Kentucky Research Foundation
Lexington, Kentucky 40506-0057

REPORT DATE: January 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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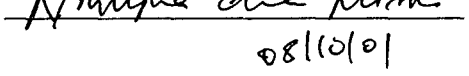

08/10/01

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2001	Annual (1 Jul 99 - 31 Dec 00)

4. TITLE AND SUBTITLE
Diphenylureas for Treatment of Prostate Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8462

6. AUTHOR(S)
Delwood C. Collins, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Kentucky Research Foundation
Lexington, Kentucky 40506-0057

E-Mail: dccoll1@email.uky.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jan 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

This progress report for the year 2000 describes the progress made on development of satisfactory mouse PC3 xenograft for testing the role of the phosphoric acid diphenylureas on PC3 tumor growth and angiogenesis. The changes in the protocol during the one year, no cost extension are described to complete the major tasks of this grant. Some additional publications, abstracts, and one patent application are also provided.

14. SUBJECT TERMS prostate cancer, angiogenesis, phosphoric acid, Diphenylureas

15. NUMBER OF PAGES 43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION:

We have synthesized and characterized a unique group of diphenylureas that are much more potent than suramin or its sulfonated analogues as inhibitors of cell growth, migration and matrix metalloproteinase activity (MMP-2 and MMP-9) in human microvascular endothelial and the human prostate cancer cell lines (PC3, LNCaP.FGC & DU145), in vitro. Toxicity studies in mice indicate no significant toxicity by the diphenylureas and a half-life 10-fold less than suramin. However, the diphenylureas do not bind the heparin-like growth factors. These results suggest that the diphenylureas may be potent therapeutic agents that will significantly reduce morbidity in man.

The experiments described in this proposal will be the first in vivo test of the ability of a unique group of diphenylureas to inhibit the growth and metastasis in human androgen dependent (LNCaP) and androgen independent (PC3) prostate tumor xenografts in nude mice. We propose that the diphenylureas are potent inhibitors of tumorigenesis, angiogenesis and metastasis in human prostate cancer growth. Furthermore, the antineoplastic activity of the diphenylureas is not related to the inhibition of the binding of the growth factors as has been demonstrated for suramin. These hypotheses will be tested by the following questions:

Question #1. Are selected diphenylureas that inhibited growth of human prostate cancer cell lines in vitro able to inhibit the growth of human androgen dependent (LNCaP) and androgen independent prostate cancer tumors in the athymic nude mouse model?

Question #2. Do the selected diphenylureas inhibit tumor angiogenesis in prostate cancers in vivo?

Question #3. Do the selected diphenylureas inhibit the metastatic ability of prostate cancers in vivo?

Positive results from these experiments will lead to translational research and clinical testing of these diphenylureas for prostate cancer therapy in man during Phase 2 of this proposal. .

BODY

The research report from 1999 indicated that we were not able to demonstrate inhibition of PC3 growth in the nude mouse xenograft because of the slow growth of the PC3 tumors and the wide variability of the growth rate. This resulted in wide variability in the control and treated nude mice. This year was devoted to developing a better protocol for determining the effect of the phosphonated diphenylureas on prostate cancer growth and angiogenesis. Several changes were made in the protocol:

1. The growth of PC3 cells obtained from ATCC was very slow, with many controls not showing a tumor after 50 days. However, if the PC3 ATCC cells were grown in a nude mouse xenograft, collected and re-grown in vitro, the resultant cells

showed a much higher growth rate both in vitro and in subsequent in vivo experiments. These cells (designated PC3-T cells) were used in PC3 xenografts.

2. Evidence described in the 1999 annual report suggested that the solubility of the diphenylurea compounds might be a problem and that their blood levels are inadequate to initiate the effects. In order to overcome this problem, the diphenylureas to be tested will be injected subcutaneously adjacent to the area where the tumor cells were implanted. In addition, the injection of NF681 in saline or saline alone were started one day after the cells were implanted. This alteration in the protocol should allow the local levels of NF681 to be adequate to initiate the effects on the growth of PC3 cells in vivo. This approach has been used by other investigators to assess the effect of poorly soluble compounds on cancer growth and angiogenesis.

Modified Work Plan and One-Year No Cost Extension

In order to accomplish the major goals of this grant, a one-year no cost extension of the grant was requested and approved. This request was necessary because of the difficulty in establishing a reliable PC3 xenograft model in nude mice and the poor solubility characteristics of the compounds being tested in the in vivo experiments.

In order to accomplish the most important tasks, we have reduced the compounds to be tested to the two most active compounds in the in vitro experiments (NF681 and NF050). We have increased the number of animals in each test group from 10-20 animals to account for the variability we have found in the PC#-T xenograft model in our laboratory.

The Task 2 studies on the androgen-dependent human prostate cancer (LNCaP) in nude mice xenografts have been excluded because our in vitro experiments suggest that the LNCaP prostate cancer cells are not inhibited as effectively as the androgen-independent PC3 prostate cancer cells by NF681 and NF050.

The modified statement of work is as follows:

Modified Statement of Work

Task 1. Determine the effect of the selected diphenylureas (NF681 and NF050) and suramin at one dose level at 50% of maximum dose on the growth of androgen independent human prostate cancer (PC3) in the athymic nude mouse xenograft. This animal protocol requires 20 animals per group and 100 animals for this experiment.

- Carry out the experimental protocol to test the ability of the selected diphenylureas to inhibit the androgen independent human prostate cancer (PC3) in the athymic nude mouse xenograft model.
- Carry out the bromodeoxyuridine (BrdU) incorporation experiments to determine the growth rate of the prostate tumor under the various treatment conditions.

- Determine the effect of NF681, NF050 and suramin on the rate of apoptosis using the Apotag Apoptosis kit.
- Determine the effect of NF681, NF050 and suramin on the presence of the matrix metalloproteinases, MMP and MMP-9, by immunohistochemistry.
- Determine the effect of NF681, NF050 and suramin on the presence of inhibitors of matrix metalloproteinases, TIMP-1 and TIMP-2, by immunohistochemistry.

Task 2. Determine the temporal effect of NF681 or NF050 and suramin on tumor angiogenesis in PC3 prostate tumors in the nude mouse xenograft model.

- Carry out the temporal animal protocol outlined in Question #2. This animal protocol requires 20 animals per group and 400 total animals.
- Quantitate the prostate cancer tumor vasculature using immunostaining for factor VIII antigen as previously described by Vukanovic et al. (1995). Point-counting morphometric analysis and computerized image analysis will be used to determine the temporal changes in microvessel counts in tumors treated with NF681, NF050 and suramin.
- Determine the temporal relationship of the response of the PC3 tumors to NF681, NF050 and suramin to tumor growth rate (BrdU incorporation), apoptosis by Apotag kit, the presence of MMP-2 and MMP-9 protein by immunochemistry, and the presence of TIMP-1 and TIMP-2 by immunochemistry. The factors to be determined will depend on the results obtained in Task 1.

Task 3. Determine the effect of NF681 or NF050 and suramin on metastasis using the PC3 prostate tumors in the athymic nude mouse model.

- Carry out the metastasis animal protocol using a research protocol similar to that described by Waters et al. (1995). This animal protocol requires 20 animals per group and 400 animals to test the effect of the two diphenylureas and suramin.
- Autopsy and determine the number of metastasis present by visual inspection and by selected histological analysis. Emphasis will be focused on the lung, spleen and lymph nodes.

KEY RESEARCH ACCOMPLISHMENTS FOR YEAR 2000:

1. Established a modified PC3-T xenograft model that will allow us to test the effect of the phosphonic acid diphenylureas on cancer cell growth and angiogenesis.

REPORTABLE OUTCOMES FOR YEAR 2000:

1. Abstracts and Presentations:

Gagliardi A., D.C. Collins and P. Nickel. Phosphonic Acid Diphenylureas, A Unique Group of Potent Antiangiogenic and Antitumorigenic Compounds. Fifth Biannual International Conference, "Angiogenesis: From the Molecular to Integrative Pharmacology", Crete, Greece, abstract, 1999.

Gagliardi, A., R.K. Munn and D.C. Collins. The Effect of Tamoxifen on the Angiogenesis Cascade. Fifth Biannual International Conference, "Angiogenesis: From the Molecular to Integrative Pharmacology", Crete, Greece, abstract, 1999.

Gagliardi, A.R.T., M. Bittencourt, P. Nickel, D.C. Collins. Inhibition of Renal Cell Carcinoma Growth and Metalloproteinase Activity by NF681, A Phosphonic Acid Diphenylurea. AACR-NCI-EORTC International Conference "Molecular Targets and Cancer Therapeutics", Washington, abstract, 1999.

Gagliardi, A.R.T., M. Bittencourt, P. Nickel, D.C. Collins. The Effect of a Phosphonic Acid Diphenylurea, NF681, on Renal Carcinoma Cell Growth and Metalloproteinase Activity. First International Kidney Cancer Symposium, Chicago, abstract, 1999.

Gagliardi, A., R. Munn and D. Collins.Estradiol-17β and Tamoxifen Induce Estrogen Receptor β in Human Microvascular Endothelial and Macrovascular Endothelial Cells In Vitro. 14th Int. Symposium of the Journal of Steroid Biochemistry and Molecular Biology, abstract, 2000.

Gagliardi, A.R.T., M. Bittencourt, P. Nickel, D.C. Collins. Inhibition of Renal Cell Carcinoma Growth and Metalloproteinase activity by NF681, a Phosphonic Acid Diphenylurea. American Association for Cancer Research, abstract, 2000.

2. Publications:

Gagliardi, A.R.T., M. Kassack, A. Kreimeyer, G. Muller, P. Nickel and D.C. Collins. Antiangiogenic and Antiproliferative Activity of Suramin Analogues. Cancer Chemotherapy and Pharmacology, 41:117-124, 1998.

Gagliardi, A.R.T., M.F. Taylor and D.C. Collins. Uptake of Suramin by Human Microvascular Endothelial Cells. Cancer Letters, 125:97-102, 1998.

Gagliardi, A.R., D.C. Collins and P. Nickel. Diphenylureas are Potent Inhibitors of Angiogenesis. Proceedings of the 17th UICC International Cancer Congress, pp. 221-225, 1998.

Gagliardi, A.R.T., P. Nickel and D.C. Collins. Inhibition of Prostate Cancer Cell Growth by Diphenylureas, A Unique Group of Antiangiogenic Compounds. Proceedings of the 17th UICC International Cancer Congress, pp. 1109-1113, 1998.

Gagliardi, A.R., M.C. Bittencourt, R.K. Munn, P. Nickel and D.C. Collins. Inhibition of Renal Carcinoma Cell Growth by Diphenylureas, a Unique Group of Antiangiogenic Compounds. Proceedings of the 17th UICC International Cancer Congress, pp.1137-1140, 1998.

Pereira-Bittencourt, M., D.D. Carvalho, A.R. Gagliardi, and D.C. Collins. The Effect of a Lectin from the Venom of the Snake, *Bothrops jararacussu,* on Tumor Cell Proliferation. Anticancer Research, 19:4023-4026, 1999.

3. Patents:

Patent application entitled "Phosphonated Agents and their Antiangiogenic and Antitumorigenic Use. Submitted by Delwood Collins, Ph.D., Antonio R.T. Gagliardi, M.D., Ph.D. and Peter Nickel, Ph.D. This patent was received in year 2000. A copy of the patent is included in the appendix.

CONCLUSIONS:

Year 2 of this grant was focused on developing a more reliable method for testing the effect of the phosphonic acid diphenylureas on PC3 prostate carcinoma cells in the nude mouse xenograft. The changes in the protocol and the extension requested should allow us to complete the major tasks of this grant.

REFERENCES:

Vukanovic J, Hartley-Asp B, Isaacs JT. Inhibition of tumor angiogenesis and the therapeutic ability of linomide against rat prostatic cancers. Prostate 26:235-246, 1995.

Waters DJ, Janovitz EB, Chen CK. Spontaneous metastasis of PC3 cells in athymic mice after implantation of orthotopic or ectopic microenvironments. Prostate 26:227-234, 1995.

APPENDIX:

Publications

Patent: Phosphonated Agents and Their Antiangiogenic and Antitumorigenic Use.

Cancer Chemother Pharmacol (1998) 41: 117–124

ORIGINAL ARTICLE

Antonio R.T. Gagliardi · Matthias Kassack
Annett Kreimeyer · Guido Muller · Peter Nickel
Delwood C. Collins

Antiangiogenic and antiproliferative activity of suramin analogues

Received: 21 October 1996 / Accepted: 8 May 1997

Abstract The purpose of this study was to test the ability of 70 polyanionic analogues of suramin to inhibit angiogenesis. The ID_{50}, the dose that produced 50% inhibition of angiogenesis, was determined for suramin and each of the analogues by measuring the ability of various amounts to inhibit angiogenesis in vivo in the chick egg chorioallantoic membrane (CAM) assay. Of the 70 analogues, 11 had antiangiogenic activities similar to suramin and an additional 7 were significantly more potent than suramin. All seven of these analogues were from the naphthalenetrisulfonic acid group and contained large urea groups. The benzene sulfonic and disulfonic acid analogues were less active inhibitors of angiogenesis than the naphthalenetrisulfonic acid analogues. Replacement of the naphthalenetrisulfonic acid groups by aliphatic carboxylic acids or benzoic acid gave analogues with very little antiangiogenic activity. In subsequent experiments, the antiproliferative activity of selected analogues on basic FGF (bFGF)-stimulated growth of immortalized human microvascular endothelial cells in vitro was determined. Analogues that inhibited angiogenesis to a greater extent than suramin in the CAM assay generally showed a greater antiproliferative effect on bFGF-induced growth of human microvascular endothelial cells. These results suggest that some of the polyanionic analogues may be potent therapeutic agents for cancers and angiogenesis-dependent diseases.

A.R.T. Gagliardi · D.C. Collins
VA Medical Center and Departments
of Obstetrics Gynecology and Physiology,
University of Kentucky College of Medicine,
Lexington, KY, USA

M. Kassack · A. Kreimeyer · G. Muller · P. Nickel
Pharmaceutical Institute, University of Bonn, Bonn, Germany

D.C. Collins (✉)
204 HSRB, UK Medical Center, Lexington,
KY 40536-0305, USA
Tel. +1 606-323-5293; Fax +1 606-257-9700

Key words Suramin · Suramin analogues · Antiangiogenesis · Antitumorigenesis

Introduction

We have previously reported for the first time [10] that suramin alone is an effective inhibitor of angiogenesis in the chick chorioallantoic membrane (CAM) assay. Our results showed that suramin inhibits angiogenesis in a dose-dependent manner. An important finding was the antagonism between suramin and heparin. These results support the hypothesis that suramin may interfere with the effects of heparin-binding growth factors, such as basic FGF (bFGF), VEGF and PDGF, on angiogenesis. Suramin has been recently shown to inhibit endothelial cell binding of bFGF, endothelial cell migration and bFGF induction of urokinase-type plasminogen activator [23]. A major problem encountered with suramin during clinical trials has been the adverse neurotoxic side effects. These effects are partly related to the prolonged half-life in vivo (45–55 days). This prolonged half-life is a consequence of being tightly bound to serum proteins, mainly albumin [2], and limited metabolism [22]. The narrow margin between the dose for antitumor activity and toxic effects prompted us to look for suramin analogues with similar or more potent antiangiogenic activity and for reduced protein binding and toxicity than suramin.

It has been known for many years that a small variation in the structure of suramin leads to great changes in the trypanocidal activity. For example, replacement of the two methyl groups of suramin by hydrogen reduces the trypanocidal activity by 95% [8]. In contrast, inhibition of HIV-1 reverse transcriptase is less sensitive to structural modifications and the structure–activity relationships are completely different from those of its trypanocidal or antifilarial activity [11]. Braddock et al. [3] investigated the structure–activity relationships for antagonism on the growth factor and angiogenic activity of bFGF by suramin and a limited number of related

polyanions. They examined 16 polyanionic analogues and found that four express bFGF-blocking activity equipotent to that of suramin in vitro. However, compounds with two bridging aromatic groups are less toxic than suramin in mice, suggesting a potential for an improved therapeutic ratio. These observations suggest that the study of a large number of suramin analogues could substantially widen the therapeutic opportunities for this class of compounds.

Microvascular endothelial proliferation is postulated to be a key event in the complex process of tumor angiogenesis [5]. Other steps include endothelial cell migration, secretion of metalloproteinases, the formation of capillaries and anastomosis [1]. Considering our findings that suramin can inhibit angiogenesis in the CAM assay and the report by Pesenti et al. [20] that suramin can inhibit tumor-induced angiogenesis, we examined some structurally related analogues of suramin for their ability to inhibit angiogenesis in the CAM assay and bFGF-stimulated human microvascular endothelial cell proliferation. A total of 70 suramin analogues synthesized by Nickel and coworkers [11, 16, 17] were purified and examined for their ability to inhibit angiogenesis in the CAM assay. Selected analogues were also tested for their ability to inhibit bFGF-stimulated endothelial cell growth in vitro. Structural features of the suramin analogues important for the expression of antiangiogenic activity were identified.

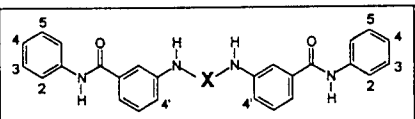
Material and methods

Structure of the analogues

The purity of the 70 suramin analogues studied was determined by high-pressure liquid chromatography before use [12]. These analogues were derivatives of naphthalenetrisulfonic acids, naphthalenedisulfonic acids, benzene sulfonic acids, benzoic acids and aliphatic carboxylic acids. The chemical structures, molecular formulae and molecular weights are shown in Figs 1–4.

Chorioallantoic membrane assay

This assay determined the ability of the suramin analogues to inhibit angiogenesis in vivo [9, 10]. Specific pathogen-free fertile eggs (Sunrise Farms, Catskill, N.Y.) were incubated for 72 h in a horizontal position in a humidified Petersine Hatching Incubator at 37 °C. After 72 h, the egg shells were broken and the egg contents were placed in 20 × 100 mm plastic petri dishes (Falcon #1005). The petri dishes containing the egg contents were then placed in a Forma Scientific incubator at 37 °C in 3% CO_2/air and 98% humidity. Each compound to be tested was dissolved in 0.45% methylcellulose in water and a 10 µl aliquot of this solution was air dried on a Teflon-coated metal tray (forming a disk around 2 mm diameter) and implanted on the outer third of a 6-day CAM where capillaries were intensively growing. The zone around the methylcellulose disk was examined 48 h after implantation with a Wild M8 stereomicroscope. A positive inhibition of angiogenesis was indicated by an avascular area of ≥4 mm. At least 20 embryos were measured for each amount of analogue tested and the results are expressed as the percentage of embryos that showed inhibition. The methylcellulose disk only and suramin (70 nmol/disk) were used as

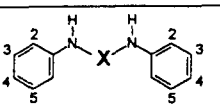


Substitution at Position

No	Code	Formula	MW	-2	-3	-4	-5	-4'	-X-	# Embryos	% Inhibition
Naphthalenetrisulfonic acid derivatives											
1	NF060 Suramin	$C_{51}H_{34}N_6O_{23}S_6Na_6$	1429.2	$-CH_3$	-H	-H	-CO-A01	-H	-B1-	67	64.2
2	NF127	$C_{53}H_{38}N_6O_{23}S_6Na_6$	1457.2	$-C_2H_5$	-H	-H	-CO-A01	-H	-B1-	23	80.0
3	NF151	$C_{55}H_{42}N_6O_{23}S_6Na_6$	1485.3	$-CH(CH_3)_2$	-H	-H	-CO-A01	-H	-B1-	20	89.0
4	NF145	$C_{57}H_{46}N_6O_{23}S_6Na_6$	1513.3	$-C(CH_3)_3$	-H	-H	-CO-A01	-H	-B1-	22	100.0
5	NF157	$C_{49}H_{28}F_2N_6O_{23}S_6Na_6$	1437.1	-F	-H	-H	-CO-A01	-H	-B1-	21	53.0
6	NF171	$C_{51}H_{34}N_6O_{23}S_6Na_6$	1429.2	$-CH_3$	-H	-CO-A01	-H	-H	-B1-	22	59.0
7	NF212	$C_{53}H_{38}N_6O_{23}S_6Na_6$	1457.2	$-CH_3$	-H	-H	-CO-A01	$-CH_3$	-B1-	21	46.0
8	NF280	$C_{49}H_{30}N_6O_{23}S_6Na_6$	1401.1	-H	-H	-CO-A01	-H	-H	-B1-	21	66.0
9	NF032	$C_{49}H_{30}N_6O_{23}S_6Na_6$	1401.1	-H	-H	-CO-A02	-H	-H	-B1-	21	66.0
10	NF061	$C_{54}H_{38}N_6O_{24}S_6Na_6$	1485.2	$-CH_3$	-H	-H	-CO-A01	-H	-B2-	21	15.0
11	NF066	$C_{54}H_{36}N_6O_{24}S_6Na_6$	1483.2	$-CH_3$	-H	-H	-CO-A01	-H	-B3-	20	37.0
12	NF299	$C_{56}H_{42}N_6O_{24}S_6Na_6$	1541.3	$-CH_3$	-H	-H	-CO-A01	-H	-B4-	23	35.0
13	NF064	$C_{56}H_{38}N_6O_{24}S_6Na_6$	1535.3	$-CH_3$	-H	-H	-CO-A01	-H	-B5-	22	76.0
14	NF059	$C_{56}H_{40}N_6O_{24}S_6Na_6$	1533.3	$-CH_3$	-H	-H	-CO-A01	-H	-B6-	24	83.0
Benzenesulfonic acid derivatives											
15	NF062	$C_{43}H_{34}N_6O_{11}S_2Na_2$	920.9	$-CH_3$	-H	-H	-CO-A11	-H	-B1-	23	7.0
16	NF250	$C_{29}H_{24}N_4O_9S_2Na_2$	682.6	-H	$-SO_3Na$	-H	-H	$-CH_3$	-B1-	20	39.0
17	NF251	$C_{29}H_{22}N_4O_{15}S_4Na_4$	886.7	$-SO_3Na$	-H	-H	$-SO_3Na$	$-CH_3$	-B1-	22	17.0
Carboxylic acid derivatives											
18	NF041	$C_{37}H_{44}N_6O_7$	684.8	-H	-H	$-CO_2Na$	-H	-H	-B1-	22	16.0
19	NF052	$C_{33}H_{28}N_4O_7Na_2$	638.6	$-CH_3$	-H	-H	$-CO_2Na$	$-CH_3$	-B1-	20	-0-
20	NF054	$C_{29}H_{20}N_4O_7Na_2$	582.5	-H	$-CO_2Na$	-H	-H	-H	-B1-	21	-0-
21	NF055	$C_{31}H_{24}N_4O_7Na_2$	610.5	$-CH_3$	-H	-H	$-CO_2Na$	-H	-B1-	23	-0-
22	NF077	$C_{41}H_{36}N_6O_{13}Na_4$	912.7	$-CH_3$	-H	-H	-CO-A15	-H	-B1-	23	-0-
23	NF092	$C_{36}H_{32}N_6O_{13}Na_4$	884.7	$-CH_3$	-H	-H	-CO-A16	-H	-B1-	20	-0-
24	NF186	$C_{35}H_{28}N_6O_{15}S_2Na_4$	928.7	-H	$-SO_2$-A14	-H	-H	-H	-B1-	21	-0-
25	NF191	$C_{35}H_{28}N_6O_{15}S_2Na_4$	928.7	-H	-H	$-SO_2$-A14	-H	-H	-B1-	24	-0-

Fig. 1 Suramin analogues containing large urea groups. The code number, chemical structures, molecular weight, number of eggs tested and the percent inhibition of angiogenesis in the CAM assay on treatment with approximately 70 nmol/disk of the analogues are shown. The structure of acidic groups -R and central bridges -X- are shown in Fig. 4. The percent inhibition of angiogenesis was determined after implanting approximately 70 nmol of each suramin analogue in 10 µl of 0.45% methylcellulose. Implants were made in day-6 CAM and read 48 h later

Fig. 2 Suramin analogues containing small urea groups. The code number, chemical structures, molecular weight, number of eggs tested and the percent inhibition of angiogenesis in the CAM assay on treatment with approximately 70 nmol/disk of the analogues are shown. The structure of acidic groups -R and central bridges -X- are shown in Fig. 4. The percent inhibition of angiogenesis was determined after implanting approximately 70 nmol of each suramin analogue in 10 µl of 0.45% methylcellulose. Implants were made in day-6 CAM and read 48 h later



No	Code	Formula	MW	Substitution at Position -2	-3	-4	-5	-X-	# Embryos	% Inhibition
Naphthalenetrisulfonic acid derivatives										
26	NF023	$C_{35}H_{20}N_4O_{21}S_6Na_6$	1162.9	-H	-CO-A01	-H	-H	-B1-	25	36.0
27	NF103	$C_{37}H_{24}N_4O_{21}S_6Na_6$	1190.9	$-CH_3$	-CO-A01	-H	-H	-B1-	20	10.0
28	NF150	$C_{41}H_{32}N_4O_{21}S_6Na_6$	1247.0	$-CH(CH_3)_2$	-H	-H	-CO-A01	-B1-	22	44.0
29	NF170	$C_{37}H_{24}N_4O_{21}S_6Na_6$	1190.9	$-CH_3$	-H	-CO-A01	-H	-B1-	23	29.0
30	NF192	$C_{47}H_{28}N_4O_{21}S_6Na_6$	1315.1	-H	$-C_6H_4$-4-CO-A01	-H	-H	-B1-	21	47.0
31	NF201	$C_{47}H_{28}N_4O_{21}S_6Na_6$	1315.1	$-C_6H_4$-3-CO-A01	-H	-H	-H	-B1-	26	62.0
32	NF013	$C_{35}H_{20}N_4O_{21}S_6Na_6$	1162.9	-H	-H	-CO-A02	-H	-B1-	23	19.0
33	NF248	$C_{37}H_{24}N_4O_{21}S_6Na_6$	1190.9	$-CH_3$	-H	-H	-CO-A02	-B1-	24	11.0
34	NF249	$C_{37}H_{24}N_4O_{21}S_6Na_6$	1190.9	$-CH_3$	-H	-H	-CO-A03	-B1-	21	12.0
35	NF252	$C_{37}H_{24}N_4O_{21}S_6Na_6$	1190.9	$-CH_3$	-H	-H	-CO-A04	-B1-	22	22.0
Naphthalenedisulfonic acid derivatives										
36	NF289	$C_{35}H_{22}N_4O_{15}S_4Na_4$	958.8	-H	-H	-CO-A05	-H	-B1-	20	50.0
37	NF290	$C_{37}H_{26}N_4O_{15}S_4Na_4$	986.8	$-CH_3$	-H	-H	-CO-A05	-B1-	22	11.0
38	NF298	$C_{37}H_{26}N_4O_{15}S_4Na_4$	986.8	$-CH_3$	-H	-H	-CO-A07	-B1-	20	-0-
39	NF326	$C_{35}H_{22}N_4O_{17}S_4Na_4$	990.8	-H	-CO-A09	-H	-H	-B1-	22	19.0
40	NF340	$C_{37}H_{26}N_4O_{15}S_4Na_4$	986.8	$-CH_3$	-H	-H	-CO-A06	-B1-	23	41.0
41	NF291	$C_{35}H_{22}N_4O_{15}S_4Na_4$	958.8	-H	-H	-CO-A08	-H	-B1-	23	30.0
42	NF294	$C_{37}H_{26}N_4O_{15}S_4Na_4$	986.8	$-CH_3$	-H	-H	-CO-A08	-B1-	21	20.0
43	NF338	$C_{42}H_{26}N_4O_{16}S_4Na_4$	1062.9	-H	-H	-CO-A06	-H	-B5-	20	13.0
44	NF339	$C_{42}H_{26}N_4O_{16}S_4Na_4$	1062.9	-H	-H	-CO-A06	-H	-B6-	21	41.0
45	NF341	$C_{44}H_{30}N_4O_{16}S_4Na_4$	1090.9	$-CH_3$	-H	-H	-CO-A06	-B5-	22	28.0
46	NF342	$C_{44}H_{30}N_4O_{16}S_4Na_4$	1090.9	$-CH_3$	-H	-H	-CO-A06	-B6-	23	30.0
47	NF383	$C_{44}H_{30}N_4O_{16}S_4Na_4$	1090.9	$-CH_3$	-H	-H	-CO-A07	-B6-	20	58.0
48	NF293	$C_{42}H_{26}N_4O_{16}S_4Na_4$	1062.9	-H	-H	-CO-A08	-H	-B6-	24	75.0
49	NF324	$C_{44}H_{30}N_4O_{16}S_4Na_4$	1090.9	$-CH_3$	-H	-H	-CO-A08	-B6-	23	58.0
Benzenesulfonic acid derivatives										
50	NF110	$C_{41}H_{28}N_6O_{17}S_4Na_4$	1096.9	-H	-CO-A11	-H	-CO-A11	-B1-	32	65.0
51	NF442	$C_{45}H_{36}N_6O_{17}S_4Na_4$	1153.0	-CO-A12	-H	-H	-CO-A12	-B1-	20	37.0
52	NF109	$C_{20}H_{15}N_3O_8S_2Na_2$	535.4	-H	-H	$-SO_3Na$	-H	-B7-	23	-0-
53	NF241	$C_{18}H_{18}N_4O_8S_2Na_2$	528.5	-H	-H	$-SO_3Na$	-H	-B4-	24	26.0
54	NF440	$C_{52}H_{40}N_6O_{18}S_4Na_4$	1257.1	-H	-CO-A12	-H	-CO-A12	-B6-	23	62.0
55	NF443	$C_{52}H_{40}N_6O_{18}S_4Na_4$	1257.1	-CO-A12	-H	-H	-CO-A12	-B6-	20	20
Carboxylic acid derivatives										
56	NF042	$C_{23}H_{18}N_4O_{11}Na_4$	618.4	-H	-CO-A14	-H	-H	-B1-	23	-0-
57	NF072	$C_{23}H_{18}N_4O_{11}Na_4$	618.4	-H	-H	-CO-A14	-H	-B1-	24	-0-
58	NF076	$C_{27}H_{26}N_4O_{11}Na_4$	674.5	$-CH_3$	-H	-H	-CO-A15	-B1-	22	-0-
59	NF091	$C_{25}H_{22}N_4O_{11}Na_4$	646.4	$-CH_3$	-H	-H	-CO-A16	-B1-	21	-0-
60	NF178	$C_{25}H_{22}N_4O_{11}Na_4$	646.4	$-CH_3$	-H	-H	-CO-A14	-B1-	22	4.0
61	NF230	$C_{23}H_{10}F_2N_4O_{11}Na_4$	654.3	-F	-H	-H	-CO-A14	-B1-	20	23.0

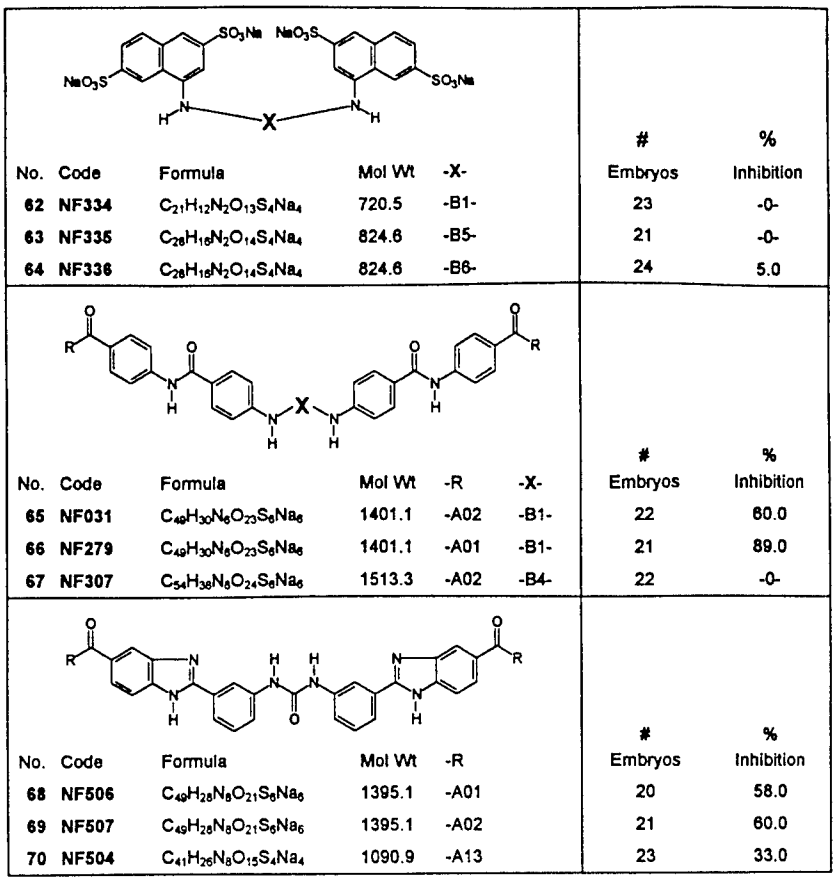
negative and positive controls, respectively. The range of dose levels was chosen to fit a response curve for each suramin analogue that showed inhibition the same or more than suramin at 70 nmol/disk.

Standard statistical software (Procedure Probit on the SAS system) allows fitting parametric families of dose response curves with the normal probit model. The detailed application of the method conforms with that given by Finney [6]. The ID_{50}, the dose that produced 50% inhibition, was calculated by separate response curves to the logarithm of dose. A subsequent plot of the data suggested a parallel lines assay model could be used for comparing the dose response curves. In a parallel lines assay model, the slope of the regression on the log of the dose was assumed to be the same for suramin and its analogues. The response curves differed most in their intercepts. The chi-square goodness of fit test for this model was not significant ($P = 0.30$), indicating that this model is a reasonable fit for the combined data. A statistical comparison of intercepts in this model is equivalent to a comparison of percentiles, such as the ID_{50}, since two response curves have different ID_{50} values only if they have different intercepts under the parallel lines assay model.

In vitro human microvascular endothelial cells

This cell line (a generous gift of Dr. Thomas J. Lawley, Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia) was transfected and immortalized by simian virus 40 larger T antigen and used for in vitro studies of the effect of suramin and selected analogues on bFGF-stimulated growth of these endothelial cells. These cells retained the characteristics of endothelial cells. The culture medium was endothelial basal medium (Clonetics, Santa Anna, Calif.) with 15% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 1 m*M* glutamine, 0.5 m*M* dibutyl cyclic AMP (Sigma Chemical Co., St Louis, Mo.), 1 µg/ml hydrocortisone acetate (Sigma Chemical Co.), 1 ng/ml epidermal growth factor (Clonetics), 100 U/ml penicillin, 100 U/ml streptomycin and 250 µg/ml amphotericin B (Sigma Chemical Co.).

Subconfluent human microvascular endothelial cells were grown in the presence of 20 ng/ml of bFGF with various amounts of each analogue for 72 h at 37 °C in 5% CO_2/air. Stock solutions of the analogues in water were prepared and various aliquots were added to the culture to establish an inhibition curve and to determine the IC_{50}, the concentration of the analogue in the cell culture



No.	Code	Formula	Mol Wt	-X-	# Embryos	% Inhibition
62	NF334	$C_{21}H_{12}N_2O_{13}S_4Na_4$	720.5	-B1-	23	-0-
63	NF335	$C_{26}H_{16}N_2O_{14}S_4Na_4$	824.6	-B5-	21	-0-
64	NF336	$C_{26}H_{16}N_2O_{14}S_4Na_4$	824.6	-B6-	24	5.0

No.	Code	Formula	Mol Wt	-R	-X-	# Embryos	% Inhibition
65	NF031	$C_{49}H_{30}N_6O_{23}S_6Na_6$	1401.1	-A02	-B1-	22	60.0
66	NF279	$C_{49}H_{30}N_6O_{23}S_6Na_6$	1401.1	-A01	-B1-	21	89.0
67	NF307	$C_{54}H_{38}N_6O_{24}S_6Na_6$	1513.3	-A02	-B4-	22	-0-

No.	Code	Formula	Mol Wt	-R	# Embryos	% Inhibition
68	NF506	$C_{49}H_{28}N_8O_{21}S_6Na_6$	1395.1	-A01	20	58.0
69	NF507	$C_{49}H_{28}N_8O_{21}S_6Na_6$	1395.1	-A02	21	60.0
70	NF504	$C_{41}H_{26}N_8O_{15}S_4Na_4$	1090.9	-A13	23	33.0

Fig. 3 Miscellaneous suramin analogues with various bridge structures. The code number, chemical structures, molecular weight, number of eggs tested and the percent inhibition of angiogenesis in the CAM assay on treatment with approximately 70 nmol/disk of the analogues are shown. The structure of acidic groups -R and central bridges -X- are shown in Fig. 4. The percent inhibition of angiogenesis was determined after implanting approximately 70 nmol of each suramin analogue in 10 µl of 0.45% methylcellulose. Implants were made in day-6 CAM and read 48 h later

that produced 50% inhibition. The IC_{50} was calculated by the statistical methods described above. Protein levels were determined by a modification of the Lowry procedure in which sodium dodecyl sulfate (SDS) was added to dissolve the proteolipids [14].

Results

The antiangiogenic activities of the 70 suramin analogues were determined in the CAM assay in the presence of 70 nmol of the suramin analogues. The chemical structure, molecular weight and percent inhibition of angiogenesis on treatment with 70 nmol of each suramin analogue tested are shown in Figs. 1–4. Statistical analysis of the results indicated that an inhibition of 50% or higher in the CAM assay with 70 nmol of the analogue had an antiangiogenic activity equal to or greater than suramin. Of the 70 analogues tested, 21 showed an inhibition of 50% or greater in the CAM assay in the presence of 70 nmol of the analogues. The chemical group with the highest activity was the naphthalenesulfonic acid analogues group (6 sulfonic acid groups) with large urea groups (Fig. 1) where 10 of 14 analogues showed an inhibition of 50% or higher at 70 nmol in the CAM assay. Only 1 of 10 naphthalenesulfonic acid analogues with a small urea group (Fig. 2) showed an inhibition ≥ 50% at 70 nmol/disk. Four of 14 naphthalenedisulfonic acid analogues and 3 of 7 benzene sulfonic acid analogues showed ≥ 50% inhibition at 70 nmol/disk (Fig. 2). This includes analogue no. 70 (NF504), which has a different structure (Fig. 3). None of the 14 carboxylic acid analogues (Figs. 1 and 2) showed any significant inhibition in the CAM assay at 70 nmol of the analogue.

Dose response curves for the inhibition of angiogenesis were established for the analogues in the CAM assay for suramin and 19 of the 21 analogues which showed antiangiogenic activity the same or more than suramin at a dose of 70 nmol/disk (see Figs. 1–4). Inadequate amounts of analogues no. 5 (NF157) and no. 9 (NF032) were available for this experiment. The ID_{50} was calculated from the dose response curves (see Table 1). The ID_{50} value of suramin was 75 nmol/disk. All except no. 47 (NF383) of the analogues shown in Table 1 had ID_{50} values similar to or less than suramin, indicating that their ability to inhibit angiogenesis was the same or more than that of suramin.

Statistical analysis of the dose response curves and the calculated ID_{50} values for suramin and the 19 analogues indicated that analogues with ID_{50} values in the range of 56–94 nmol/disk showed antiangiogenic activity equivalent to suramin (75 nmol/disk). An ID_{50} of less than 55 nmol/disk indicated that the analogue was significantly more potent than suramin as an inhibitor of angiogenesis in the CAM assay ($P \geq 0.05$). Of the 19 analogues, 7 had ID_{50} values ≤ 55 nmol/disk (range

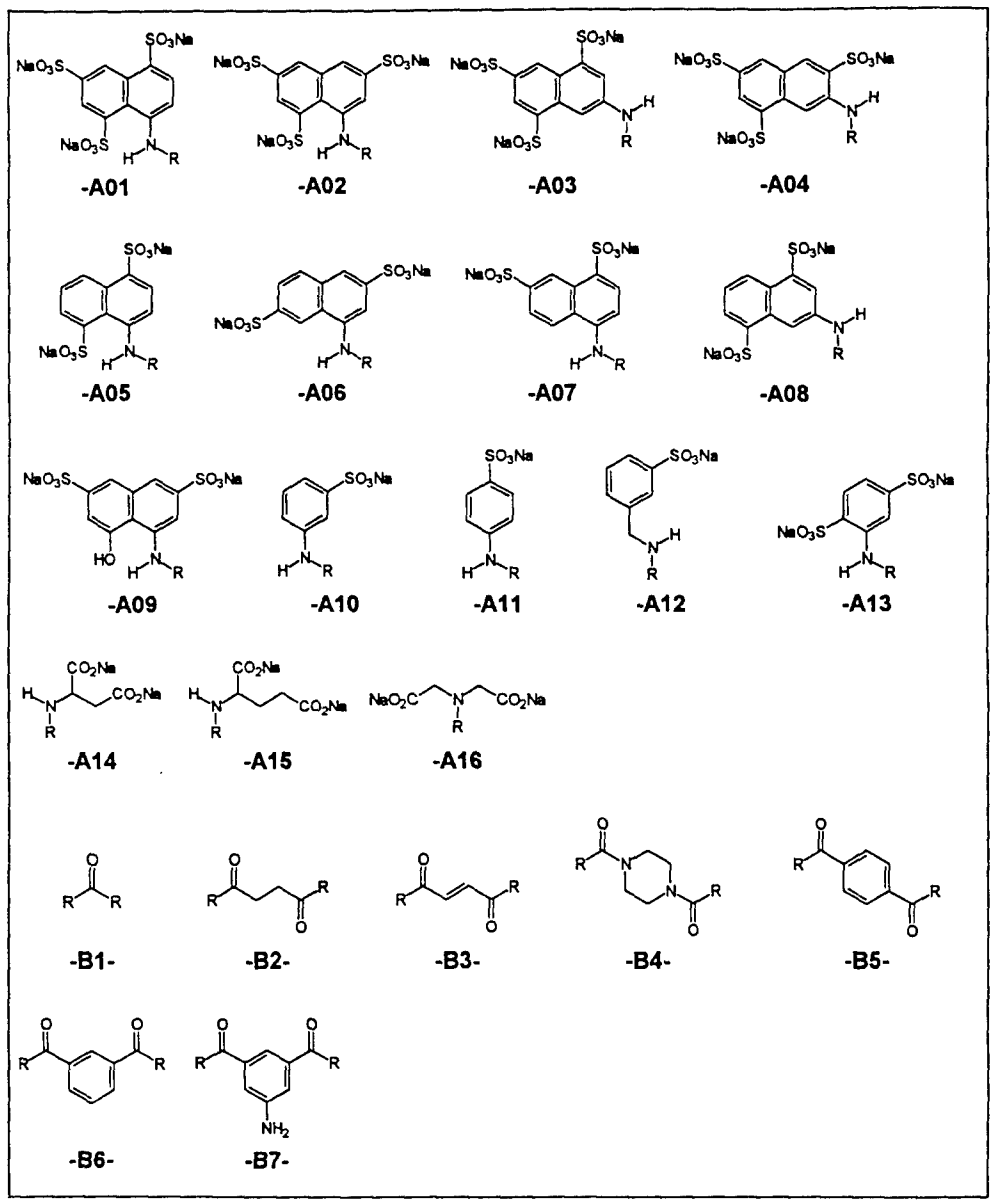


Fig. 4 The structure of acidic groups -R and central bridges -X- in Figs. 1–3

35–55), indicating antiangiogenic activity significantly greater than that of suramin. All seven analogues were naphthalenetrisulfonic acid derivatives of the large urea type (nos. 2, 3, 4, 6, 13, 14, 66; NF127, NF151, NF145, NF171, NF064, NF059, NF279, respectively). All the other analogues shown in Table 1 except no. 47 (NF383) with an ID_{50} of 137 nmol/disk had antiangiogenic activities similar to that of suramin.

Seven analogues and suramin were tested for their ability to inhibit the growth of human microvascular endothelial cells in the presence of 20 ng/ml bFGF. The IC_{50} values for the subconfluent microvascular endothelial cells in the presence of bFGF treated with the analogues are shown in Table 2. Four of the seven aminonaphthalenetrisulfonic acid analogues that showed greater potency than suramin in the CAM assay were tested. Two analogues, no. 4 (NF145) and no. 6 (NF171), were better inhibitors of endothelial growth than suramin as they were in the CAM assay. Another analogue, no. 14 (NF059), which was an excellent inhibitor of angiogenesis in the CAM assay, was not as effective as suramin as an inhibitor of endothelial cell growth. Analogue no. 65 (NF031) was similar to suramin as an inhibitor of both angiogenesis in the CAM assay and endothelial cell growth. The benzene sulfonic acid analogue tested, no. 50 (NF110), was a better inhibitor of human microvascular endothelial cell growth in vitro than suramin (ID_{50} of 280 vs 438 nmol/ml for suramin). A similar pattern was seen for no. 48 (NF293), the only disulfonic acid analogue tested.

Table 1 The ID_{50} of angiogenesis in the CAM assay for suramin and a series of analogues (ID_{50} dose in nmol/disk that induced 50% inhibition of angiogenesis)

Suramin analogue[a]		ID_{50} (nmol/disk)
No.	Code	
Naphthalenetrisulfonic acid derivatives		
1	NF060 (suramin)	75
2	NF127	54
3	NF151	45
4	NF145	36
6	NF171	35
8	NF280	68
13	NF064	55
14	NF059	40
31	NF201	71
65	NF031	75
66	NF279	55
68	NF506	70
69	NF507	65
Naphthalenodisulfonic acid derivatives		
36	NF289	72
47	NF383	137
48	NF293	60
49	NF324	92
Benzenesulfonic acid derivatives		
50	NF110	70
54	NF440	70
70	NF504	60

[a] The corresponding chemical structures are shown in Figs. 1–4

Table 2 ID_{50} for inhibition of cell growth in basic FGF-stimulated human microvascular endothelial cells by suramin and a series of analogues (ID_{50} dose in nmol/disk that induced 50% inhibition of angiogenesis)

Suramin analogue[a]		IC_{50} (nmol/ml)
No.	Code	
Naphthalenetrisulfonic acid derivatives		
1	NF060 (suramin)	438
4	NF145	143
6	NF171	170
14	NF059	800
26	NF023	750
65	NF031	440
Naphthalenedisulfonic acid derivatives		
48	NF293	150
Benzenesulfonic acid derivatives		
50	NF110	280

[a] The corresponding chemical structures are shown in Figs. 1–4

Discussion

The chemical structure of suramin was systematically varied in the suramin analogues used in this study. The naphthalenetrisulfonic acid residues -A01 were replaced by the acidic groups -A02 to -A16 shown in Fig. 4. The central urea bridge -B1- of suramin was replaced by the dicarboxylic acid diamide bridges -B2- to -B7- shown in Fig. 4. The size of the molecules was varied by modifying the number of benzoyl residues from zero (Fig. 3, nos. 62–64) via two (small urea type, Fig. 2) to four (large urea type, Figs. 1 and 3). Further, the rigidity of the molecules was modified. Thus, two aminobenzoyl residues of suramin were replaced by the 2-phenylbenzimidazole residue (Fig. 3, nos. 68–70). These suramin analogues have a similar size to suramin but a reduced flexibility.

The following molecular features seemed to be important for high antiangiogenic activity. A structure with two agglomerations of highly acidic groups in a certain distance was essential. Asymmetric molecules with only one highly acidic group (e.g. only one naphthalenetrisulfonic acid residue -A1) were inactive (data not shown in this report). The number of anionic groups was also important. The most active compounds were found among the naphthalenetrisulfonic acid derivatives. Naphthalenedisulfonic acid derivatives were, in general, less active. Among the benzene sulfonic acid derivatives, only those with four sulfonic acid residues (Figs. 2 and 3; nos. 50, 54 and 70; NF110, NF440 and NF504, respectively) showed significant antiangiogenic activity. None of the 13 carboxylic acid derivatives having two or four carboxylate residues (Fig.1, 18–25; Fig. 2, nos. 56–61) had significant antiangiogenic activity. The distance between the acidic groups was also important. Analogues with small bridges without benzoyl groups between the naphthalenesulfonic acid residues had no antiangiogenic activity (Fig. 3, nos. 62–64) and analogues of the small urea type with only two benzoyl residues (Fig. 2) were, in general, less active than those of the large urea type with four benzoyl residues (Fig. 1). It seems that steric factors and the rigidity of the molecule also had an important influence on the antiangiogenic activity. In the suramin analogues 10–14 (Fig. 1), the central urea bridge of suramin is replaced by dicarboxylic acid diamides. In the case of no. 13 (NF064) and no. 14 (NF059), the bridges are diamides of terephthalic (-B5-) and isophthalic acid (-B6-), respectively. The resulting analogues are, like suramin (no. 1, NF060), rigid and flat molecules. Both showed an inhibitory activity (76% and 87%, respectively) superior to that of suramin. Analogue no. 10 (NF061) had a much lower inhibitory activity (15%) than suramin. The central bridge of no. 10 is formed by succinic acid diamide. This is a very flexible bridge which allows many conformations of the molecule. Analogue no. 12 (NF299) has a dicarbamic acid diamide (-B4-) as the central bridge. This bridge contains two urea groups. The two-dimensional formula for this bridge -B4- shown in Table 2 seems to be very similar to the terephthalic diamide bridge -B5-, but the three-dimensional structure of -B4- differs greatly from that of -B5-. Two preferred conformations of analogue no. 12 can be expected. In neither of these conformations can the aromatic ring systems of the molecules be arranged in the same plane.

In the series of naphthalenedisulfonic acids, only one analogue, no. 48 (NF324), showed an inhibitory activity superior to that of suramin (75% versus 64%).

Interestingly, this compound is a 2-aminonaphthalenedisulfonic acid derivative, whereas the 2-aminonaphthalenetrisulfonic acid derivatives, no. 34 (NF249) and no. 35 (NF252), had very low activity (12% and 14%, respectively).

When the ID_{50} values were calculated from the 21 analogues shown to inhibit angiogenesis in the CAM assay by 50% or more, 17 were found to have antiangiogenic activity the same or greater than suramin, as indicated by ID_{50} values $\leq$ 75 nmol/disk (see Table 1). The ID_{50} value of 92 nmol/disk for no. 49 (NF324) was not significantly different from suramin, whereas the ID_{50} of 137 nmol/disk for no. 47 (NF383) indicates that no. 47 was significantly less antiangiogenic than suramin. The ID_{50} values were not calculated for no. 5 (NF157) and no. 9 (NF032) because inadequate amounts were available. The distribution of the most active suramin analogues was not uniform in different groups, showing that there was a clear relationship between the chemical structure and the inhibition of angiogenesis. When the partial structure of suramin was altered, 7 of the 18 analogues showed ID_{50} values that were significantly lower ($\leq$55 nmol/disk), indicating that their antiangiogenic activity was significantly greater than that of suramin. All of these analogues were from the naphthalenetrisulfonic acid group and contained large urea groups. In general, the naphthalenedisulfonic acid and benzene sulfonic analogues were less active than the naphthalenetrisulfonic acid analogues. Replacement of the trisulfonic acid groups by carboxylic acids resulted in analogues with very low antiangiogenic activity in the CAM assay.

Our results show that the suramin analogues were effective inhibitors of bFGF-stimulated growth of human microvascular endothelial cells. The polyanionic structure of the analogues seems to be an important factor for the interaction with various growth factors, such as bFGF, VEGF, epidermal growth factor, PDGFβ and IGF-1 [13, 15, 18, 19, 21]. These results are supported by results from other polyanions such as polysulfonated distamycin-A derivatives, which inhibit PDGFβ [4], and pentosan polysulfate, which inhibits bFGF-stimulated growth of SW13 cells [24] and blocks tumor growth in nude mice [25].

In studying the naphthalenetrisulfonic acids, great differences in antiangiogenic activity were noted, suggesting that other structural properties also influence this activity. Firsching et al. [7] indicated a similar pattern for antiproliferative activity with nine similar analogues synthesized by Dr. Peter Nickel. Structural modifications of suramin have been reported to markedly influence trypanosomal and antifilarial activity. Alterations of methyl groups leads to a significant decrease in trypanosomal activity [16]. The antifilarial activity of suramin analogues is also sensitive to structural changes and symmetry is essential for antifilarial activity [16]. For HIV-1 reverse transcriptase activity, no clear relationship between chemical structure and inhibitory activity has been demonstrated [11]. Two of the most active inhibitors of HIV-1 reverse transcriptase, analogues no. 65 (NF031) and no. 32 (NF013), have no trypanocidal or antifilarial activity. Firsching et al. [7] found that no. 65 (NF031) was the most potent antiproliferative compound against five tumor cell lines and had antiangiogenic activity similar to suramin in the CAM assay. We found that no. 65 (NF031) was similar to suramin with regard to antiangiogenic activity in the CAM assay and the inhibition of bFGF-induced growth of human microvascular endothelial cells in vitro. However, we found seven analogues that showed significantly greater antiangiogenic activity than suramin and no. 65 (NF031). Analogue no. 32 (NF031) did not significantly inhibit angiogenesis in our CAM assay.

Our results, in general, agree with the findings of Braddock et al. [3], who studied the effects of ten different suramin analogues on endothelial cells in vitro and in the CAM assay using analogues different from those used in our study. In conclusion, we identified seven analogues which have significantly greater antiangiogenic activity than suramin in the CAM assay. These analogues also appear to have significantly greater inhibitory activity against bFGF-stimulated growth in human microvascular endothelial cells in vitro. These results suggest that these more potent analogues may be developed into therapeutic agents for angiogenesis-dependent and proliferative diseases.

Acknowledgements This work was supported by the Department of Veterans Affairs, the American Cancer Society (Award DHP 117A) and CAPES (Brazil) – PROC:901/90-3. The authors gratefully acknowledge the contribution of Ursula Ardanuy, Barbara Boing, Christiane Gurgel, Hans-Joachim Haack, Erich Schott, Hanady Widjaja and Margit Stabler to the synthesis of the 70 suramin analogues, the excellent technical assistance of Robin King and the editorial assistance of Patricia Collins.

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ELSEVIER

Cancer Letters 125 (1998) 97–102

CANCER LETTERS

Uptake of suramin by human microvascular endothelial cells

Antonio R.T. Gagliardi[a], Matthew F. Taylor[b], Delwood C. Collins[a,*]

[a]*Veterans Affairs Medical Center and the Department of Obstetrics and Gynecology, University of Kentucky Medical Center, Lexington, KY, USA*

[b]*School of Biological Sciences, University of Manchester, Manchester, UK*

Received 24 August 1997; received in revised form 12 November 1997; accepted 12 November 1997

Abstract

We have demonstrated for the first time that suramin is taken up by human dermal microvascular endothelial (HMEC-1) cells by an active process involving the caveolae system. The uptake of suramin was time-dependent and reduced by more than 90% when incubated in the presence of albumin or at 4°C. Suramin uptake was also inhibited when incubated in the presence of filipin and digitonin, both potent cholesterol-binding agents, but not in the presence of probenecid. The [^{3}H]suramin taken up by the HMEC-1 cells was located primarily within the nucleus, followed by the cytoplasmic fraction. The presence of suramin in these cellular compartments suggests that this drug may act through intracellular mechanisms. Published by Elsevier Science Ireland Ltd.

Keywords: Suramin; Caveolae; Human microvascular endothelial cells

1. Introduction

Suramin, a symmetrical polysulfonated naphthylamine derivative of urea, was found to be very valuable for the treatment of human trypanosomiasis and onchocerciasis in Africa [1]. Recent studies have concentrated on this compound as a potential therapy for advanced human malignancies [2–4]. We have previously reported that suramin inhibits angiogenesis in the 6-day chick chorioallantoic membrane assay in a dose-dependent manner [5]. In addition, we found that suramin and some of its analogues directly inhibit human microvascular endothelial cell growth in vitro [6]. Suramin blocks the interaction of many growth factors with their receptors on the cell surface [7] but it has also been shown to express different intracellular actions [8]. Powis et al. [9] found no direct relationship between the inhibition of growth factor binding or calcium signaling and cell growth inhibition by suramin and six related azo dyes. These results suggested that although inhibition of growth factor binding may contribute to the inhibition of cell growth, other actions appear to be important.

Hawking [10] showed that a significant amount of suramin was absorbed by living but not dead trypanosomas. Fairlamb and Bowman [11] reported that 14% of the administered dose was found in rat liver 2 days after intravenous injection of suramin (250 mg/kg). They also found that suramin was bound in the lyso-

* Corresponding author. 204 HSRB, UK Medical Center, Lexington, KY 40536-0305, USA. Tel.: +1 606 3235293; fax: +1 606 2579700.

0304-3835/98/$19.00 Published by Elsevier Science Ireland Ltd.
PII S0304-3835(97)00496-5

somes and the activities of the enzymes, β-glycerophosphatase and β-*N*-acetylglucosaminidase, were significantly decreased. Constantopoulos et al. [12] showed that suramin caused a marked accumulation of two glycosaminoglycans in rat liver and excessive excretion in the urine (heparan sulfate and dermatan sulfate). Three enzymes required for degradation of the glycosaminoglycans were reduced in the liver of rats treated with suramin. Similar effects were observed on arylsulfatase and heparin sulfatase activities after intracerebral injection of suramin into the rat brain.

Baghdiguian et al. [13] showed morphological evidence for the uptake of [^{3}H]suramin for the first time using polarized colon adenocarcinoma (HT29-D4) cells. The autoradiographic data showed that suramin was mainly concentrated in the apical cytoplasm (lysosomes) of HT29-D4 cells, followed by the nucleus. In later studies [14], the intracellular localization of suramin in these cells was significantly altered when albumin was added to the culture medium. In the absence of serum albumin, the radioactive suramin was widely distributed in the nucleus, the Golgi apparatus and the mitochondria, while it was restricted to the lysosomal system in the presence of albumin.

In this study, we have investigated the uptake of [^{3}H]suramin by human microvascular endothelial (HMEC-1) cells in culture. The effects of temperature and albumin concentration in the medium on the uptake of [^{3}H]suramin by HMEC-1 cells were also determined. We have also provided evidence that the caveolae system plays an essential role in the uptake of [^{3}H]suramin by HMEC-1 cells.

2. Materials and methods

2.1. [^{3}H]Suramin uptake by microvascular endothelial cells

[^{3}H]Suramin sodium (specific activity 47 Ci/mM, 15.2 μg/ml) was obtained from Moravek Biochemicals (Brea, CA). Human microvascular endothelial cells, a gift from Dr T. Lawley (Emory University School of Medicine, Atlanta, GA), were grown in MCDB 131 + 10% fetal bovine serum (FBS) in T225 cm^2 tissue culture flasks as previously described [15] and allowed to grow to confluency. Confluent cultures were washed twice with Hank's solution [16]. Then, 30 ml of MCDB 131 medium without FBS containing 5.0 μCi of [^{3}H]suramin and 20 μg of cold suramin were added to each culture. Triplicate cultures were incubated at 4 or 37°C in 5% CO_2/air for different periods of time (2–72 h). After incubation, each cell culture was washed twice with Hank's solution at 4°C, followed by Hank's solution containing 100 μg/ml of cold suramin at 4°C. The endothelial cells were trypsinized and resuspended (twice) with cold MCDB 131 with 15% FBS, centrifuged at 800 × *g* for 25 min at 4°C and resuspended in cold DNA buffer (0.05 M sodium phosphate dibasic, 2.0 M sodium chloride and 2.0 mM EDTA). The cells were then centrifuged at 800 × *g* for 25 min at 4°C and the supernatant discarded. The pellet was resuspended in cold DNA buffer, sonicated for 2 min on ice and the suspension was centrifuged at 800 × *g* at 4°C for 10 min. Aliquots of the supernatant were taken for protein analysis. The supernatant was centrifuged at 800 × *g* for 45 min and the supernatant and the pellets were transferred to separate scintillation vials, solubilized in liquid scintillation cocktail (Ultima Gold XR, Packard, Meriden, CT) and counted in a 2000 CA TRICARB Liquid Scintillation Counter (Packard, Meriden, CT).

The effect of protein in the medium on [^{3}H]suramin uptake was determined by adding 2% of fatty acid-free bovine serum albumin (BSA) (Sigma, St. Louis, MO) to the MCDB 131 medium and repeating the experiments as described above. In some experiments, inhibitors of the caveolae system (filipin and digitonin) and probenecid were added and incubated for 2 h at 37°C in 5% CO_2/air in phosphate buffered saline (pH 7.4) as described by Schnitzer et al. [17] and Hinshaw et al. [18]. [^{3}H]Suramin (0.33 μCi/ml) was added to each flask. Triplicates were carried out for experimental and control samples. Cell viability was tested by Trypan Blue extrusion. After 2 h incubation, the cells were treated as previously described to determine the total uptake of [^{3}H]suramin.

2.2. Effect of protein concentration on the inhibition of microvascular endothelial cell growth by suramin

HMEC-1 cells were grown in Falcon culture dishes

Table 1

The uptake of [^{3}H]suramin (mean DPM ± SD × 10^3/mg protein) by human microvascular endothelial (HMEC-1) cells cultured in MCDB 131 without fetal bovine serum from 0 to 72 h at 37 or 4°C in 5% CO_2/air

Incubation time (h)	Total cells	% of DPM	Total cells	% of total DPM	Supernatant	% of uptake	Nuclear	% of uptake
Temperature (°C)	4		37		37		37	
2	9 ± 1	0.1	122 ± 7	1.0	86 ± 5	71.7	34 ± 3	28.3
4	11 ± 1	0.1	178 ± 8	1.4	109 ± 8	61.2	64 ± 5	39.8
8	38 ± 2	0.3	408 ± 26	3.2	167 ± 12	41.5	235 ± 16	58.5
24	97 ± 3	0.8	1982 ± 123	15.4	731 ± 43	37.8	1204 ± 69	62.2
48	178 ± 8	1.4	2981 ± 172	23.1	981 ± 49	33.4	1958 ± 133	66.6
72	193 ± 9	1.4	2949 ± 105	22.9	997 ± 55	34.0	1934 ± 126	66.0

The cells incubated at 37°C were fractionated as described in Section 2 and the DPM/mg protein was presented for the [^{3}H]suramin uptake by the total cell, supernatant and 800 g precipitate (nuclear fraction). Experiments were carried out in duplicate with triplicates of each sample.

(60 × 15 mm) in MCDB 131 + 10% FBS. Experiments were carried out with subconfluent cell cultures. Cells were washed once with Hank's solution. The medium was changed to experimental MCDB 131 medium containing either 2.5 or 15% FBS and various concentrations (0–560 μM) of suramin. Four parallel cultures were carried out for each suramin concentration. After 72 h, the cells were washed, harvested, sonicated and analyzed for total protein [16] and total DNA [19]. Experiments were carried out in duplicate and the data were analyzed by analysis of variance (ANOVA).

3. Results

3.1. [^{3}H]Suramin uptake

Table 1 shows the uptake of [^{3}H]suramin by human microvascular endothelial cells in culture from 0 to 72 h. Confluent cell cultures were incubated with [^{3}H]suramin in MCDB 131 serum-free medium at 4 or 37°C in 5% CO_2/air. [^{3}H]Suramin uptake into the cells increased from 1% after 2 h of incubation and reached a plateau with 23% of the total radioactivity present by 48 h. The distribution of radioactivity in the cells changed over the incubation period. Over the first 4 h, more than 60% of the [^{3}H]suramin in the cells incubated at 37°C was in the supernatant fraction. This ratio had reversed by 8 h with about 60% of the radioactivity in the nuclear fraction of the cell.

3.2. Effect of albumin in the medium on [^{3}H]suramin uptake

The effect of protein in the incubation medium is shown in Table 2. When 2% albumin was added to the MCDB 131 medium, the [^{3}H]suramin uptake was reduced to less than 10% of that in the medium without albumin, regardless of the incubation time (4–24 h).

3.3. Effect of inhibitors of the caveolae system

The effects of filipin, digitonin and probenecid on [^{3}H]suramin uptake are shown in Table 3. Treatment with 5 μg/ml of filipin, an inhibitor of the caveolae system, showed a significant reduction in [^{3}H]suramin uptake ($P < 0.05$). Digitonin, another cholesterol-binding agent, also showed a significant inhibitory effect ($P < 0.05$) on the [^{3}H]suramin uptake. On the

Table 2

The uptake of [^{3}H]suramin (mean DPM ± SD × 10^3/mg protein) by human microvascular endothelial (HMEC-1) cells in culture from 0 to 24 h at 37°C in 5% CO_2/air

Incubation time (h)	In 2% albumin	% of DPM	Without albumin	% of DPM
4	13 ± 9	0.1	164 ± 68	1.3
8	25 ± 13	0.2	338 ± 16	2.6
12	38 ± 15	0.3	790 ± 28	6.1
24	63 ± 25	0.5	1853 ± 115	14.4

Experiments were carried out in duplicate with triplicates of each sample.

Table 3

The uptake of [^{3}H]suramin (mean DPM ± SD × 10^3/mg protein) in confluent human microvascular endothelial (HMEC-1) cells alone and after 2 h of incubation at 37°C in 5% CO_2/air in the presence of filipin (5 μg/ml), digitonin (5 μg/ml) and probenecid (5 mM/ml)

Treatment	Mean DPM ± SD × 10^3/mg	*P*-value
Control	124 250 ± 7	
Filipin	56 378 ± 5	<0.05
Digitonin	85 599 ± 6	<0.05
Probenecid	115 547 ± 8	NS

NS, not significant.
Experiments were carried out in duplicate with triplicates of each sample. Data were analyzed by analysis of variance (ANOVA).

other hand, probenecid did not show a significant inhibition on [^{3}H]suramin uptake.

3.4. Effect of protein concentration on cell growth

Table 4 shows the results of incubating endothelial cells with increasing amounts of suramin for 72 h in the presence of 2.5 or 15% FBS in the medium. There was no detectable effect of suramin on total DNA and total protein in cells incubated in 15% FBS, even at the highest concentration of suramin (560 μM). Reducing the serum concentration to 2.5% in the experimental medium resulted in a significant ($P < 0.05$) inhibition of total DNA and protein at all suramin concentrations.

4. Discussion

Albumin, a major protein component in blood, appears to have three contiguous domains and at least two specific binding sites for drugs [20]. Suramin has a high affinity for albumin and induces profound conformational changes in this protein [21]. Clinical pharmacokinetic studies showed that 99.7% of suramin is bound to plasma proteins, mainly albumin, with a plasma half-life of 44–55 days. Thus, it was not surprising to find that the addition of albumin to the culture medium reduced the [^{3}H]suramin uptake by more than 90% compared to the endothelial cells incubated without albumin. Stein et al. [8] demonstrated that many classes of polyanionic oligodeoxynucleotides (e.g. phosphodiesters and phosphorothioates) similar to suramin and pentosan polysulfate cannot passively diffuse through lipophilic cell membranes but can be internalized into HL60 cells. Cellular internalization, assessed after removal of cell surface oligomer, revealed a concentration- and time-dependent process consistent with pinocytosis. Pinocytosis, as a process of internalization for polyanions, has also been described for heparin [22,23] and dextran sulfate [24].

The role of caveolae or non-coated plasmalemma vesicles in the transport of macromolecules into and across the endothelium has been studied [25]. In endothelial cells, caveolae appear to pinch off and form vesicles that sequester low molecular weight tracer molecules introduced into the blood vessel [26,27]. Interestingly, considering that suramin has a high affinity for nucleotide-binding sites [7], it has recently been shown that caveolae are enriched with nucleotide-binding proteins, suggesting that in certain cells, caveolae may function as storage and release sites for cyclic AMP [27]. Caveolae are more abundant in certain endothelial cells but are also present in many, if not all, cell types.

Cholesterol is an important component of caveolae and appears to be required to maintain the structural integrity of the vesicular complex [28]. Caveolae disappeared in cells depleted of cholesterol and the exposure of cells to sterol-binding agents such as filipin. Filipin, a polyene antibiotic, and digitonin, a cardiac glycoside, are potent cholesterol-binding agents that bind cholesterol and remove it from cell membranes.

Table 4

The effect of the concentration of fetal bovine serum (FBS) in MCDB 131 medium on the inhibition of total protein and total DNA/well by various concentrations of suramin

Suramin concentration (μM)	Total protein ± SD (μg/well)		Total DNA ± SD (μg/well)	
	15% FBS	2.5% FBS	15% FBS	2.5% FBS
0	123 ± 6	86 ± 6	12.6 ± 0.9	8.0 ± 0.4
70	129 ± 9	51 ± 5*	13.3 ± 1.5	4.5 ± 0.4*
140	128 ± 9	42 ± 4*	13.1 ± 0.9	3.9 ± 0.3*
280	121 ± 4	37 ± 3*	12.3 ± 1.0	3.6 ± 0.2*
420	133 ± 7	29 ± 2*	12.8 ± 0.8	2.5 ± 0.3*
560	126 ± 4	17 ± 2*	12.4 ± 0.5	1.5 ± 0.2*

The values given are the mean values ± SD of the average of two experiments with four repeats per sample.
*Significantly different from samples with no suramin ($P < 0.001$).

They have been reported to inhibit caveolae-mediated transport by more than 60% in bovine microvascular endothelial cells [17].

Morphological and functional studies indicate that the clathrin-coated vesicular pathway is not disrupted by treatment of endothelial cells. Furthermore, the selectivity of filipin in the disruption of caveolae provides a useful tool for separating intracellular transport mediated by coated versus non-coated vesicles [17,29]. Bovine pulmonary artery macrovascular endothelial cells possess a probenecid inhibitable organic anion transporter [18]. Probenecid inhibitable anion transporters have been studied in a variety of polarized epithelia and have been best characterized in the renal proximal tubule [30]. Our results (see Table 3) with filipin and probenecid clearly suggest that the inhibition of caveolae by filipin is associated with significant inhibition ($P < 0.05$) in the uptake of [^{3}H]suramin in HMEC-1 cells. Digitonin, another cholesterol-binding agent, also showed a significant inhibitory effect ($P < 0.05$). However, probenecid was not effective in inhibiting [^{3}H]suramin uptake. These results clearly indicate that the caveolae system is important in the uptake of suramin by the human microvascular endothelial cell.

The results presented in Table 1 clearly indicate that the uptake of [^{3}H]suramin by cultured human microvascular endothelial cells is an active process. This is shown by the fact that the uptake of suramin in cultures incubated at 37°C is 10–15 times greater than cultures incubated at 4°C. The radioactivity found in the cells is associated with [^{3}H]suramin because this compound is poorly metabolized with a half-life of 45–60 days [8].

A majority of the [^{3}H]suramin that entered the HMEC-1 cells was found in the cell nucleus after 8 h of incubation at 37°C. These results suggest that suramin may have actions in both the cytoplasm and nucleus of the HMEC-1 cells. Baghdiguian et al. [13] reported similar results in polarized human adenocarcinoma cells where [^{3}H]suramin was accumulated in the apical cytoplasm and the nucleus. Suramin has also been shown to inhibit DNA topoisomerase II in Chinese hamster fibrosarcoma cells [31]. These workers also showed that heparin sulfate, another polysulfonated compound, was found primarily in the nucleus of cultured hepatocytes.

The results reported here suggest for the first time that [^{3}H]suramin is taken up by human microvascular endothelial cells by an active process involving the caveolae system. The localization of [^{3}H]suramin in the nucleus and cytosolic fractions suggests that suramin may inhibit cell growth and angiogenesis via intracellular effects.

Albumin and serum proteins have been shown to bind large amounts of suramin in vivo. This binding is a factor in the long half-life in man. The binding of suramin to heparin-binding growth factors is the likely mechanism for its antitumorigenic effect [7]. Using FBS, we have shown that the serum protein concentration is also an important factor in determining the ability of suramin to inhibit growth of HMEC-1 cells in vitro (Table 4). Suramin significantly reduced growth as indicated by the reduced total DNA and total protein in the presence of 70 μM of suramin in medium containing 2.5% FBS. This inhibition did not occur, even at 560 μM of suramin, in medium containing 15% FBS. Schnitzer and Oh [32] identified three different membrane-associated proteins. Albondin binds native albumin and serves as a carrier for transcytosis of small ligands. Modified albumin is probably bound more avidly to gp 30- and gp 18-binding protein and is directed primarily to endosomes and lysosomes for degradation. Albumin modified by suramin binding is bound more avidly to gp 30- and gp 18-binding proteins and is directed to endosomes and lysosomes. Thus, we expect the serum protein concentration to be a factor on suramin cytostatic effects both in vivo and in vitro.

Acknowledgements

This work was supported by the Department of Veterans Affairs, the American Cancer Society and the University of Kentucky Research Foundation.

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REPRINTED FROM:

17th International Cancer Congress

Rio de Janeiro (Brazil), August 24-28, 1998

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Diphenylureas are potent inhibitors of angiogenesis

A. GAGLIARDI, D. COLLINS and P. NICKEL*

VA Medical Center and University of Kentucky
College of Medicine, Lexington, KY (USA)
** Institute of Pharmaceutical Chemistry, University of Bonn (D)*

SUMMARY

We have synthesized and characterized a unique group of phosphonated diphenylureas that are potent inhibitors of angiogenesis in the chick chorioallantoic membrane (CAM) assay. These compounds were potent inhibitors of cell growth, migration and tube formation in human microvascular endothelial cells (HMEC-1 and HMVEC) in vitro. These diphenylureas did not inhibit basic FGF (bFGF) binding to its receptor, suggesting that the mechanism for angiogenesis inhibition is different from suramin. In vivo studies in mice injected i.p. with the three most active diphenylureas showed no significant clinical or histological evidence of toxicity whereas suramin and its sulfonated analogues showed typical toxicity previously described for suramin. These results suggest that these diphenylureas are potent inhibitors of angiogenesis and may be important therapeutic agents for "angiogenesis-dependent" diseases.

INTRODUCTION

The search for inhibitors of angiogenesis was stimulated by the concept of "antiangiogenic therapy" that an inhibitor of blood vessel growth limits tumor growth. Angiogenic factors increase cell division of vascular endothelial cells, secretion of extracellular matrix metalloproteinases and migration and organization of endothelial cells into vessels. Angiogenesis inhibitors may act on any of the components of the angiogenic cascade.

We have synthesized and characterized a unique group of phosphonated diphenylureas with molecular weights about half that of suramin. In this study, the ability of these compounds to inhibit angiogenesis was determined in the chick chorioallantoic membrane (CAM) assay and in microvascular endothelial cells. In addition, we determined the effect of the diphenylureas on the components of the angiogenesis cascade, such as endothelial cell migration and metalloproteinase activity. The toxicity of these compounds was also determined.

MATERIALS AND METHODS

CAM Assay: The ability of the diphenylureas to inhibit angiogenesis was determined using a modification of the CAM assay (Gagliardi et al., 1992). The 50% inhibitory dose (ID_{50}) was determined from dose response curves.

Cell Lines: HMEC-1, a SV40 large T antigen immortalized human dermal microvascular endothelial cell line (foreskin), was a gift from Dr. J.T. Lawley (Emory University). HMVEC-d, a normal dermal (foreskin) microvascular endothelial cell, was obtained from Clonetics, Santa Ana, CA and used in passages 5-12. Both cells were cultured as described by Ades et al. (1992).

Effect on Growth In Vitro: Endothelial cells were harvested by trypsinization, resuspended in fresh medium and 100 µl (3000-5000 cells/well) of the cell suspension were plated in 96 well flat bottom microtiter plates (Corning, NY) and incubated in a humidified atmosphere with 5% CO_2 at 37° C. Suramin or the diphenylureas (0-140 µM) were added to the cell culture after 24 hr and incubated for 3 days. Cell growth curves were used to calculate the 50% inhibitory concentration (IC_{50}). The MTT assay described by Carmichael et al. (1987) was used to determine growth rate.

Metalloproteinase activity: SDS-polyacrilamide gel electrophoresis (PAGE) using 8% gels containing 0.1% gelatin was used to determine metalloproteinase activity in endothelial cells in vitro as described by Lim et al. (1996).

Endothelial cell migration assay: The effect of the diphenylureas and suramin on endothelial cell migration was determined as described by Burk (1973).

Tube/cord formation in Matrigel: Human microvascular endothelial cells (HMVEC) were grown in P100 tissue culture plates (Falcon) in MCDB-131 with 15% FCS. Cells between passage 3 and 5 were used for experiments. Matrigel (Collaborative Biomedical Products, Bedford, MA) was prepared in serum free MCDB-131 (1:3), plated in 96 well microplates (100 µl/well), and seeded with HMVEC (1 X 10^4 cells) and harvested without trypsin in 200 µl MCDB-131 with 15% FCS. Cells were then treated with suramin or the diphenylureas for 18 hr and photographed for tube analysis.

In vivo toxicity experiments: The toxicity of suramin, sulfonic acid analogues and 3 diphenylureas was determined in Balb-C female mice injected i.p. with equimolar doses (35, 70 and 140 µmol/kg body weight) every other day for 10 days. The mice were observed daily and weighed every 3 days. After 22 days of observation, they were killed, and tissues were subject to histological analysis (heart, lung, liver, kidney, adrenal gland, soleus muscle).

RESULTS

CAM Assay: The 50% inhibitory dose (ID_{50}) for suramin, its sulfonic analogues and the diphenylureas were calculated from dose response curves. The ID_{50} for suramin was 75 nmol/disk. The ID_{50} values for the 8 phosphonated diphenylureas were 2-35 nmol/disk (NF681=2, NF069=9, NF167=20, NF050=20). The ID_{50} dose for NF681 was 35 times lower than suramin. Increasing amounts of heparin to the CAM inhibited the antiangiogenic activity of suramin but this inhibitory effect was not seen in the diphenylureas.

Inhibition of endothelial cell growth: The IC_{50} values for HMEC-1 and HMVEC-d cells by the diphenylureas were 2-75 μM, with NF681 showing the greatest activity. The IC_{50} value for suramin was 438 μM.

Inhibition of endothelial cell metalloproteinase activity: Culture media from both HMEC-1 and HMVEC-d cells showed significant levels of matrix metalloproteinase activity (MMP-2 and MMP-9). The gelatinolytic activity was significantly increased in the culture media when bFGF (10 ng/ml) was added to the culture media. The most active diphenylurea, NF681, showed complete inhibition of metalloproteinase activity at a concentration of 35μM.

Endothelial cell migration assay: NF681 inhibited HMEC-1 cell migration in a dose-dependent manner in the in vitro wound healing model (Burk, 1973). The migration was inhibited 50% when incubated with 25 μM of NF681. Suramin showed no inhibitory effect on HMEC-1 migration at concentrations of 150 μM. At higher suramin concentrations (280 μM), migration was inhibited by 40%.

In vivo toxicity: Balb-C female mice (6 weeks old) were injected i.p. every other day for 10 days at doses of 140, 70 and 35 μmol/kg body weight. Animals injected with suramin showed acute toxicity, weight loss, scruffy hair coat, secretion from the eye and other clinical signs of toxicity whereas the diphenylureas, NF681, NF050, NF167 and NF161, all showed normal weight gain and no significant clinical signs of toxicity. These animals were euthanasized 22 days after the last injection and tissue samples were taken for histological analysis from animals treated with 70 μmol/kg body weight of suramin or the diphenylureas. Suramin-treated animals showed a characteristic toxicity with a high frequency of lesions in the liver, kidney and adrenal. No significant tissue lesions or histological signs of toxicity were detected in the animals treated with the diphenylureas.

DISCUSSION

These results clearly show that the diphenylureas are potent inhibitors of angiogenesis in the 6-day CAM, where the level of bFGF is high and a high rate of angiogenesis is occurring. The ID_{50} was 35-fold lower than suramin and more potent than pentosan polysulfate and TNP-470, a fumagilin analogue (Liekens et al., 1997; Gagliardi et al., 1992; Kusaka et al., 1991). Increasing amounts of heparin decreased the antiangiogenic activity of suramin, suggesting that heparin protected the angiogenic heparin binding growth factors from suramin binding. The presence of heparin did not change the antiangiogenic activity of the diphenylureas, suggesting that they inhibit angiogenesis by a different mechanism.

The diphenylureas inhibited endothelial cell growth in vitro in a dose-related manner. There was a strong positive correlation between the antiangiogenic activity of the diphenylureas in the CAM assay and their inhibitory effects on bFGF-induced endothelial cell growth in culture, suggesting that a direct effect of the diphenylureas on endothelial cell growth might account for a significant part of their antiangiogenic activity. However, the diphenylureas did not inhibit the binding of I^{125} bFGF to endothelial cells as has been described for suramin and other polyanionic compounds (Ciomei et al., 1994), pentosan polysulfate, and the sulfonic acid analogues of suramin (Liekens et al., 1997).

Metalloproteinases have an important role in endothelial cell migration, vascular remodeling and angiogenesis (Mignatti et al., 1989; Schapner et al., 1993; Fisher et al., 1994). HMEC-1 expressed MMP-2 activity, which was significantly enhanced in the presence of bFGF and inhibited by the diphenylureas in a dose-dependent manner. Endothelial cell migration was also inhibited in the presence of the diphenylureas in a dose-dependent manner.

These results suggest that a unique group of diphenylureas are potent inhibitors of many fundamental steps in angiogenesis, such as endothelial cell growth, migration and metalloproteinase activity. These compounds have lower molecular weights, are less bound to albumin and have shorter half life and are much less toxic than suramin. The diphenylureas may be effective therapeutic agents for angiogenesis dependent diseases and cancer.

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REPRINTED FROM:

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Rio de Janeiro (Brazil), August 24-28, 1998

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International Proceedings Division

17th International
CANCER CONGRESS
Rio de Janeiro, Brazil
24-28 August 1998

Inhibition of prostate cancer cell growth by diphenylureas, a unique group of antiangiogenic compounds

A. GAGLIARDI, D. COLLINS, R. MUNN* and P. NICKEL**

VA Medical Center and University of Kentucky College of Medicine, Lexington, KY (USA)
** University of Kentucky, College of Medicine, Lexington, KY (USA)*
** Institute of Pharmaceutical Chemistry, University of Bonn (D)*

SUMMARY

We have synthesized and characterized a unique group of phosphonated diphenylureas that are potent inhibitors of angiogenesis. The three most active phosphonated diphenylureas were equal to or more potent than suramin or its sulfonated analogues as inhibitors of growth by human prostate cancer cell lines (LNCap, PC3, and DU145). They were are also potent inhibitors of the metalloproteinases, gelatinase A (MMP-2) and gelatinase B (MMP-9) in human prostate cancer cell lines. These results suggest that these unique compounds may be effective therapeutic agents for prostate cancer.

INTRODUCTION

Our laboratory first showed that suramin is a potent inhibitor of angiogenesis (Gagliardi et al., 1992). Suramin is currently undergoing phase II and III trials in prostate cancer and various solid tumors. Since the initial reports of suramin activity in metastatic hormone refractory prostate cancer, studies have confirmed that suramin has independent antitumor activity in hormone refractory prostate cancer (Myers et al. 1992). A major

problem with suramin during clinical trials has been the adverse neurotoxicity related to its prolonged half-life in vivo (45-55 days), its strong binding to serum proteins, mainly albumin (Bos et al., 1990), and its limited metabolism (Stein et al., 1989).

We have synthesized and characterized a unique group of phosphonated diphenylurea compounds with molecular weights about half that of suramin. The diphenylureas are up to 30 times more active than suramin as inhibitors of angiogenesis in the chick chorioallantoic membrane (CAM) assay. Furthermore, nonspecific binding of these compounds to serum proteins is lower and the half life shorter than for suramin or its sulfonated analogues.

In this study, the effect of selected phosphonated diphenylureas was determined on the growth of human prostate cancer cell lines in vitro. In addition, their effect on matrix metalloproteinase activity in the human prostate cancer cells was determined.

MATERIALS AND METHODS

Cell Culture: Human prostate cancer cell lines, PC3, DU145 and LNCaP.FGC, were obtained from ATCC (Rockville, MD). PC3 and LNCaP.FGC cell lines were grown in RPMI 1640 (GIBCO, Grand Island, NY) with 10% fetal bovine serum (GIBCO), 1% L-glutamine (GIBCO) and 1% penicillin-streptomycin-amphotericin (GIBCO). DU145 cancer cells were grown in DMEM (GIBCO), 1% glutamine, 1% penicillin-streptomycin-amphotericin and non-essential amino acids solution (GIBCO).

Effect on Growth In Vitro: Prostate cancer cells were harvested by trypsinization, resuspended in fresh medium and 100 µl (3000-5000 cells/well) of the cell suspension were plated in 96 well flat bottom microtiter plates (Corning, NY) and incubated in a humidified atmosphere with 5% CO_2 at 37° C. Suramin and the diphenylureas (0-140 µM) were added to the cell culture after 24 hr and incubated for 5 days. Cell growth curves were used to calculate the 50% inhibitory concentration (IC_{50}) for suramin and the diphenylureas. The MTT assay described by Carmichael et al. (1987) was used to determine growth rate. Because suramin in the medium reduced formazan production in the MTT assay, wells were washed twice with PBS before MTT was added for 3 hr. Both the diphenylureas and suramin inhibited prostate cancer cell proliferation in a dose-related manner.

Metalloproteinase activity: SDS-polyacrilamide gel electrophoresis (PAGE) using 8% gels containing 0.1% gelatin was used to determine metalloproteinase activity in prostate
cancer cells in vitro as described by Lim et al. (1996).

RESULTS

Effects of the diphenylureas on growth of prostate cancer cell lines in

vitro. We have compared the inhibitory activity of the diphenylureas and suramin on proliferation of some prostate cancer cell lines in vitro. The IC_{50} values for suramin and the most active diphenylureas on the classical prostate cancer cell lines, LNCaP (androgen dependent), PC3 and DU145, are shown in **Table 1**. The three most active diphenylureas (NF681, NF162 and NF050) showed IC_{50} values equal to or greater than suramin and its sulfonated analogues in LNCaP, PC3 and DU145 human prostate cancer cells.

Table 1: The IC_{50} values for growth of human prostate cancer cell lines treated with suramin and selected diphenylureas as determined with the MTT assay by incubating with various concentrations of suramin and diphenylureas for five days.

Cell Lines	PC3	LNCaP	DU145	
Compound (μM)				
Suramin	125	300	>500	
NF681	62	225		250
NF167	150	500		500
NF162	50	320		500
NF069	62	350		500
NF050	50	350	500	

Inhibition of matrix metalloproteinases (MMP) by diphenylureas. Polyacrilamide gel electrophoresis zymography of serum free media of cultures from PC3 prostate cancer cells contained significant amounts of 72-KD gelatinase A (MMP-2) activity. DU145 prostate cancer cells secreted 92-KD gelatinase B (MMP-9). The secretion of these metalloproteinases was enhanced in both cell types by the addition of Epidermal Growth Factor (EGF). NF681, NF167 and NF050 were potent inhibitors of gelatinase A in the PC3 cells and gelatinase B in DU145 cells.

DISCUSSION

We have compared the inhibitory activity of the diphenylureas and suramin on proliferation of some tumor cell lines in vitro. The three most active diphenylureas (NF681, NF162 and NF050) showed IC_{50} values equal to or greater than suramin and its sulfonated analogues in LNCaP, PC3 and DU145 human prostate cancer cells. These results clearly indicate that the diphenylureas are potent inhibitors of the growth of prostate cancer cell lines. Several recent reports have shown a correlation between MMP expression and tumor invasiveness in prostate cancer. Pajouh et al. (1991) found that MMP-7 was increased in malignant compared to benign prostatic tissue but absent in stroma. Boag and Young (1993) found increased levels of gelatinase A (MMP-2) in malignant prostate and metastatic tissue. Stearns and Wang (1993) analyzed prostate cancer tissue extracts for gelatinase A (MMP-2) and found that the enzyme is selectively over-expressed by malignant pre-invasive epithe-

lial cells with very low levels in benign tissue and the stroma surrounding the tumor. Wilson et al. (1993) reported gelatinolytic proteinase activities in human prostate secretions with an increased level of expression in neoplastic disease. Hamdy et al. (1994) reported that MMP-9 activity is increased in malignant compared to benign prostatic tissue. Gelatinase B (MMP-9) was not expressed in benign tissue but was detected in 42% of prostate carcinomas. Samples that exhibited MMP-9 activity in vitro had particularly unfavorable clinical parameters, such as high Gleason scores, serum PSA levels and primary tumor ploidy. Furthermore, 100% of the patients that did not respond to treatment expressed MMP-9. Recent data suggests that MMP-2 and MMP-9 are important contributors to the initial growth of metastasis by regulating access to growth factors from the extracellular matrix and increasing angiogenesis (Chambers and Matrisian, 1997).

We have shown that the diphenylureas are potent inhibitors of MMP-9 activity in PC3 cells and MMP-2 activity in DU145 cells in culture. Our results suggest that the diphenylureas may act as therapeutic agents by inhibiting MMP activity in tumors.

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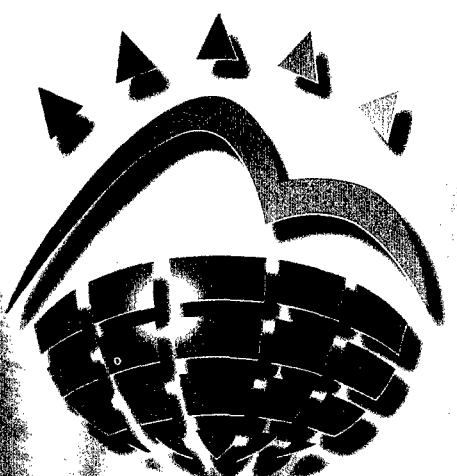
REPRINTED FROM:

17th International Cancer Congress

Rio de Janeiro (Brazil), August 24-28, 1998



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17th International
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Rio de Janeiro, Brazil
24-28 August 1998

Inhibition of renal carcinoma cell growth by diphenylureas, a unique group of antiangiogenic compounds

A. GAGLIARDI, D. COLLINS, M. BITTENCOURT*, R. MUNN* and P. NICKEL**

VA Medical Center and University of Kentucky College of Medicine, Lexington, KY (USA)
** University of Kentucky, College of Medicine, Lexington, KY (USA)*
** Institute of Pharmaceutical Chemistry, University of Bonn (D)*

SUMMARY

We have synthesized and characterized a unique group of phosphonated diphenylureas that are potent inhibitors of angiogenesis. We found that four of the most active phosphonated diphenylureas were equal to or more potent than suramin as inhibitors of growth by human renal cancer cell lines (A-498, 786-0, Caki-1 and Caki-2). They were also potent inhibitors of the metalloproteinase, gelatinase A (MMP-2), in human 786-0 renal cancer cells. These results suggest that these unique compounds may be effective therapeutic agents for prostate cancer.

INTRODUCTION

We have synthesized and characterized a unique group of phosphonated diphenylurea compounds with molecular weights about half that of suramin. The diphenylureas are up to 30 times more active than suramin as inhibitors of

angiogenesis in the chick chorioallantoic membrane (CAM) assay. Furthermore, nonspecific binding of these compounds to serum proteins is lower and the half lives shorter than for suramin or its sulfonated analogues. In this study, the effect of selected phosphonated diphenylureas was determined on the growth of human renal cancer cell lines (A-498, 786-0, Caki-1, Caki-2) in vitro. In addition, their effect on matrix metalloproteinase activity in the human renal cancer cells was determined.

MATERIALS AND METHODS

Cell Culture: Human renal cancer cell lines, A-498, 786-0, Caki-1 and Caki-2, were obtained from ATCC (Rockville, MD). Cells were cultured in a complete growth media. A-498 was cultured in DMEM containing 10% heat inactivated FCS, 1% NEAA, 1mM L-glutamine, 100 units/ml streptomycin. 786-0 cells were cultured in RPMI medium supplemented with 10%FCS, 1 mM sodium pyruvate, 1 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Caki-1 and Caki-2 cells were cultured in McCoy's medium containing 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin.

Effect on Growth In Vitro: Renal cancer cells were harvested by trypsinization, resuspended in fresh medium and 100 μl (3000-5000 cells/well) of the cell suspension were plated in 96 well flat bottom microtiter plates (Corning, NY) and incubated in a humidified atmosphere with 5% CO_2 at 37° C. Suramin or the diphenylureas (0-500 μM) were added to the cell culture after 24 hr and incubated for 5 days. Cell growth curves were used to calculate the 50% inhibitory concentration (IC_{50}) for suramin and the diphenylureas. The MTT assay described by Carmichael et al. (1987) was used to determine growth rate. Because suramin in the medium reduced formazan production in the MTT assay, wells were washed twice with PBS before MTT was added for 3 hr.

Metalloproteinase activity: SDS-polyacrilamide gel electrophoresis (PAGE) using 8% gels containing 0.1% gelatin was used to determine metalloproteinase activity in renal cancer cells in vitro as described by Lim et al. (1996).

RESULTS

Effects of the diphenylureas on the growth of renal cancer cell lines in vitro. We have compared the inhibitory activity of the diphenylureas and suramin on proliferation of some renal cancer cell lines in vitro. The IC_{50} values for suramin and the most active diphenylureas on the classical renal cancer cell lines, A-498, 786-0, Caki-1 and Caki-2, are shown in Table 1. The two most active diphenylureas (NF681and NF162) showed IC_{50} values equal to or greater than suramin and its sulfonated analogues in the renal cancer cell lines. NF681 showed the greatest inhibitory activity with IC_{50} values of 15 and 13 μM in A-498 and 786-0 renal cancer cells, respectively. However, the values for Caki-1 and Caki-2 cells were >500 μM, indicating relatively little inhibitory activity. The IC_{50} values for NF162 ranged from 138-226 μM in the four renal cancer cell lines. These results compared favorably with the values for suramin and seemed to inhibit all of the renal cancer cells tested. These results clearly indicate that the diphenylureas are potential therapeutic agents for inhibiting the growth of human renal cancer in vitro.

Table 1: The IC_{50} values for growth of human renal cancer cell lines treated with suramin and selected diphenylureas as determined with the MTT assay by incubating with various concentrations of suramin and diphenylureas for five days.

Cell Lines	A-498	786-0	Caki-1	Caki-2
Compound (μM)				
Suramin	>500	>254	56	>500
NF050	>500	>500	31	>500
NF069	166	>500	370	>500
NF162	138	200	100	226
NF681	15	13	>500	>500

Inhibition of matrix metalloproteinases (MMP) by diphenylureas.
Polyacrilamide gel electrophoresis zymography of serum free media conditioned by cultures of 786-0 cells contained 72-KD gelatinase A activity (MMP-2) but no 92-KD gelatinase B activity (MMP-9). The gelatinase A activity was enhanced by Epidermal Growth Factor (EGF) (40 ng/ml). Treatment with NF681 and NF050 (35, 70, 140 μM) for 24 hr induced a significant inhibition of gelatinase A activity and abolished the stimulation of EGF. This inhibition was not seen when suramin was added. The A498 cell line did not express detectable metalloproteinase activity in vitro.

DISCUSSION

Renal cell carcinoma remains one of the major challenges for oncologists (Young, 1997). Over the past years, progress has been made in understanding the molecular biology of this disease as new insights in prognostic factors for outcome following nephrectomy. The results of systemic therapy for metastatic renal cell carcinoma are disappointing and currently available chemotherapeutic agents have not been effective. Even promising new anti-metabolite compounds resulted in responses of less than 10% of the treated patients. The most important characteristics of renal cell carcinoma include hypervascularity, high frequency of metastasis and poor prognosis. The role of angiogenesis in growth of renal cancer cells and metastasis has been shown in clinical studies (Yoshino et al., 1995). Microvessel count is a significant predictor of survival and the density of microvessels correlates with metastasis.

Our results show that diphenylureas are potent inhibitors of renal cell carcinoma growth in vitro. These compounds are effective inhibitors of angiogenesis in the chick chorioallantoic membrane (CAM) assay and inhibit human microvascular endothelial cell growth and tube/cord formation in Matrigel.

The diphenylureas also inhibit gelatinase activity in conditioned media from renal cancer cells. Recent studies provided evidence that progelatinase A mRNA expression in cell lines derived from tumors in patients with renal cancer correlates inversely with survival (Walther et al., 1997). Gelatinase A activity correlates with tumor invasiveness in vivo and synthetic inhibitors of gelatinase activity prolonged survival and decreased the number of metastasis. This

suggests that the inhibition of metalloproteinase activity may be a suitable target for therapeutic intervention in human renal cancer.

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ANTICANCER RESEARCH *19:* 4023-4026 (1999)

The Effect of a Lectin from the Venom of the Snake, *Bothrops jararacussu*, on Tumor Cell Proliferation

MARISTELA PEREIRA-BITTENCOURT, DANIELA D. CARVALHO*, ANTONIO R. GAGLIARDI and DELWOOD C. COLLINS

*VA Medical Center and College of Medicine, University of Kentucky, Lexington, KY, USA ; *IB-Departamento de Bioquímica, UNICAMP, Campinas-SP, Brazil*

Abstract *Lectins have been used extensively as histochemical probes to describe changes in tumor cell surface and are known to influence the growth of cancer cells. In this study, we determined the effect of a lectin from the venom of Bothrops jararacussu (BJcuL) on the proliferation of a number of established human cancer cell lines. The growth of eight cancer cell lines was inhibited in a dose-related manner in the presence of BJcuL lectin. This lectin was most potent as an inhibitor of growth in renal (Caki-1 and A-498) and pancreatic (CFPAC-1) cancer cell lines with 50% inhibitory concentrations (IC_{50}) of 1-2 mM. Melanoma (Wm115) and prostate (PC-3) cancer cells showed IC_{50} values of 7.9 and 8.5 mM, respectively, in the presence of BjcuL lectin whereas colon (Caco-2) and breast (MCF7) cancer cell lines showed no effect. Our results suggest that BJcuL lectin is an effective inhibitor of cell growth in some cancer cell lines.*

Lectins are polyvalent carbohydrate-binding proteins that are present in a wide range of plant and animal cells, including snake venoms (1-6). Studies of the effect of lectins on cancer have focused on the biological and biochemical characterization of endogenous lectins from tumor cells. Lectins also affect the growth of normal and cancer cells (3). Peanut lectin (PNA) binding sites have been reported in breast cancer cell lines (5). The binding of PNA to recognized cell surface glycoproteins inhibited cell proliferation of estrogen-sensitive human breast cancer cell lines (7). *Griffonia simplicifolia* lectin and wheat germ agglutinin (WGA) inhibited the growth of a number of tumor cell lines (8) and several plant lectins were effective inhibitors of cell proliferation in three prostatic cancer cell lines (LNCaP.FGC, PC-3, DU 145) (9).

Lectin-like compounds have been isolated from snake venoms (2). These proteins bind to lactose moieties and induce agglutination of erythrocytes, aggregation of platelets (10) and mitogenesis in lymphocytes (11). BJcuL, a lectin from the venom of the snake, *Bothrops jararacussu*, is a disulfide dimer composed of 15 kDa subunits with a high affinity for glycoproteins containing β-D-galactosides (2). In this study, we investigated the effect of BJcuL lectin on cell growth in eight human cancer cell lines (kidney, pancreas, prostate, and colon). Our results suggest that BJcuL lectin is an effective inhibitor of cancer cell growth in vitro, especially against renal and pancreatic cancer cell lines.

Correspondence to: Dr. D.C. Collins, 204 Health Sciences Research Building, University of Kentucky Medical Center, Lexington, KY 40536-0305, USA. Telephone Number: 606-323-5293, Fax Number: 606-257-9700. E-Mail Address: dccoll1@pop.uky.edu

Key Words: Snake venom, lectin, cancer cells, proliferation.

Materials and Methods

Lectin. The lectin (BJcuL) from *Bothrops jararacussu* venom was isolated by affinity chromatography on an immobilized D-galactose column, dialyzed exhaustively against distilled water and lyophilized as recently described (2). The BJcuL lectin was stored at -4°C until used.

Cells and media. The eight cell lines used in this study were obtained from the American Type Collection (ATCC, Rockville, MD). They were maintained as monolayers cultured at 37°C with 5% CO_2 in closed Falcon plastic dishes (GIBCO, Grand Island, NY) containing media supplemented with 5% or 10% heat inactivated fetal bovine serum (FBS) (HYCLONE LAB, Inc). Cells were routinely grown in their respective complete growth media: RPMI 1640 medium for PC-3 cells (human prostate carcinoma); DMEM medium for Caco-2 (human colon carcinoma), CFPAC-1 (human pancreatic adenocarcinoma), MCF7 (human breast carcinoma), A-498 (human kidney carcinoma) and Wm-115 (human melanoma); and McCoy's 5A medium for Caki-1 and Caki-2 cells (human renal carcinoma). DMEM was supplemented with 1 mM L-glutamine and 1% non-essential amino acids (NEAA); RPMI medium was supplemented with 1 mM sodium pyruvate and 1 mM L-glutamine. All media also contained antibiotics (10,000 units/L penicillin and 100 mg/L streptomycin).

The cells used in this study were harvested by trypsination (trypsin/EDTA for 2 min), resuspended in fresh medium and plated (3,000 – 5,000 cells/well) in 96-well flat microtiter plates. After 24 hr, various concentrations of BJcuL lectin (0-10 μM) were added and the cells were incubated for five days (37°C with 5% CO_2).

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

Table I. *The 50% inhibitory concentration (IC_{50}) of cell growth by BJcuL-lectin (mM) in eight cancer cell lines.*

Cell line	IC50 (mM)
Renal	
Caki-1	1.06
Caki-2	7.10
A-498	1.63
Pancreas	
CFPAC-1	1.13
Prostate	
PC-3	7.90
Melanoma	
Wm-115	8.50
Colon	
Caco-2	>10.0
Breast	
MCF7	>10.0

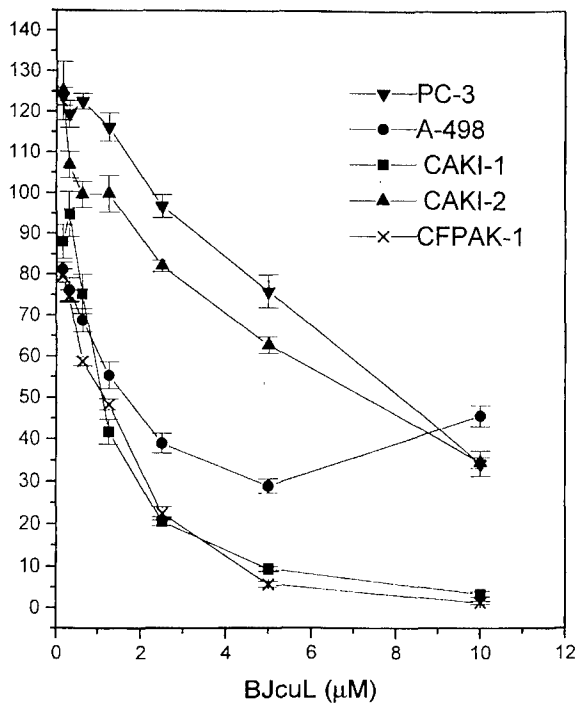

Figure 1. *The effect of various concentrations of BJcuL lectin (0-12 μM) on cell growth in five cancer cell lines, human renal carcinoma (A-498, Caki-1 and Caki-2), prostate carcinoma (PC-3) and human pancreatic adenocarcinoma (CFPAC-1). Results are expressed as percent of the control.*

bromide) assay measured cell growth as described by Carmichael *et al* (12). The effect of BJcuL lectin on cell growth was determined at each concentration from at least three different experiments with four replicate samples. This data was used to calculate the 50% inhibitory concentration (IC_{50}) for BJcuL lectin. A linear relationship between the MTT assay and cell number was established as previously described (12).

Statistical analysis. Data was analyzed by ANOVA and Dunnett's procedure. T tests were used to ascertain statistical differences. Statistical probability of $p \leq 0.05$ was considered significant.

Results

Figure 1 shows the effect of different concentrations of BJcuL lectin on cell proliferation in renal (Caki-1, Caki-2 and A-498), pancreas (CFPAC-1) and prostate (PC-3) cancer cell lines. BJcuL lectin inhibited cell proliferation in a dose-related manner for these cancer cell lines. For most of the cancer cell lines, a transient stimulation of cell proliferation was observed at low doses (<0.5 μM) of BJcuL lectin. Incubations reported in these studies were carried out in 5% FBS. Incubation with 10% FBS decreased the inhibitory activity of BJcuL lectin by approximately 50% (data not shown). The potency of BJcuL lectin as an inhibitor of cell proliferation is reflected by the IC_{50} values in the eight cancer cells lines shown in Table I. The IC_{50} values in renal cancer cell lines were 1.06 μM for Caki-1, 1.63 μM for A-498 and 7.10 μM for Caki-2. BJcuL lectin was also a potent inhibitor of the pancreas (CFPAC-1) and prostate (PC-3) cancer cell lines. On the other hand, BJcuL lectin was not as effective as a growth inhibitor of melanoma (Wm-115), colon (Caco-2) and breast (MCF7) cancer cell lines (IC_{50} values < 8.5 μM).

Discussion

Lectins are multivalent carbohydrate-binding proteins that are widely distributed in plants and animals (1,6). Previous reports have shown that, depending on the tissue target, type and concentration of the lectin, they may either stimulate or inhibit cell growth (9). Most studies of the effect of lectins on cancer cell growth have focused on plant lectins. PNA (peanut lectin) inhibited cultured breast cancer cell proliferation (7), but stimulated benign and malignant colorectal cells (HT29 and Caco-2) (13) as well as smooth muscle and pulmonary artery cells (14). A more recent study assessed the effect of five plant lectins on the growth of three colorectal cancer cell lines (LoVo, HCT-15 and SW837) (15). Wheat germ (WGA) and concanavalin A (Con A) had significant inhibitory effects on growth of all three cell lines

whereas PNA had modest stimulatory effects on cell growth. Similar results were reported in three melanoma cell lines (SK-MEL-28, HT-144 and C32) where four plant lectins inhibited and PNA stimulated cell growth (16).

We report here the first evidence that snake venom lectins were potent inhibitors of cell growth in selected cancer cell lines. Lectin-like proteins have been found in the venom of four snake species (Elapidae, Viperidae, Crotalidae and Bothrops) (2). Snake venom lectins have properties intermediate between the S and C families of animal lectins. They are CA^{2+}-dependent galactose-binding proteins that show a common n-terminal amino acid sequence. Our data clearly demonstrated a marked inhibition of cell proliferation in renal (Caki-1 and A-498) and pancreas (CFPAC-1) cancer cell lines (IC_{50} values = 1-2 μM) by BJcuL lectin. It was also an effective inhibitor in renal (Caki-2), melanoma (Wm-115) and prostate (PC-3) cell lines. However, BJcuL lectin did not show significant inhibition in breast (MCF7) and colon (Caco-2) cancer cells. The lack of inhibition of estrogen positive breast cancer (MCF7) proliferation by BJcuL lectin suggests that its mechanism of action differs from PNA. A high correlation has been shown between estrogen receptor (ERα), PNA binding and inhibition of proliferation in MCF7 breast cancer cells by PNA (7). The growth response of colon (Caco-2) cancer cell lines to BJcuL lectin was similar to plant lectins.

In the A-498 cells, the higher cell concentrations of BJcuL lectin (10 μM) resulted in increased cell proliferation (Figure 1). This observation is difficult to explain. We hypothesize that BjcuL lectins stimulated cell proliferation. In fact, lectins have been shown to enhance the secretion of cytokines (16). Other investigators have reported a significant serum protective effect on cell toxicity in plant lectins (9,16). The explanation for this effect is that FBS contains significant amounts of glycoligands specific for the plant lectins that interfere with the binding of the plant lectins to the cell surface (16). BJcuL lectin showed high levels of cell toxicity when incubated in the presence of 5% FBS. This is 5-fold higher than the plant lectins which were incubated in 0 or 1% FBS. This suggests that FBS contains much less BJcuL-specific glycoligands compared to plant glycoligands. Thus, the BJcuL lectin is more available for binding to the cancer cells.

Acknowledgements

This work was supported by the Department of Veterans Affairs, the American Cancer Society (Award DHP 117A) and CAPES (Brazil). The editorial assistance of Patricia Collins is gratefully acknowledged.

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Received May 11, 1999
Accepted June 3, 1999

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