PHARMACEUTICAL COMPOSITIONS COMPRISING DISULFIRAM FOR INHIBITING ANGIogenesis

DISULFIRAM ENTHALTENDE ARZNEIMITTEL

COMPOSITIONS PHARMACEUTIQUES CONTENANT DU DISULFIRAME POUR INHIBER L’ANGIOGENÈSE

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BOLONTRADE M.F. ET AL: ‘Angiogenesis is an early event in the development of chemically induced skin tumors’ CARCINOGENESIS vol. 19, no. 12, 1998, OXFORD, pages 2107 - 2113

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**Description**

**Field of the Invention**

The present invention relates to the use of disulfiram as an angiogenesis inhibitor for the preparation of pharmaceutical compositions as mentioned in the appended claims for the treatment of angiogenesis-dependent disorders.

**Background of the Invention**

Disulfiram is administered simultaneously with said drugs without compromising their anti-cytotoxicity against murine leukemia at 3 mg/kg (Habs, 1981). Disulfiram was also shown to potentiate the anti-toxic side effects of 1-(2-hydroxyethyl)3-(2-chloroethyl)-3-nitrosourea (HECNU), without inhibiting its anti-tumor potency (Habs, 1981). Disulfiram was also shown to potentiate the anti-cancer activity of some agents such as to nitrogen mustard (HN2) cytotoxicity against murine leukemia at 3 mg/mouse (Valeriote, 1989).

Disulfiram, together with ascorbic acid, augmented inhibition of Meth A tumor cell proliferation in vitro by increasing the intracellular oxygen free radicals (Mashiba, 1990). In addition, disulfiram inhibited superoxide dismutase in vivo (Forman, 1980; Ohman, 1986). All of these could result in an increase in oxygen species toxic to the cell thereby making the cell more sensitive to damage by a variety of chemotherapeutic agents or radiation that produce superoxide anions species (Goodman, 1977). Resistance to cyclophosphamide and oxazaphosphorines is related to aldehyde dehydrogenase activity (Magni, 1996; Rekha, 1994), and inhibition of this enzyme by disulfiram thus increases sensitivity to these chemotherapies.

**US Patent No. 4,870,101 (Ku and Doherty, 1989) discloses a method for inhibiting the release of interleukin-1 in animals which comprises administering to said animals an amount of disulfiram effective to inhibit the release of interleukin-1, thus proposing disulfiram for the treatment of IL-1 mediated inflammations such as psoriasis, rheumatoid arthritis, diabetes and atherosclerosis.**

**Document WO95/30415 describes the use of a dithiocarbamate including disulfiram for the treatment of cardiovascular diseases.**

Disulfiram was shown to protect mice against ifosfamide- and cyclophosphamide-induced urotoxicity when administered simultaneously with said drugs without compromising their anti-tumor activity against Sarcoma 180, EL-4 leukemia or L1210 murine leukemia (Hacker, 1982; Ishikawa, 1991; Ishikawa, 1994). On the other hand, disulfiram did not show any protection against cisplatin nephrotoxicity in humans (Verma, 1990). Disulfiram protected rats against the cytotoxicity of large bowel induced by 1,2-dimethylhydrazine (Wattenberg, 1978), and in neoplasia of the forestomach induced by benzo(a)pyrene in mice (Borchert, 1976). Disulfiram inhibited the metabolism of the carcinogen azoxymethane thus offering protection from the metabolite neoplasia (Fiala, 1977). Disulfiram was also shown to inhibit tumor progression - from papilloma to squamous cell carcinoma - in the murine skin multistage carcinogenesis model (Rotstein, 1988), to inhibit transmammary carcinogenesis induced in mice by 7,12-dimethylbenz (a)anthracene (Rao, 1989) and to reduce the incidence of mammary tumors induced in rats by N-2-fluorenylacacetamide or N-hydroxy-N-2-fluorenylacacetamide through inhibition of their metabolic activation (Malejka Giganti, 1980). Since a cytotoxic aldehyde dehydrogenase is induced during rat hepatocarcinogenesis (Allen, 1982), the inhibitory effect of disulfiram on various carcinogens may be related to its inhibitory effect on aldehyde dehydrogenases.

**Document WO95/30415 describes the use of a dithiocarbamate including disulfiram for the treatment of cardiovascular diseases.**

Disulfiram, given 0.05% in diet for 2 years, did not increase any tumor type in rats (Cheever, 1990). The toxic dose for disulfiram in normal mice is about 6-10 mg/mouse/day. The LD50 of disulfiram given orally in rats is 8.6 g/Kg.

[0010] None of the above publications describes or suggests the use of disulfiram as an inhibitor of angiogenesis.

Summary of the Invention

[0011] It has now been found in accordance with the present invention that disulfiram inhibits angiogenesis and is able to block neovascularization induced subcutaneously in nude mice.

[0012] The present invention thus relates to the use of disulfiram for the preparation of a pharmaceutical composition for treatment of angiogenesis-dependent diseases selected from ophthalmologic disorders such as diabetic retinopathy, corneal graft neovascularization, neovascular glaucoma, trachoma and retinopathy of prematurity also known as retrolental fibroplasia, neurologic cerebrovascular disorders such as arteriovenous malformation, neoplasms such as leukemia and solid tumors, and treatment of hypertrophic scars.

[0013] Examples of solid tumors that can be treated with disulfiram according to the invention include bladder, breast, cervix, ear, esophagus, kidney, larynx, liver, lung, ovary, pancreas, prostate, skin, stomach, thyroid, urethra and uterus carcinomas.

[0014] For the preparation of the pharmaceutical compositions of the invention, disulfiram is mixed with pharmaceutically acceptable carriers and conventional excipients to produce unit dosage formulations suitable for administration. Any suitable mode of administration is envisaged by the invention, but oral administration is preferred.

[0015] The dosage of disulfiram to be administered daily will depend on the disorder being treated and the age, weight and condition of the patient being treated, and can be determined without difficulty by skilled physicians. Based on the examples herein performed in animals, it can be deduced that dosages between 1-50 mg/person are suitable for humans.

[0016] In another aspect, the invention relates to the use of disulfiram for the preparation of a pharmaceutical composition to prevent cell hyperproliferation and formation of clots along or around medical devices such as stents, catheters, cannulas, electrodes, and the like. In one embodiment, disulfiram may be systemically administered to a patient in which such a device has been inserted. In another embodiment, the medical device is coated with disulfiram before insertion in the patient, and such disulfiram-coated medical devices are also envisaged by the present invention.

[0017] Abbreviations: BCE: bovine capillary endothelial cells; bFGF: basic fibroblast growth factor; BSMC: bovine vascular smooth muscle cells; DMEM: Dulbecco’s Modified Eagle’s Medium; EGF: epidermal growth factor; FCS: fetal calf serum; GPS: glutamine/penicillin/streptomycin; HB-EGF: heparin-binding epidermal growth factor.

Brief Description of the Drawings

[0018] Figs. 1A-1C show inhibition of neovascularization in nude mice by disulfiram (D). Agarose beads containing the angiogenic compound bFGF (10 μg/bead) were implanted subcutaneously in nude mice and the angiogenic potential of bFGF in vivo was demonstrated 4 days after implantation, in skin specimens (Fig. 1B). Saline, as a negative control, did not induce neovascularization around the bead (Fig. 1A). Disulfiram (D) introduced systemically at 60 μg/mouse inhibited almost completely neovascularization induced by bFGF inside and around the bead (Fig. 1C). Bar is one mm.

[0019] Figs. 2A-2D show in vitro inhibition of DNA synthesis by disulfiram in a dose-dependent manner in BCE, BSMC, BALB/MK and C6 rat glioma cells, respectively, as measured by incorporation of 3H-thymidine into the cells. Experiments were done in triplicates and the inhibition was calculated as percentage of DNA synthesis of non-treated control.

[0020] Figs. 3A-3D show inhibition of DNA synthesis in a non-reversal manner by disulfiram at various periods of time (1, 2, 4 and 24 hours) in BCE, BSMC, BALB/MK and C6 rat glioma cells, respectively, as measured by the incorporation of 3H-thymidine into the cells. Experiments were done in triplicates and the inhibition was calculated as percentage of DNA synthesis of non-treated control.

[0021] Fig. 4 shows disulfiram-induced apoptosis in endothelial cells. BCE cells were incubated with 0.5-5 μM disulfiram (D) for 20 hours and analyzed by FACS for the DNA content of the cells. Disulfiram induced in a dose-dependent manner a sub-diploid apoptotic population of endothelial cells, that was not apparent in control-treated cells. Experiments were
repeated twice with triplicates.

[0022] Figs. 5A-5B show disulfiram-induced apoptosis in BCE, BSMC and C6 glioma cells, following incubation with the indicated concentrations of disulfiram (D) for 6 hours, as analyzed by the TUNEL method. The nuclei of BCE cells (Fig. 5A, bottom) treated with disulfiram were labeled using the FUNEL staining method, while the nuclei of control BCE (Fig. 5A, top) and of BSMC Fig. 5B, top) and C6 glioma cells (Fig. 5B, bottom) treated with disulfiram were not labeled using the TUNEL staining method. Experiments were repeated twice with triplicates.

[0023] Fig. 6 shows inhibition by disulfiram of Lewis lung tumor metastasis in the lungs of mice in the footpad model. Lungs from C57/BL mice fed systemically 3 times a week with disulfiram (30 μg) were weighed 24 days following removal of the tumor-bearing leg. The weight of the lungs from normal mice was subtracted from that of the metastasized lungs. Compared with water-fed control, metastasis in the lungs of disulfiram-treated animals was significantly smaller (n=6) p=0.005.

[0024] Fig. 7 shows inhibition by disulfiram of Lewis lung tumor metastasis in the lungs of mice in the i.v. model. Lungs from C57/BL mice fed systemically 3 times a week with disulfiram (13-40 μg) were weighed 24 days following injection of D122 tumor cells i.v.. The weight of the lungs from normal mice was subtracted from that of the metastasized lungs. Compared with water-fed control, metastasis in the lungs of disulfiram-treated animals was significantly smaller (n=6) (p=0.023-0.037). At 13-40 μg/mouse disulfiram decreased metastasis to the lungs 6-19 fold accordingly. Experiments were repeated twice.

**Detailed Description of the Invention**

[0025] Vascular smooth muscle cells and endothelial cells are the two cell types constituting the blood vessel walls. Angiogenesis, the growth of new blood vessels by sprouting from established vessels, requires the growth of vascular endothelial cells and vascular smooth muscle cells. According to the data of the present invention, disulfiram is clearly identified as an effective inhibitor of angiogenesis.

[0026] Thus, as shown herein, disulfiram inhibited in vivo the induction of new blood vessels in the mouse skin and was effective when administered orally. The ability of disulfiram to inhibit at low concentrations the growth of cultured capillary endothelial cells (BCE) suggests that the drug acts directly on capillary endothelial cells. Moreover, the inhibition of endothelial cell growth was shown to be non-reversible. The growth of vascular smooth muscle cells (BSMC), another cell constituent of the blood vessel wall, was also inhibited by disulfiram at low concentrations (0.5 μM). The fact that the drug induces apoptosis in capillary endothelial cells and fails to induce apoptosis in other cell types such as vascular smooth muscle cells, keratinocytes (MK), fibroblasts and C6 rat glioma cells, indicates that it has some specificity for capillary endothelial cells. Indeed, when disulfiram was administered systemically at low doses of 25-60 μg/mouse, the formation of new blood vessels was specifically disrupted, while no evidence for damage in other tissues was observed. The low concentration of disulfiram administered systemically when calculated for the volume of a mouse (3 μM), was in the range of that used in vitro for endothelial cells (0.1-0.2 μM), especially when the metabolic processing of the drug in the body is taken into account.

[0027] As might be expected from its ability to inhibit capillary endothelial cells and BSMC at concentrations achievable in vivo, systemic treatment of mice with disulfiram inhibited neovascularization in the skin. The growth of C6 rat glioma cells in vitro was inhibited by disulfiram. Taken together with the fact that active angiogenesis is essential for the progressive growth of solid tumors (Folkman, 1990) and that C6 glioma tumor development is angiogenesis-dependent (Abramovitch, 1995; Ikeda, 1995; Niida, 1995; Plame, 1992), one could expect that C6 glioma tumor growth would be affected by disulfiram. Indeed, disulfiram significantly reduced both Lewis lung metastasis in the lungs and C6 tumor development in vivo when administered systemically per os at low concentrations similar to those observed to be effective in vitro, both for endothelial and C6 glioma cells, suggesting that the inhibitory activity for C6 tumor growth and for metastasis in the lungs in vivo is induced through inhibition of angiogenesis and of C6 glioma cell growth. Once ingested and absorbed through the intestinal tract or injected intraperitoneally, disulfiram is extensively distributed throughout the body including the brain (Faiman, 1978), but the mechanism through which disulfiram induces its inhibitory effects in vitro or in vivo is not known. Also the reason for capillary endothelial cells being more liable to disulfiram than other cell types for induction of apoptosis, is not known.

[0028] The results shown here demonstrate that disulfiram inhibits capillary endothelial and vascular smooth muscle cell growth and induces apoptosis in capillary endothelial cells and that, when used systemically in mice, disulfiram inhibits angiogenesis and decreases C6 glioma tumor growth, clearly defining disulfiram as a new inhibitor of angiogenesis and showing its potential use for therapy in angiogenesis-dependent diseases such as pathologies in which neovascularization is involved, including neoplasia.

[0029] The invention will now be illustrated by the following non-limiting examples.
EXAMPLES

Material and Methods

(a) Materials:

[0030] Disulfiram (Sigma) and mouse EGF (Collaborative Biomedical Products, Bedford, MA, USA) were purchased. Recombinant b-FGF and recombinant HB-EGF were kindly provided by Prof. Gera Neufeld and by Dr. Judith A. Abraham (Scios Nova Inc., Mountain View, CA), respectively.

(b) Cell lines:

[0031] C6 rat glioma cells were routinely cultured in DMEM supplemented with 5% FCS (Biological Industries, Israel), GPS (100 U/ml penicillin, 100 mg/ml streptomycin (Biological Industries, Israel) and 2 mM glutamine (Biolab Ltd. Israel)) and 125 μg/ml fungizone (Biolab Ltd, Israel).

[0032] Brain bovine capillary endothelial cells (BCE) and bovine vascular smooth muscle cells (BSMC), kindly provided by Prof. Israel Vlodavsky (Hadassah Medical School, Jerusalem, Israel), were cultured at 37°C in low glucose DMEM (1 g/liter) supplemented with 10% calf serum (HyClone, Logan, Utah, USA), a serum-free supplement: biogro-1 (Beth Haemek, Israel) and GPS.

[0033] Bovine aortic vascular smooth muscle cells (BSMC) were cultured in low glucose DMEM (1 g/liter) supplemented with 10% FCS (HyClone, Logan, Utah) and GPS.

[0034] The BALB/MK epidermal keratinocyte cell line, kindly provided by Dr. S. Aaronson (National Cancer Institute, Bethesda, MD, USA), was cultured (37°C, 10% CO₂ humidified atmosphere) in calcium-free MEM (Beth Haemek, Israel) supplemented with 10% dialyzed FCS and murine EGF (5 ng/ml).

(c) Measurement of DNA synthesis

[0035] C6 rat glioma cells were plated in 96-well plates (Nunc, Denmark) (5000 cells per well) in DMEM with 5% FCS. After 6 hours the cells were rinsed and incubated for 48 hours in serum free medium. 5% FCS or growth factors were then added to the cells for 24 hours (triplicates). ³H-methyl-thymidine (5μCi/ml) (Rotem Ind. Ltd., Israel) was added to the cells for the last 14 hours. The cells were rinsed with 100 μl methanol for 10 minutes, followed by 200 μl 5% trichloroacetic acid, and then rinsed and lysed with 150 μl 0.3M NaOH. Radioactive thymidine incorporated into the DNA was determined for 1 min with 3 ml scintillation liquid (ULTIMA GOLD Packard) in a β-counter. DNA synthesis assays were performed in triplicates.

[0036] Bovine capillary endothelial cells (BCE) and bovine aortic BSMC were plated in 24-well plates (6000 cells per well) in 500 μl DMEM medium supplemented with 10% Colorado calf serum (CCS) (GIBCO, USA) and GPS. After 24 hours, medium was changed to starvation medium (2% CCS, 0.5% BSA, GPS) for 24 hours. ³H-methyl-thymidine (5μCi/ml) was added for the last 6 hours. DNA synthesis assays were performed as described above, in triplicates. DNA synthesis assays in BALB/MK keratinocytes were performed as previously described (Marikovsky, 1995). DNA synthesis assays were performed in triplicates.

[0037] Disulfiram was prepared in 0.1 mM stock solutions in DMSO. Control samples were incubated with the appropriate concentration of DMSO. Inhibition was calculated as percentage of DNA synthesis of control.

(d) Subcutaneous angiogenesis in nude mice

[0038] Spherical agarose beads of approximately 1 mm in diameter were formed from 4% low gelling temperature agarose (Sigma) in PBS containing b-FGF or HB-EGF as angiogenic agent. The candidate angiogenic agent (10 μg/bead) was warmed in sterile microtest tubes to 40°C in a dry-bath for a few seconds. 10 μl of the angiogenic compound and beads were formed above ice using a 20 μl pipette tip. Beads were implanted subcutaneously 1 cm away from the incision site as reported previously for multicellular spheroids (Abramovitch, 1995) in mice anesthetized with a single dose of 75 mg/kg ketamine + 3 mg/kg xylazine (i.p.). Experiments were carried out for 4 days in CD 1 nude male mice. Each day one ml of aqueous solutions with or without 0.1-0.25 mM (25-60 μg) disulfiram was introduced per os to the mice using a feeding needle. Treatment was for three days starting from the day of bead implantation until one day before termination. Experiments were done in quadruplicates and repeated three times.
(e) Growth of C6 glioma tumors

[0039] C6 rat glioma cells (10⁶) were injected subcutaneously into the back of the neck of CD1 nude male mice. After 3 days, 1 ml of aqueous solutions with or without 0.1-0.5 mM disulfiram (25-120 μg) was introduced per os to the mice using a feeding needle. Mice were orally fed three times per week. Tumors were removed 30 days following C6 cells injection, weighed, fixed in buffered formalin and histological sections were prepared. Each experimental group included 8 animals, and experiments were repeated twice.

(f) Growth of Lewis lung carcinoma tumors

[0040] The Lewis lung carcinoma (3LL), which originated spontaneously in a C57/BL/6J (H-2⁷) mouse, is a malignant tumor that produces spontaneous lung metastases. The metastatic clone D122, kindly provided by Prof. Lea Eisenbach (Weizmann Institute of Science, Rehovot, Israel), was used herein for tissue culture and for in vivo experiments. The cell cultures were maintained in DMEM supplemented with 10% heat-inactivated FCS, glutamine, antibiotics, sodium pyruvate and nonessential amino acids.

[0041] Two metastasis models were used: 1. The footpad model. 2. The i.v. model. The assay of tumor development in the footpad model and evaluation of lung metastases was done as previously described (Eisenbach, 1983). Briefly, eight mice in each experimental group were inoculated with 2x10⁵ D122 cells in 0.05 ml PBS in the right hind footpad. Three days following tumor cells injection, mice were treated per os by disulfiram or saline 3 times per week. The tumors became palpable within 11-19 days. Local tumor growth was determined by measuring the footpad diameter with a calipers. At 26-40 days following inoculation, when local tumor reached a diameter of 6-7 mm, mice were anesthetized with a single dose of 75 mg/kg ketamine + 3 mg/kg xylazine (i.p.) and the tumor-bearing leg was removed by amputation above the knee joint. To measure progress of metastases the mice were killed 24 days following amputation by injecting 20 mg/mouse xylazine i.p. and lungs were weighed.

[0042] To distinguish between the effect on the migration of the cells from the main tumor and the development of a tumor from a metastatic foci, the i.v. model was used. D122 cells (5x10⁵) were injected i.v. to the tail of C57/Bl male mice and after 24 days mice were killed and their lungs weighed. Treatment with disulfiram was started 3 days following the injection of D122 cells. Each experimental group included 8 animals, and experiments were repeated twice.

(g) Analysis of apoptotic cells by FACS

[0043] Cells were cultured in plastic tissue culture dishes for 48 hours in presence of growth media as described above, until reaching 40-50% confluency. Disulfiram was then added to the cells for 20 hours. The cells were removed from the plates by EDTA-trypsin and fixed in ice-cold 70% ethanol (BioLab, Israel) in -20°C for 2 hr to overnight. The fixed cells (2.5x10⁵) were washed once with HEPES-buffered saline (HBSS) and incubated with 0.5 mg/ml RNaseH (Boehringer Mannheim). Afterwards the cells were resuspended in HBSS containing 50 μg/ml propidium iodide (Sigma) and subsequently analyzed on a FACSort flow cytometer (Beckton Dickinson Inc.) using Lysis II software. For the analysis, 10,000 cells were examined from each sample. The percentage of the hypodiploid cells was measured (Darzynkiewicz, 1992; Afanasyev, 1993). The cell cycle histogram was divided into four regions according to the cell cycle phases: Ap, apoptotic cells; G₁, diploid cells; S, intermediate cells; andG₂/M, tetraploid cells.

(h) TUNEL assay for apoptosis

[0044] Cells were cultured on microscope slides for 48 hours in presence of growth media as described above, until reaching 40-50% confluency. Disulfiram was then added to the cells for 6 hours or for 20 hours in the case of BALB/MK keratinocytes, fixed with 4% paraformaldehyde and washed three times with PBS. Apoptosis was analyzed by the in situ TUNEL staining carried out as described (Wride, 1994). Briefly, microscope slides were incubated for 15 min in 2xSSC buffer at 60°C, washed in DDW and incubated with 20 μg/ml proteinase K (Boehringer Mannheim) for 15 min at room temperature. After a was with DDW, endogenous peroxidases were inactivated by incubating the slides with 2% H₂O₂ in PBST (PBS with 0.05% Tween 20) for 10 min at room temperature. Slides were then incubated in TdT buffer (Boehringer Mannheim) for 5 min at room temperature, and a reaction mixture containing 5xTdT buffer and 1 μl biotin-21-dUTP (Clontech, 1 mM stock) and 8 units of the TdT enzyme (Boehringer Mannheim) in total volume of 50 μl was subsequently added. The reactions were carried out at 37°C for 1.5 hr in a humid chamber. The slides were washed in 2xSSC, DDW and finally with PBS and covered with 10% skim milk in PBST for 15 min. After removal of the skim milk, the sections were incubated with ABC solution from ABC kit (Vector Laboratories, Inc.) for 30 min at room temperature, washed with PBS and stained using AEC procedure (Sigma) The slides were then washed x3 in DDW and stained with haematoxylin for 30 sec and mounted using Kaiser’s glycerol gelatin (Merck).
EXAMPLE 1: In vivo inhibition of neovascularization by disulfiram.

[0045] Agarose beads containing the angiogenic factor bFGF (10 μg/bead) were implanted subcutaneously into CD1 nude mice as described in section (d) of "Materials and Methods" above. The results are shown in Fig. 1. After 4 days new blood vessels clearly developed around and inside the beads containing bFGF (Fig. 1B) while the control beads containing only saline appeared clear and without any new blood vessels being formed around or within the beads (Fig. 1A). However, when the mice were daily fed per os during 3 days with 1 ml aqueous solution (0.1-0.25 mM) of disulfiram (D) (25-60 μg/ mouse /day), angiogenesis around the beads containing bFGF was clearly inhibited (Fig. 1C).

EXAMPLE 2: Disulfiram inhibits capillary endothelial cell proliferation.

[0046] To determine whether disulfiram acts directly on endothelial cells rather than on accessory cells such as macrophages and mast cells that can be responsible for the development of an angiogenic response in vivo, the effect of the drug on the growth of BCE cells was examined in vitro as described in section (c) of "Materials and Methods" above. DNA synthesis in BCE cells was measured in presence of increasing concentrations of disulfiram incubated for 24 hours with the cells. The results are shown in Fig. 2. At concentrations ranging from 0.1-0.5 μM, disulfiram was able to inhibit DNA synthesis in BCE cells in a dose dependent manner, complete inhibition being achieved at 0.5 μM disulfiram (Fig. 2A) BCE cells were shown to be more sensitive to the inhibitory activity of disulfiram than other cell types such as BALB/MK keratinocytes (Fig. 2C), C6 rat glioma cells (Fig. 2D) or bovine aortic vascular smooth muscle cells (BSMC) (Fig. 2B). Maximal inhibitory activity (80%) for BSMC was at 0.5-1 μM disulfiram, while maximal inhibitory activity for C6 glioma cells or BALB/MK keratinocytes was at concentrations 10 fold higher (2-3 μM) than those for endothelial cells (0.2-0.5 μM). At higher concentrations (>10 μM), disulfiram became less inhibitory for all cell types examined (not shown).

[0047] To examine the time course of the effect of disulfiram and whether the effect was reversible, cells were incubated with disulfiram for various periods of time (1, 2, 4 and 24 hours), then washed away and DNA synthesis was determined. One to 4 hours of exposure to disulfiram were enough to induce maximal inhibitory effect at concentrations ranging from 0.5 μM for BCE cells (Fig. 3A) to 5 μM for BALB/MK keratinocytes (Fig. 3C) and C6 glioma cells (Fig. 3D). As shown, for BCE, MK and C6 glioma cells the inhibitory effect seemed to be maintained even 24 hours following short exposure to the drug. BCE cells were most sensitive: 1-2 hours of incubation with as low as 0.5 μM disulfiram were enough to induce near maximal inhibition of DNA synthesis (Fig. 3A) BSMC were less sensitive as far as time course is concerned when incubated with 1 μM disulfiram. Following 4 hours of incubation with 1 μM disulfiram, DNA synthesis inhibition in BSMC reached only 50% (Fig. 3B). The data shown here indicate that the damage for capillary endothelial cells, following a short incubation of 1 to 2 hours, was maximal and that, at least in a time scale of 24 hours, this damage was non-reversible.

EXAMPLE 3: Inhibition of endothelial cell proliferation by disulfiram is via apoptosis.

[0048] To determine whether the non-reversible inhibition of capillary endothelial cells was induced by the programmed cell death pathway, apoptosis in BCE cells was examined by means of the FACS analysis and by the TUNEL method.

[0049] Capillary endothelial cells were grown during 48 hours to 40-50% confluency and disulfiram was then added to the cells for 20 hours as described in section (g) of "Materials and Methods" above. The results are shown in Fig. 4. FACS analysis of the DNA content of endothelial cells incubated with 0.5-5 μM disulfiram demonstrated the appearance of a sub-diploid apoptotic population of cells. The abundance of apoptotic cells was dose-dependent. In contrast, control non-treated endothelial cells did not undergo this DNA degradation process and most of the cells were in the G0/G1 phase and some in the S and G2/M phase. Unlike capillary endothelial cells, BALB/MK keratinocytes treated with 4 μM disulfiram and analyzed by FACS, did not exhibit induction of apoptosis (not shown). Typically for cells undergoing apoptosis, endothelial cells treated with disulfiram quickly became rounded. In contrast, 3T3 fibroblasts, C6 glioma cells or BSMC treated with 1 μM, 2 μM or 5 μM, respectively, of disulfiram, did not change their shape to the rounded form.

[0050] The TUNEL method was used to label the nuclei of cells undergoing apoptosis as described in section (h) of "Materials and Methods" above. The results are shown in Fig. 5. As shown in Fig. 5A, bpttom, capillary endothelial (BCE) cells incubated for 6 hours with 1 μM disulfiram were induced into apoptosis. In contrast, disulfiram did not induce BSMC (1 μM) (Fig. 5B, top), BALB/MK keratinocytes (5 μM) (not shown) and C6 rat glioma cells (5 μM) (Fig. 5B, bottom) into apoptosis following incubation for 6 hours, as measured by the TUNEL method. Capillary endothelial cells are, thus, distinct in their apparent sensitivity to disulfiram-induced apoptosis.

EXAMPLE 4: Disulfiram inhibits Lewis lung carcinoma and C6 glioma tumor growth in vivo.

[0051] Since disulfiram was shown to be an effective inhibitor of neovascularization in vivo as well as inhibitory to C6 glioma cell growth in vitro, one could expect that systemic treatment with the drug may slow tumor development, since
active angiogenesis is essential for the progressive growth of solid tumors beyond a diameter of a few millimeters (Folkman, 1990).

[0052] The effect of disulfiram was thus examined in the Lewis lung carcinoma footpad model in C57/BL mice as described in section (f) of "Materials and Methods" above, using concentrations that were shown to be inhibitory for angiogenesis in vivo. Twenty-four days following the removal of the tumor-bearing leg, lungs were weighed. Metastasis in the lungs was significantly retarded by systemic treatment with disulfiram. In the control non-treated group, 50% of the mice died before or on the 24th day following the removal of the tumor-bearing leg, while no disulfiram-treated mice died. Moreover, two out of six mice in the disulfiram-treated group had no metastases at all. As shown in Fig. 6, treatment with disulfiram at concentrations of 30 μg/mouse lowered the metastatic load in the lungs by almost 10-fold. At higher concentrations (120 μg/mouse) there was only slight decrease in the metastatic load in the lungs (not shown).

[0053] Similarly, the effect of disulfiram was examined in a C6 rat glioma model in CD1-nude mice as described in section (e) of Materials and Methods above, using concentrations that were shown to be inhibitory for angiogenesis in vivo. Tumors from mice fed systemically 3 times a week with disulfiram were weighed 30 days following administration of C6 rat glioma cells to CD1 nude mice. The growth of the tumors was significantly retarded by systemic treatment with disulfiram. Compared with water-fed control, tumors from animals treated with disulfiram were significantly smaller. Experiments were repeated twice (n=8).

[0054] As shown in Table 1, at disulfiram (DSF) concentrations of 25-120 μg/mouse, tumor development was retarded by 57-38%, respectively. Interestingly, the most effective concentration was the lowest one. This is in agreement with the data observed that disulfiram became less inhibitory for in vitro cell growth at high concentration (not shown). Since disulfiram inhibits angiogenesis both in vivo in mice and in vitro in C6 glioma cells, it can be assumed that tumor growth inhibition observed for disulfiram is the result of its dual action, one on the neovascularization of the tumor and one on the C6 glioma cells. Pathological examination of various tissues (kidney, liver, stomach, lungs and spleen), including histological sections prepared from these tissues, revealed no effect on these tissues in the treated animals. Blood vessels examined in these tissues were also not affected.

| Table 1: Disulfiram inhibits C6 rat glioma tumor growth in nude mice |
| (n) | Tumor weight (g)a | %inhibition | p valueb |
| Control (8) | 1.37 ± 0.21 | 0 |  |
| DSF (25μg) (8) | 0.59 ± 0.08 | 57 | p=0.003 |
| DSF (120μg) (8) | 0.84 ± 0.18 | 38 | p<0.05 |

a Tumors were weighed 30 days following administration of C6 rat glioma cells into nude mice. Indicated values are mean of (n) animals ± SEM.
b Significance of difference between control and treated animals as determined by Student’s test.

EXAMPLE 5: Disulfiram inhibits Lewis lung metastasis in the i.v. model.

[0055] To distinguish between the effect on the migration of the cells from the main tumor and the development of a tumor from a metastatic foci, the i.v. metastasis model was used as described in section (f) of materials and Methods above. D122 cells were injected i.v. to the tail of C57/B1 male mice. Three days after, disulfiram was administered to the mice per os 3 times per week, at concentrations of 13-40 μg/mouse. As shown in Fig. 7, disulfiram inhibited 83-95% of the development of metastatic foci in the lungs. The fact that disulfiram was highly inhibitory to lung metastasis even in the i.v. model indicates that its inhibitory effect does not occur on the migratory phase of the cells but rather during foci development.

References


by a human colon carcinoma cell line expressing relatively large amounts of a class-3 aldehyde dehydrogenase. Biochim. Pharmacol 48, 1943-52.
multistage carcinogenesis model. Carcinogenesis 9, 1547-51.
39. Wattenberg, L. W., and Fiala, E. S. (1978). Inhibition of 1,2-dimethylhydrazine-induced neoplasia of the large 
some regions of programmed cell death in the chick embryo. Int. J. Dev. Biol 38, 673-82.

Claims

1. Use of disulfiram for the preparation of a pharmaceutical composition, for the treatment of an angiogenesis-dependent 
disease selected from an ophthalmologic, or a neurologic cerebrovascular disorder, a neoplasm, or hypertrophic 
scars, wherein the neoplasm is a leukaemia or a solid tumor selected from bladder, breast, cervix, ear, esophagus, 
kidney, larynx, liver, lung, ovary, pancreas, prostate, skin, stomach, thyroid, urethra and uterus carcinomas.

2. The use according to claim 1, wherein the ophthalmologic disorder is diabetic retinopathy, corneal graft neovas-
cularization, neovascular glaucoma, trachoma, or retrolental fibroplasia.

3. The use according to claim 1, wherein the neurologic cerebrovascular disorder is arteriovenous malformation.

4. The use according to claim 1, for the prevention of tumor metastasis.

5. The use according to any one of claims 1 to 4, wherein the pharmaceutical composition is suitable for oral admin-
istration.

6. Use of disulfiram for the preparation of a pharmaceutical composition for preventing cell hyperproliferation and 
formation of clots along or around medical devices.

7. The use according to claim 6, wherein the medical device is a stent, catheter, cannula, or an electrode.

8. The use according to claim 6 or 7, wherein disulfiram is systematically administered to a patient in which such a 
device has been inserted.

9. The use according to claim 6 or 7, wherein the medical device is coated with disulfiram prior to its insertion into a 
patient.

10. A disulfiram-coated medical device.

11. The disulfiram-coated medical device according to claim 10, wherein the medical device is a stent, catheter, cannula, 
or an electrode.
12. The use according to any one of claims 1 to 5, wherein the pharmaceutical composition comprises as an active ingredient 1 to 50 mg disulfiram in unit dosage form.

13. The use according to any one of claims 1 to 5, wherein the daily dosage to be administered to a human is 1 to 50 mg.

14. A pharmaceutical composition comprising 1 to 50 mg disulfiram as an active ingredient in oral unit dosage form.

Patentansprüche


2. Verwendung nach Anspruch 1, wobei die ophthalmologische Erkrankung diabetische Retinopathie, Neovaskularisation bei Hornhautübertragung, ein neovaskuläres Glaukom, ein Trachom oder eine retrolentale Fibroplasie ist.

3. Verwendung nach Anspruch 1, wobei die neurologische cerebrovaskuläre Erkrankung eine arteriovenöse Fehlbildung ist.

4. Verwendung nach Anspruch 1 zur Vorbeugung vor Tumormetastasen.

5. Verwendung nach einem der Ansprüche 1 bis 4, wobei das Arzneimittel zur oralen Verabreichung geeignet ist.


7. Verwendung nach Anspruch 6, wobei die medizinische Vorrichtung ein Stent, ein Katheter, eine Kanüle oder eine Elektrode ist.

8. Verwendung nach Anspruch 6 oder 7, wobei Disulfiram systemisch an einen Patienten verabreicht wird, dem solch eine Vorrichtung eingepflanzt wurde.


10. Medizinische Vorrichtung, welche mit Disulfiram überzogen ist.

11. Medizinische Vorrichtung, welche mit Disulfiram überzogen ist, nach Anspruch 10, wobei die medizinische Vorrichtung ein Stent, ein Katheter, eine Kanüle oder eine Elektrode ist.

12. Verwendung nach einem der Ansprüche 1 bis 5, wobei das Arzneimittel als Wirkstoff 1 bis 50 mg Disulfiram in einer Einzeldosisform enthält.

13. Verwendung nach einem der Ansprüche 1 bis 5, wobei die tägliche Dosis, welche an einen Menschen verabreicht werden soll, 1 bis 50 mg beträgt.

14. Arzneimittel, umfassend 1 bis 50 mg Disulfiram als Wirkstoff in einer oralen Einzeldosisform.

Revendications

1. Utilisation du disulfiram pour la préparation d’une composition pharmaceutique destinée au traitement d’une maladie dépendante de l’angiogenèse sélectionnée parmi un trouble ophthalmique ou cérébrovasculaire neurologique, un néoplasme ou des cicatrices hypertrophiques, dans laquelle le néoplasme est une leucémie ou une tumeur solide

2. Utilisation selon la revendication 1, dans laquelle le trouble ophtalmique est une rétinopathie diabétique, une néovascularisation d’une greffe de la cornée, un glaucome néovasculaire, un trachome, ou une fibroplasie rétrolentale.

3. Utilisation selon la revendication 1, dans laquelle le trouble cérébrovasculaire neurologique est une malformation artério-veineuse.

4. Utilisation selon la revendication 1 pour la prévention d’une métastase tumorale.

5. Utilisation selon l’une quelconque des revendications 1 à 4, dans laquelle la composition pharmaceutique est adaptée pour une administration orale.

6. Utilisation du disulfiram pour la préparation d’une composition pharmaceutique destinée à la prévention de l’hypertrofie cellulaire et de la formation de caillots le long ou autour des dispositifs médicaux.

7. Utilisation selon la revendication 6, dans laquelle le dispositif médical est un stent, un cathéter, une canule, ou une électrode.

8. Utilisation selon la revendication 6 ou 7, dans laquelle le disulfiram est administré de manière systémique à un patient dans lequel un tel dispositif a été introduit.

9. Utilisation selon la revendication 6 ou 7, dans laquelle le dispositif médical est recouvert de disulfiram avant son introduction dans un patient.

10. Dispositif médical recouvert de disulfiram.

11. Dispositif médical recouvert de disulfiram selon la revendication 10, dans lequel le dispositif médical est un stent, un cathéter, une canule, ou une électrode.

12. Utilisation selon l’une quelconque des revendications 1 à 5, dans laquelle la composition pharmaceutique comprend en tant que principe actif 1 à 50 mg de disulfiram sous forme de dosage unitaire.

13. Utilisation selon l’une quelconque des revendications 1 à 5, dans laquelle le dosage journalier à administrer à un humain est de 1 à 50 mg.

14. Composition pharmaceutique comprenant 1 à 50 mg de disulfiram en tant que principe actif sous forme de dosage unitaire oral.
control (saline)

FGF (10 ug)

D (60 ug)

Fig. 1
Fig. 2

- **A**: BCE
- **B**: BMMC
- **C**: MK
- **D**: C6 glioma

DNA SYNTHESIS as a function of concentration (%) of control.
Fig. 4
Fig. 6
**Fig. 7**

- **Tumor Weight (mg)**
  - CONTROL
  - D(13ug)
  - D (40 ug)

- *P = 0.037*
- *P = 0.023*
REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

• US 4870101 A, Ku and Doherty [0008]
• US 1989 A [0008]
• WO 9530415 A [0009]

Non-patent literature cited in the description

• COHEN, J. D. ; ROBINS, H. I. Cytotoxicity of diethylthiocarbamate in human versus rodent cell lines. Invest. New Drugs, 1990, vol. 8, 137-42 [0056]
• FIALA, E. S. Investigations into the metabolism and mode of action of the colon carcinogens 1,2-dimethylhydrazine and azoxymethane. Cancer, 1977, vol. 40, 2436-45 [0056]
• FOLKMAN, J. What is the evidence that tumors are angiogenesis dependent?. J. Natl. Cancer Inst, 1990, vol. 82, 4-6 [0056]
- ROTSTEIN, J. B.; SLAGA, T. J. Effect of exogenous glutathione on tumor progression in the murine skin multistage carcinogenesis model. Carcinogenesis, 1988, vol. 9, 1547-51 [0056]
• **WATTENBERG, L. W.; FIALA, E. S.** Inhibition of 1,2-dimethylhydrazine-induced neoplasia of the large intestine in female CF1 mice by carbon disulfide. *J. Natl. Cancer Inst*, 1978, vol. 60, 1515-7 [0056]