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Suramin, an anti-cancer drug, inhibits protein kinase C and induces differentiation in neuroblastoma cell clone NB2A

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Protein kinase C purified from rat brain was found to be inhibited by suramin, a substance used originally in the therapy of antitrypanosomic infections and more recently proposed as antineoplastic agent. The inhibition of suramin was competitive with one of the substrates of the enzyme, ATP with a K_i of 10 μM. At concentrations adequate to inhibit the isolated enzyme, suramin was shown to slow the rate of proliferation of neuroblastoma NB2A cells in vitro and to induce their differentiation as evidenced by typical morphological changes.

Protein kinase C; Neuroblastoma; Suramin; Differentiation; Antineoplastic therapy; Proliferation inhibition

1. INTRODUCTION

Suramin, a polysulphonated naphthylurea, was the first widely accepted antiparasitic drug to be developed and is still one of the most commonly prescribed antitrypanosomal drugs. The inhibition of protein kinases in Trypanosomatida by suramin has been demonstrated [1,2]. In recent studies suramin was used in the chemotherapy of acquired immune deficiency syndrome (AIDS) [3] but host toxicity precluded its usefulness. The drug is currently under investigation for the treatment of advanced malignancy and has exhibited antitumor activity in a number of systems [4]. Protein kinase C[1], a Ca^{2+}- and phospholipid-dependent protein kinase has been implicated in numerous biological processes including tumor promotion and differentiation (for review see [5,6]). Since suramin has been shown to interact with other kinases we have considered and tested the possibility that suramin antitumor activity is due to its inhibition of protein kinase C.

2. MATERIALS AND METHODS

2.1. *Materials*

Suramin, a generous gift from the World Health Organization (Geneva, Switzerland), was prepared as a stock solution of 10 mg/ml in distilled water and stored at −20°C. Human erythrocytes were obtained from the Swiss Red Cross and calf thymus histone III-S was isolated by the procedure of De Nooij and Westenbrink [7]. Leupeptin was from Fluka (Buchs, Switzerland) and phosphatidylserine from Lipid Products (S. Nutfield, Surrey, England). Cell culture medium and serum were from Gibco (Grand Island, NY). [γ-^{32}P]ATP was supplied by Amersham (Buckinghamshire, England) and all other products were reagent grade from Merck (Darmstadt, FRG) or Fluka.

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Abbreviations: PKC, protein kinase C; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; EGTA, [ethylenebis(oxyethylenenitrilo)]tetra-acetic acid; EDTA, ethylenediamine tetra-acetic acid; DTT, dithiothreitol

2.2. *Purification of protein kinase C*

Protein kinase C was purified from rat brain by a modification of the procedure described by Wolf et al. [8]. Purification was carried out by adsorption to and elution from inside-out erythrocyte vesicles followed by HPLC on a Mono Q 5/5 column.

Inside out erythrocyte vesicles were prepared from human erythrocytes by hypotonic lysis in 10 mM Tris-HCl, pH 7.6 at 4°C followed by incubation in 0.1 mM EDTA for 45 min at 37°C.

Five rat brains were homogenized in a Ca^{2+} buffer (20 mM Tris-HCl, pH 7.4 at 4°C, 20 μg/ml leupeptin, 10 mM DTT, 1 mM $CaCl_2$). The enzyme was then released from the particulate fraction in EGTA-EDTA buffer (20 mM Tris-HCl, pH 7.6 at 4°C, 20 μg/ml leupeptin, 10 mM DTT, 5 mM EGTA, 2 mM EDTA) and this soluble enzyme preparation was incubated with inside-out erythrocyte vesicles (20 mg/ml protein) in the presence of 1 mM $CaCl_2$ and 3 mM $MgCl_2$, in a Tris buffer (20 mM Tris-HCl, pH 7.6 at 25°C, 20 μg/ml leupeptin, 2 mM DTT and 1 mg/ml polyethylene glycol 20000). After incubation at room temperature for 15 min the vesicles with protein kinase C bound were resuspended in EGTA-EDTA buffer (20 mM Tris-HCl, 1 mM DTT, 2 mM EGTA, 1 mM EDTA) causing release of protein kinase C from the membranes. Following removal of the vesicles by centrifugation the supernatant, enriched in protein kinase C, was applied to a Mono Q 5/5 column equilibrated with a 20 mM Tris-HCl buffer, pH 7.6 at 4°C containing 1 mM DTT, 2 mM EGTA and 1 mM EDTA. Protein kinase C was eluted using a discontinuous NaCl gradient with peak activity eluted between 160 mM and 170 mM NaCl. The protein was stable for several months when stored at −70°C in the presence of 1 mg/ml polyethylene glycol 20000 and 10% glycerol.

Protein concentrations were determined by the method of Bradford [9] using bovine serum albumin as a standard.

2.3. *Assay of PKC activity*

Ca^{2+} and phospholipid dependent protein kinase activity was

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determined using an assay mixture (total volume: 0.25 ml) containing 20 mM Tris-HCl, pH 7.5 at 30°C, 5 mM Mg-acetate, 0.2 mg/ml histone III-S, 1 mM $CaCl_2$, 0.04 mg/ml phosphatidylserine and 10 μM [γ-^{32}P]ATP (100000 cpm/nmol). The reaction was started by the addition of 20 μl of enzyme fraction to the assay mixture and carried out at 30°C for 8 min. After termination of the reaction with 0.35 ml of cold 12% trichloroacetic acid, 2% sodium pyrophosphate and 10 mg/ml bovine serum albumin, the precipitated protein was quantitatively transferred to filters (Millipore HA, 0.45 μM) which were then counted in 4 ml liquid scintillation cocktail. One unit of kinase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol ^{32}P into histone III-S per min. The enzyme preparation had a specific activity of 1000 nmol/min/mg.

2.4. *Cell culture*

Mouse neuroblastoma cells, clone NB2A, derived from the C-1300 tumor cell line [10] were kindly supplied by Dr U. Wiesmann (Kinderklinik, Universität Bern). Cells were cultured in DMEM supplemented with 5% FCS and antibiotics (penicillin 60 U/ml and streptomycin 60 μg/ml). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Differentiation and growth curves: To study the effect of suramin on morphological changes, cells (5×10^4/ml) were plated in Falcon 6-well tissue culture plates and the drug (100 μM) was added 24 h later. The medium or drugs were not changed during the course of the experiment. Viable cells were determined by the trypan blue dye exclusion method.

To study the effect of suramin on growth, cells (2×10^4/ml) were plated in Falcon 24-well tissue culture plates in DMEM containing 0.2% FCS and allowed to recover for 24 h before treatment. Growth of cells was stimulated by changing the medium for that supplemented with 5% FCS, in the presence or absence of suramin at 100 μM. Cells were removed from dishes every 12 h and counted in a haemocytometer.

3. RESULTS AND DISCUSSION

The inhibition of purified rat brain protein kinase C by suramin is shown in fig.1. A half-inhibitory concentration of 30 μM, for the assay conditions employed, was calculated. The lack of inhibition, and sometimes the slight activation, of the enzyme at concentrations below 10 μM is, at present, not clear. A kinetic analysis of the inhibition of PKC by suramin is shown in fig.2. The type of inhibition appears to be competitive with respect to ATP (K_i = 10 μM). The data points were not fitted optimally by a straight line, suggesting the existence of heterogeneity in the preparation. Such a phenomenon may be consistent with a different degree of inhibition by suramin of the different isoforms of the enzyme. The lack of inhibition at low concentrations of suramin may be related to the same phenomenon. The inhibition of the Na^+,K^+-ATPase [11] and Ca-ATPase [12] by suramin, together with the present results are consistent with previous kinetic and computer modelling studies of protein kinases from Trypanosomatida. In these studies suramin was shown to inhibit protein kinase I by competing for the ATP binding site [1] and computer modelling of phosphoglycerate kinase have shown how suramin could be fitted to the ATP binding site and thereby inhibit kinase activity [13].

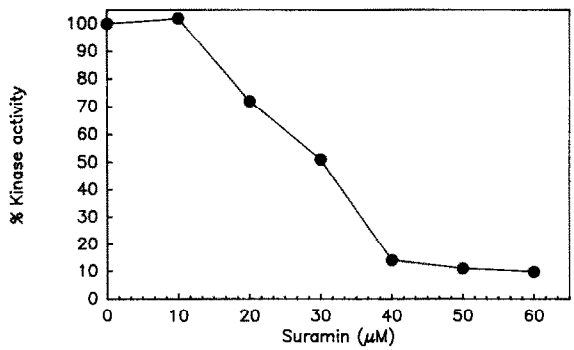

Fig.1. Concentration-dependent inhibition of protein kinase C activity by suramin. Results are the mean of triplicate experiments. Assay conditions were as described in section 2.

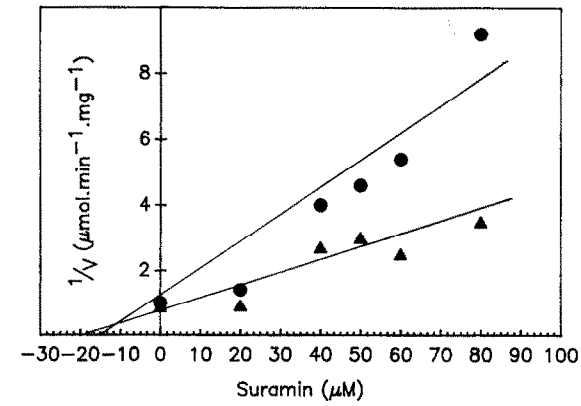

Fig.2. Dixon plot of the inhibition of PKC by suramin. PKC activity was measured in the presence of different concentrations of suramin at two ATP concentrations, 10 μM (●) and 40 μM (▲). Results are the mean of triplicate experiments. Assay conditions were as described in section 2.

To investigate the effect of suramin on intact cells, quiescent cultures of neuroblastoma cells (clone NB2A) maintained in 0.1% FCS were stimulated to growth by the addition of 5% FCS to the culture medium. In untreated cultures an increase of 20-fold in the cell

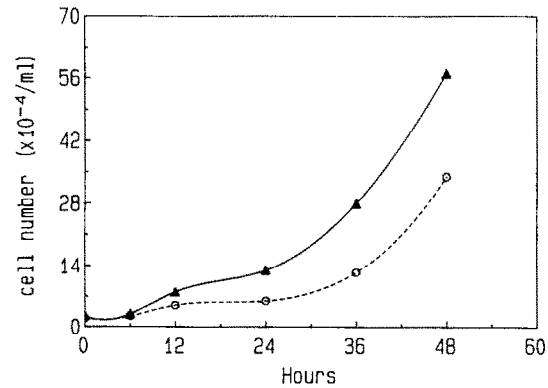

Fig.3. Effect of suramin on serum-induced proliferation of NB2A cells in culture. The quiescent cultures were stimulated by changing the medium to one containing 5% FCS, with (○) or without (▲) suramin (100 μM). At the indicated times, the number of cells was determined as described in section 2.

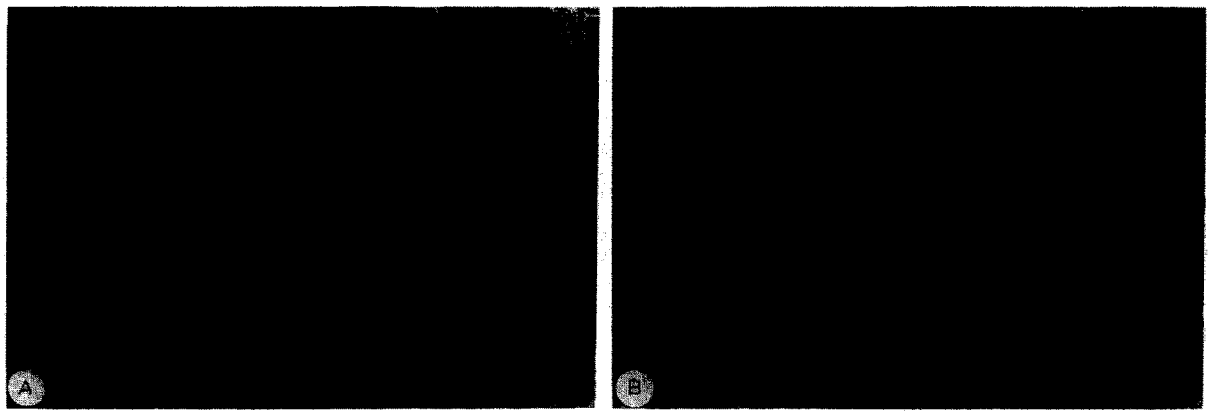

Fig.4. Effect of suramin on the morphology of NB2A neuroblastoma cells. Cells were plated at 1×10^5/well in 6-well plates and suramin was added 24 h later in the presence of 2% FCS. After 48 h of treatment, the degree of differentiation, as evidenced by the enlargement of neurites, is observed in suramin-treated cells (B), but not in control cells (A).

number was observed after 48 h incubation. The presence of 100 μM suramin in the culture medium produced a diminution of approximately 50% in the rate of cell multiplication in the first 30 h. Subsequently, however, the rate of growth of the cells, in presence and absence of suramin, became equal (fig.3). The conclusion which can be drawn from this experiment is that suramin slows down, at least in the first 30 h of incubation, the process of cell multiplication. Why the effect is not prolonged in time is not, at present, clear. What is, however, evident is that at 48 h of the treatment the cells have changed rather dramatically their morphology. The spherical and generally separated cells emit neurites, which in many cases are branched, indicating a certain degree of differentiation. The cells acquire a polygonal shape and tend to associate more frequently in lumps (fig.4). Whether suramin produces the above described morphological changes by solely interacting with protein kinase C or by multiple interactions with different proteins (possibly utilizing ATP as a substrate) remains to be elucidated. The analogous findings obtained by staurosporine (manuscript in preparation), a potent PKC inhibitor, suggest a central role of PKC in the effects of suramin at a cellular level. The newly described inhibition of PKC through the interaction of suramin with the catalytic domain of the enzyme suggests that the inhibition of growth, and induction of differentiation in neuroblastoma cells, could be mediated through the inhibition of protein kinase C. However, other mechanisms by which this drug exhibits antitumor activity have been reported. Suramin was shown to block the binding of a range of tumor growth factors to their cell surface receptors thereby inhibiting tumor growth [14], and another report suggests the antitumor effect may be due to the drug causing an increase in tissue glycosaminoglycans which have important effects in tumor biology including differentiation [4]. However, since suramin is an inhibitor of protein kinase C we see this as an alternative reason for suramin antitumor activity.

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