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Eriochrome Black T inhibits endothelial cell growth through S-phase blockade

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Abstract

We used human umbilical vein endothelial cells (HUVEC) cultures to investigate in vitro the antiproliferative effects of suramin and of its analogue, Eriochrome Black T. The cell cycle phases of interest were characterised with specific immune sera raised against cyclin D_1, cyclin E and proliferating nuclear cell antigen (PCNA). Simultaneous detection of two cell cycle markers was ensured by double colour immunofluorescence. Both compounds inhibited the endothelial cell growth while Eriochrome Black T was more potent than suramin. Suramin induced HUVEC to accumulate in G1-phase as an increase of the number of cells expressing both cyclin D_1 and PCNA was observed. Eriochrome Black T preferentially blocked them in the early S-phase, as it increased the proportion of cyclin E positive cells. These results suggest that in addition of its more potent antiproliferative effect on endothelial cell growth, Eriochrome Black T acts at another molecular level than suramin.

Keywords: Suramin; Eriochrome Black T; Endothelial cell; Cyclin; Cell cycle

1. Introduction

Malignant solid tumours depend on neovascularisation for their survival, growth and metastasis (Folkman, 1990, 1995). Angiogenesis inhibitors are therefore expected to be potential anticancer agents, able to control angiogenesis-dependent tumour growth and metastasis (Folkman, 1990; Gastl et al., 1997). Suramin, a polysulfonaphtyl urea compound developed in the 1920s for the treatment of protozoan infections (Voogd et al., 1993), is antiangiogenic both in vitro and in vivo (Danesi et al., 1993; Gagliardi et al., 1992). Its mechanism of action is complex. It has indeed been shown to inhibit growth factor binding (La-Rocca et al., 1990), DNA topoisomerase II (Bojanowski et al., 1992), DNA polymerase (La-Rocca et al., 1990), heparanase (Nakajima et al., 1991) and protein kinase C (Hensey et al., 1989). In addition, it behaves as a G-protein coupled receptors' antagonist (Beindl et al., 1996) and as a purinoceptor antagonist (Ralevic and Burnstock, 1998). Numerous side effects are, thus, likely limiting its therapeutic use (Voogd et al., 1993; La-Rocca et al., 1990). Therefore, the search for suramin analogues with less toxicity and better activity seems to be a promising approach for the development of new angiogenesis inhibitors. In this framework, Morris et al. (1997) demonstrated that Eriochrome Black T, that is structurally related to suramin, inhibited endothelial cell proliferation in vitro and, using the chorioallantoic membrane assay, observed antiangiogenic effects in vivo (Morris et al., 1997). However, these authors did not investigate whether this growth inhibition was the consequence of an increased cell death, or the result of interference with the cell cycle.

Blockade of the cell cycle may occur at many different steps, each of these having its own cytological implications. Indeed, intercinesis is under the control of several intracellular enzymes, formed of a regulatory subunit (cyclin) and a catalytic subunit, cyclin-dependent kinase (cdk) (Johnson and Walker, 1999; Yang and Kornbluth, 1999). Each cyclin–cdk complex is specific for a given cell cycle phase. The D- and E-type cyclins and the corresponding cdks are recognised to be key regulators of

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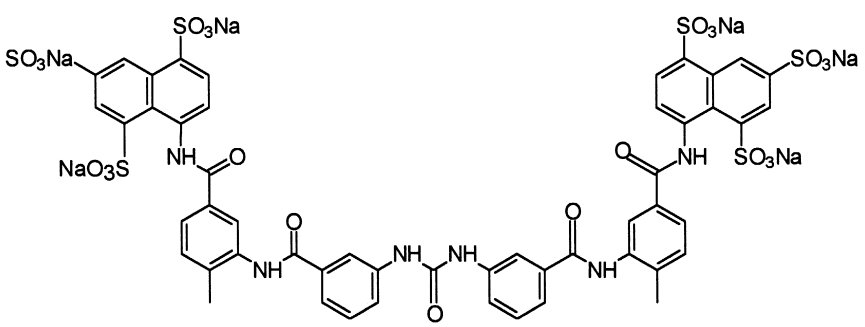
G1 to S-phase progression in mammalian cells (Johnson and Walker, 1999; Yang and Kornbluth, 1999). Cyclin D's are the first cyclins induced when cells in G0 are stimulated to enter the cell cycle (Johnson and Walker, 1999). Their expression is controlled by the presence of growth factors (Yang and Kornbluth, 1999) and their intracellular distribution varies during the different steps of intercinesis. The level of nuclear cyclin D–cdk complexes increases during G1 but when the S-phase begins, these complexes are not further exported to the nucleus and accumulate within the cytoplasm (Yang and Kornbluth, 1999). D-type cyclins bind to and activate cdk4, cdk5 or cdk6 (Johnson and Walker, 1999). Cyclin E is crucial for initiation of DNA synthesis (Johnson and Walker, 1999), and association with cdk2 is required for the transition from G1 to S-phase (Johnson and Walker, 1999). In contrast with D-type cyclins, cyclin E is concentrated in the cells' nucleus during the S-phase only, reflecting its role in promotion and maintenance of DNA replication (Yang and Kornbluth, 1999). A third protein, the proliferating nuclear cell antigen (PCNA), is a component of DNA replication complex, functioning as the accessory protein for DNA polymerase δ responsible for the replication of chromosomal DNA (Kelman, 1997). Originally isolated as a protein with elevated levels during S phase, PCNA is constitutively expressed in actively growing cells, but undetectable in quiescent and senescent cells (Kelman, 1997). Its intranuclear distribution varies along the S-phase (Kelman, 1997). As a consequence, the expression of these control proteins can be used to characterise the amount of cells that are, respectively, in G1 or S phase in a given population.

The aim of the present study was to investigate whether suramin and Eriochrome Black T inhibited endothelial cell growth inhibition by interfering with the cell cycle, and to identify the affected step. We used human umbilical vein endothelial cell cultures. These are the most common source of human endothelial cells (Jaffe et al., 1973), and as they are widely used to evaluate in vitro antiproliferative effects of new potential antiangiogenic compounds, the results are easy to compare with data obtained with other antiproliferative agents. In our experiments, the cell cycle phases of interest were characterised using specific immune sera raised again cyclin D_1, cyclin E and PCNA. Simultaneous detection was ensured by double colour immunofluorescence. We demonstrated that suramin induced human umbilical vein endothelial cells (HUVEC) to accumulate in G1-phase, while Eriochrome Black T preferentially acted by blocking them in the early S-phase.

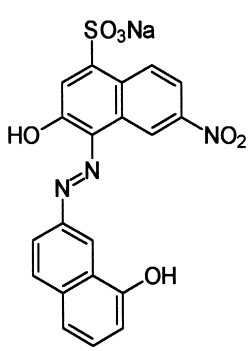
2. Materials and methods.

2.1. Chemicals

Suramin was a gift from Bayer (Germany). Eriochrome Black T was a gift from Institut de Recherches Servier (France; Fig. 1). Being a polyanionic substituted poly-



Suramin



Eriochrome Black T

Fig. 1. Chemical structure of suramin and Eriochrome Black T.

phenyl compound, Eriochrome Black T is considered as structurally related to suramin.

2.2. Cells

HUVEC were isolated according to the method of Jaffe et al. (1973). Briefly, endothelial cells were detached from blood vessel by collagenase treatment for 15 min at 37°C (0.5 mg/ml in RPMI medium; Gibco) and seeded in gelatine-coated flasks (Nunc), in ECGM2 medium supplemented with 10 ng/ml of epidermal growth factor (EGF; PromoCell). All experiments were carried out between passages one and three.

2.3. Cell proliferation assay

Inhibition of cell proliferation by test compounds was measured using the microculture tetrazolium assay. In brief, cells were seeded in 96-well gelatine-coated microplates, at the appropriate densities to maintain exponential proliferation in control cells and linearity between optical density (OD) and the number of viable cells. Plates were exposed to graded concentrations of test compounds (eight serial dilutions in triplicate) for 72 h. Medium was replaced with fresh RPMI medium (Gibco) and 15 μl of a 5 mg/ml MTT solution was added to each well. After 4 h of incubation, medium is eliminated and formazan crystals dissolved in dimethysolfoxid (DMSO; Merck). OD of each well solution is then determined using a microplate reader (Dynatech) at 570 nm. IC_{50} values corresponded to the compound concentration that reduced the treated cells OD by 50% as compared to control cells.

2.4. Immunohistochemistry

Rabbit polyclonal antibody to cyclin D_1, goat polyclonal antibody to cyclin E and mouse monoclonal antibody to PCNA were obtained from Santa-Cruz (CA, USA). Texas Red-conjugated horse anti-mouse anti-Immunoglobulin (anti-IgG; $H+L$), biotinylated goat anti-rabbit IgG ($H+L$), biotinylated rabbit anti-goat IgG ($H+L$) and Fluorescein avidin D were obtained from Vector Laboratories (CA, USA). HUVEC were cultured on gelatine-coated glass slides in ECGM2 medium with or without test compounds. After 72 h of treatment, the cell cultures were washed in phosphate buffer saline (PBS) and fixed with −20°C absolute methanol for 10 min. Unspecific protein binding was prevented by 15 min incubation with blocking reagent (Roche Diagnostics). The cells were then incubated for 36 h at 4°C with primary antisera. Primary antibodies were either a mixture of mouse anti-PCNA (1:1000) and goat anti-cyclin D_1 (1:500) or anti-PCNA and rabbit anti-cyclin E (1:100) diluted in PBS. After washing, cells were incubated for 30 min at room temperature with Texas Red-conjugated anti-mouse IgG (1:100) and biotinylated anti-goat IgG (1:100) or with Texas Red-conjugated anti-mouse IgG (1:100) and biotinylated anti-rabbit IgG (1:100). All the secondary antibodies were diluted in PBS. Cells were finally incubated for 30 min at room temperature with Fluorescein avidin D and mounted with vectashield (Vector). Slides were washed three times in PBS between the different steps. Fluorescent controls were incubated without the primary or secondary antibodies and/or without Fluorescein avidin D. No significant cross-labelling or non specific labelling was observed.

A minimum of 400 cells was counted for each slide. Six slides were examined for each experimental condition. Nuclear staining only was taken into account.

2.5. Statistical analysis

For cell proliferation assay, curves analysis, S.E.M. and IC_{50} were calculated using GraphPad Prism software. For immunohistochemistry, statistical analysis (means, S.E.M.) were also performed using GraphPad Prism software, p value was calculated using the non parametric Mann–Whitney test.

3. Results

3.1. Inhibition of cell proliferation

Antiproliferative effects of suramin and Eriochrome Black T were evaluated on HUVEC after 72 h of treatment. As shown in Fig. 2, both drugs were able to reduce cellular growth. Suramin reduced HUVEC proliferation

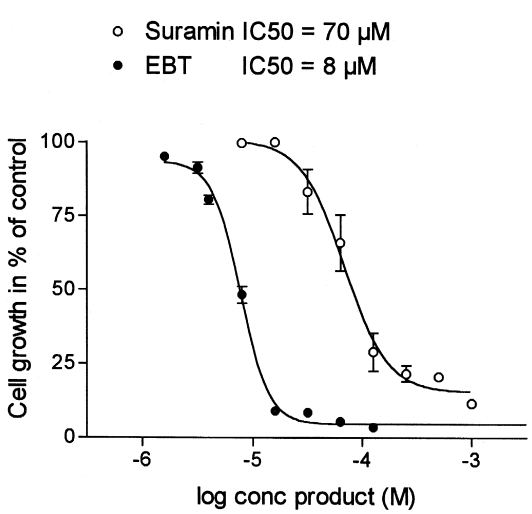


Fig. 2. Growth of HUVEC after 72 h of treatment with increasing concentrations of suramin (open circle) and Eriochrome Black T (black circle). Results represent the means ± S.E.M. of three independent experiments.

with an IC_{50} value of 70 μM, while for Eriochrome Black T, more active than suramin, the IC_{50} value was 8 μM.

3.2. Immunohistochemistry

Double colour immunohistochemistry was performed on HUVEC sub-cultures, cultured with normal medium or with the same medium plus suramin or Eriochrome Black T.

3.2.1. Co-expression of cyclin D_1 and PCNA

The labelled cells were subdivided in five categories based on the relative nuclear staining intensities: cyclin D_1 immunoreactivity alone (a), double labelling with promi-

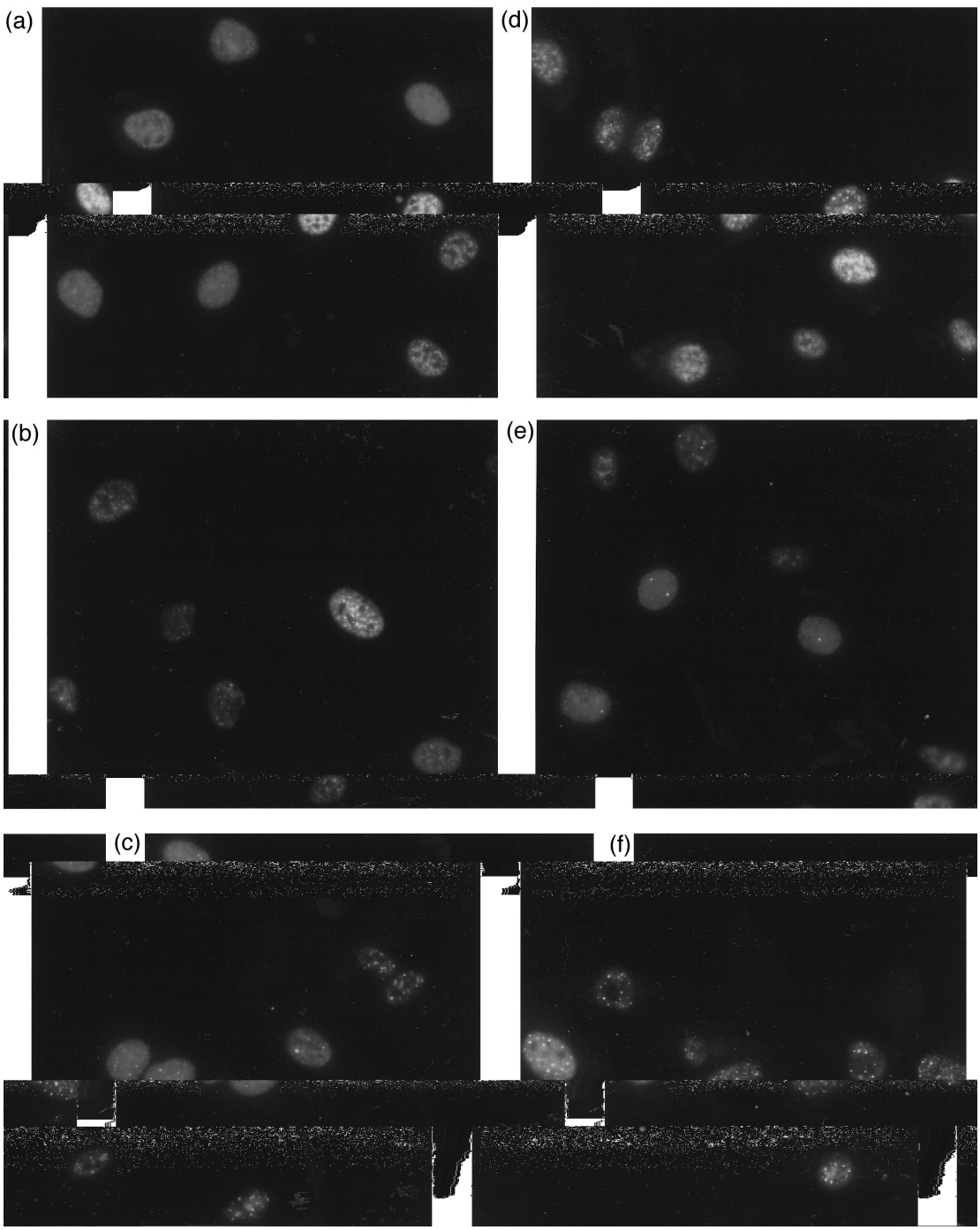


Fig. 3. Visualisation by double colour immunofluorescence of cyclin D_1 (green) and PCNA (red) expression in HUVEC cells (left panel). Pictures show representatives fields of control cells (a) and treated cells with suramin 50 μM (b) or Eriochrome Black T 5 μM (c) during 72 h. Visualisation by double colour immunofluorescence of cyclin E (green) and PCNA (red) expression in HUVEC cells (right panel). Pictures show representatives fields of control cells (d) and treated cells with suramin 50 μM (e) or Eriochrome Black T 5 μM (f) during 72 h.

Table 1
Expression of cyclin D_1 and PCNA in HUVEC cells after 72 h of treatment with suramin 50 μM or Eriochrome Black T 5 μM. Results represent percent of positive nuclei

	Cyclin D_1 + PCNA −	Cyclin D_1 + PCNA +			PCNA + Cyclin D_1 −
		Cyclin D_1 > PCNA	Cyclin D_1 = PCNA	Cyclin D_1 < PCNA	
Control	0	6.2 ± 0.54	27 ± 1.7	8.3 ± 1.3	58 ± 1.5
Suramin (50 μM)	0	15 ± 3.5**	28 ± 2.9	15 ± 1.9*	41 ± 5.5*
Eriochrome Black T (5 μM)	0	14 ± 1.5**	47 ± 2.8**	15 ± 1.5**	26 ± 1.7**

* $p < 0.05$ measured with the Mann–Whitney test, $n = 6$, means ± S.E.M.
** $p < 0.01$ measured with the Mann–Whitney test, $n = 6$, means ± S.E.M.

nent cyclin D_1 immunoreactivity (b), equal cyclin D_1 and PCNA immunoreactivities (c), prominent PCNA (d), and lastly, PCNA immunoreactivity without cyclin D_1 (e). In control cultures (Fig. 3a), most of the cells were expressing PCNA alone or prominently. About one third of the cells showed comparable intensities of cyclin D_1 and PCNA immunoreactivities and none of them were only cyclin D_1 immunoreactive. Suramin (Fig. 3b) induced a slight increase in the proportion of cells expressing simultaneously high cyclin D_1 levels and low PCNA levels. It also induced a comparable decrease of the number of cells expressing PCNA alone that was observed in these cultures. The results obtained with Eriochrome Black T were clearly different (Fig. 3c): about half of the cells had comparable cyclin D_1 and PCNA immunoreactivities, while the percentage of cells expressing PCNA alone was markedly decreased. No difference was observed between suramin- and Eriochrome Black T-treated cultures when comparing the percentage of cells that were more immmunoreactive for cyclin D_1 than for PCNA (Table 1 and Fig. 3).

3.2.2. Co-expression of PCNA and cyclin E

Most control cells were immunoreactive for PCNA alone, but only 10% of the cells expressed in the addition of cyclin E (Fig. 3d). Suramin did not modify this distribution (Fig. 3e). In contrast, Eriochrome Black T increased significantly the number of HUVEC with double immunoreactivity (Fig. 3f, Table 2).

Table 2
Expression of cyclin E and PCNA in HUVEC cells after 72 h of treatment with suramin 50 μM or Eriochrome Black T 5 μM. Results represent percent of positive nuclei

	Cyclin E + PCNA −	Cyclin E + PCNA +	PCNA + Cyclin E −
Control	0	10 ± 1.8	90 ± 1.8
Suramin 50 μM	0	10 ± 1.7	90 ± 1.7
Eriochrome Black T 5 μM	10 ± 1.0**	32 ± 2.2**	58 ± 1.6**

** $p < 0.01$ measured with the Mann–Whitney test, $n = 6$, means ± S.E.M.

4. Discussion

First proposed in the 1970s by Folkman (1971), the concept of antiangiogenic therapy to cure cancer still constitutes a promising hypothesis to develop new class of anticancer agents. Several compounds, such as the fumagillin analogue TNP-470 (Ingber et al., 1990), thalidomide (D'Amato et al., 1994) and pentosan sulfate (Pluda, 1997), have been shown to be antiangiogenic both in vitro and in vivo. They are currently evaluated in phase II–III clinical trials (Nelson, 1998). Suramin also has antiangiogenic properties (Danesi et al., 1993; Gagliardi et al., 1992), but a low therapeutic index in clinical trials (La-Rocca et al., 1990; Voogd et al., 1993).

In this study, we show that Eriochrome Black T is a more potent inhibitor of human umbilical vein endothelial cell proliferation ($IC_{50} = 8$ μM) than suramin ($IC_{50} = 70$ μM), and despite some structural similarities, they have different impacts on the cell cycle.

The nuclear staining pattern of PCNA in methanol-fixed cells can be considered as a general cycle marker (Baptist et al. 1993). Quiescent cells (G0) are barely stained, while cells in G1-phase display a diffuse but gradually increasing nuclear staining that becomes speckled when cells reach S-phase. Cells in G2-phase are characterised by strong cloudy labelling of nuclei. At prophase, PCNA becomes progressively cytoplasmic (Baptist et al., 1993). In contrast, nuclear staining of cyclin D_1 is limited to the G1- and early S-phase (Yang and Kornbluth, 1999), and cyclin E is characteristic of S-phase (Yang and Kornbluth, 1999).

In the control cultures (performed in the presence of growth factors), a high number of cells are undergoing cell cycling (PCNA +). Among them, less than 10% of the cells are in early G1-phase (cyclin D_1 > PCNA), and 25% are in late G1 (PCNA + +, cyclin D_1 + +, cyclin E −). About 10% of the cells are encountered in the early S-phase (PCNA + +, cyclin D_1 + +, cyclin E +). The

65% PCNA > cyclin D_1 and PCNA + cyclin D_1 − cells represent cells in the late S-phase and in the G2-phase.

Suramin and Eriochrome Black T significantly increased the number of cells expressing both PCNA and cyclin D_1, with a labelling pattern similar to the pattern observed in G1-phase. This increase was more important with Eriochrome Black T, and accompanied by a decrease of the proportion of cells typical of late intercinesis. The PCNA-cyclin D_1 pattern and the increased proportion of cyclin E positive cells indicate that Eriochrome Black T induces, in addition, a twofold increase in the cells found between phase G1 and S. This strongly suggested that Eriochrome Black T, but not suramin, inhibited the entry or progression in the S-phase. It is well known that growth factors act during G1-phase by stimulating cell cycle progression. The results suggesting that suramin interfered in the early phase of cell cycle were in good agreement with the other observations, describing that this drug acts through inhibition of growth factor binding (La-Rocca et al., 1990). The present results also showed that, in contrast with suramin, Eriochrome Black T would inhibit DNA replication. On the other hand, as growth factors do not participate in S-phase progression, it also suggest that Eriochrome Black T would probably not interfere with growth factors. It might inhibit DNA polymerase or DNA topoisomerase II, as suggested by Morris et al. (1997), or, like the other colorants, act as a DNA intercalator.

In conclusion, we have shown that Eriochrome Black T was a more potent inhibitor of human umbilical vein endothelial cell proliferation than suramin, a structurally related compound. Moreover, we demonstrate that it acts at another molecular level than suramin, by blocking early DNA replication.

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