Binding of the Urokinase-type Plasminogen Activator to Its Cell Surface Receptor Is Inhibited by Low Doses of Suramin

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The multipotent drug suramin, which is currently being studied as an anticancer agent, was found to inhibit the interaction between the urokinase-type plasminogen activator (u-PA) and its cellular receptor. 50% inhibition of binding was obtained with a suramin concentration between 30 and 60 µg/ml when using U937 cells and a ligand concentration of 0.3 mM. This concentration of the drug is well below the serum levels found in suramin-treated patients. Inhibition of binding was also demonstrated at the molecular level, using chemical cross-linking or an enzyme-linked immunosorbent assay-type technique based on the ligand interaction. The inhibition was not caused by a mere polyanion effect since polysulfates such as heparin, heparan sulfate, and pentosan polysulfate were non-inhibitory or showed only a very weak inhibition. However, polysulfonated compounds with structures resembling suramin (i.e. trypan blue and Evans blue) did prove inhibitory. The inhibition found with suramin showed a concentration dependence consistent with a mixed competitive and noncompetitive mechanism. The off-rate of prebound ligand was accelerated by the drug. It is speculated that the present effect may contribute to the anti-invasive properties of suramin by destroying the cellular potential for localized plasminogen activation and proteolytic matrix degradation.

* This work was supported by the Danish Cancer Society, the Danish Biotechnology program, and the Danish Medical Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: u-PA, urokinase-type plasminogen activator; u-PA receptor, receptor; DFP, diisopropyl fluorophosphate; ATP, amino-terminal fragment of u-PA; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DSS, N,N'-disuccinimidylsuberate; ELISA, enzyme-linked immunosorbent assay.

2 See, e.g. Ref. 27 for formulas.
from phorbol 12-myristate 13-acetate-treated U937 cells (6), were purified by affinity chromatography on immobilized, DFP-treated u-PA (7). Monoclonal antibodies for ELISA-type assays were R2 and R4, directed against distinct epitopes on human u-PA (34), and anti u-PA clone 5, directed against the B-chain of human u-PA (35).

Ligand-binding ELISA—Details of this assay have been published (36). Brieﬂy, the wells of an ELISA plate are coated with a monoclonal anti-u-PAR antibody, clone R4, which is capable of binding u-PAR in the absence of foreign molecules other than antibodies. A suramin dose-response curve was obtained for the binding of ATF to u-PAR in the same concentration range as the ligand concentration was varied. In all experiments, each representation of bound radioactivity is the mean of a duplicate determination.

Chemical Cross-linking Assay—Human HEp-2 cells were cultured, acid-treated, and subjected to detergent lysis as described (6), except that CHAPS (0.5%) was used as the detergent component in the lysis buffer. After centrifugation, the supernatants were analyzed by chemical cross-linking to 125I-labeled ATF, using N,N'-dicycimimidylsulphonate (DSS) (7); varying concentrations of suramin were included in the incubation mixture before cross-linking.

Ligand-binding ELISA—Details of this assay have been published (36). Brieﬂy, the wells of an ELISA plate are coated with a monoclonal anti-u-PAR antibody, clone R4, which is capable of binding u-PAR without affecting subsequent ligand-binding to the receptor (34). After blocking of the remaining protein binding sites, a fixed amount of u-PA is immobilized in each well by addition of 2 mg of pure u-PA from phorbol 12-myristate 13-acetate-treated U937 cells. Subsequently, DFP-treated u-PA (0.1 nM) is added in the presence or absence of suramin. After incubation and washing, the amount of bound ligand is measured using a biotin-labeled monoclonal anti-u-PAR antibody, clone R4, which is capable of binding u-PAR without affecting subsequent ligand-binding to the receptor (34). No such displacing effect was found for suramin in the concentration range used in this work.

RESULTS

Suramin Inhibits Cellular Binding of u-PA and ATF—Cultured human U937 cells (which possess u-PA (5)) were incubated with a ﬁxed concentration of 125I-labeled AT (i.e., the receptor-binding fragment of u-PA (11)) in the presence of varying concentrations of suramin (Fig. 1). The speciﬁc binding of AT (i.e., the receptor-binding fragment of u-PA (11)) in the presence of varying concentrations of suramin (Fig. 1). The speciﬁc binding of AT (i.e., the receptor-binding fragment of u-PA (11)), was completely inhibited by the drug in concentrations above 0.5 mg/ml, with half-maximal inhibition being obtained at a suramin concentration of approximately 50 µg/ml (30–40 µM). When 125I-labeled, DFP-treated u-PA was used instead of AT (5), a similar inhibition curve was obtained for speciﬁc binding (data not shown). When using this ligand at a concentration of 0.3 nM, half-maximal inhibition was reached at a suramin concentration between 30 and 60 µg/ml. Subsequent cell binding assays were, however, performed using ATF; assays with DFP-u-PA are slightly less exact due to the need for subtraction of unspeciﬁc binding (36); see “Experimental Procedures.”

In order to study whether the inhibitory phenomenon could be explained by a simple polyanion effect, certain other polysulfonated or polysulfated compounds were examined. The structures of trypan blue and Evans blue resemble that of suramin, all being polysulfonated, symmetric dinaphthyl compounds. Heparin, heparan sulfate and pentosan polysulfate, being polysulfated polysaccharides, share with these compounds a high negative net charge but show no structural similarity. These reagents were tested for inhibition in the cell binding assay, performed as described above (Table I). None of the polysulfated polysaccharides showed appreciable inhibition in the low concentration range relevant with suramin, thus excluding a polyanion effect as the sole cause of inhibition. On the other hand, the inhibitory potency was not an exclusive property of suramin; the two dyes with structures resembling the drug did indeed show inhibition in the same concentration range.

The Inhibitory Phenomenon Is Due to a Direct Effect on the Molecular Interaction with u-PAR—The inhibitory effect of suramin could be due to either a well deﬁned molecular interference with the ligand-binding process, or to more extensive changes occurring on the cell surface. In order to distinguish between these possibilities, receptor-binding studies were done at the molecular level. Chemical cross-linking is a convenient tool for direct demonstration of the interaction between u-PAR and radiolabeled ATF (6). For examination of the role of suramin in this assay (Fig. 2A), we used detergent lysates of human HEp-2 cells as the source of u-PA. These cells contain high amounts of u-PA (6), leading to a much stronger signal in the cross-linking assay than that obtained with U937 cells. It is evident that suramin inhibits the binding of AT to u-PA in the same concentration range as observed above. The same result was obtained when puriﬁed, recombinant Su-PAR (i.e., a u-PA variant devoid of the membrane-binding part (33)) was used as the source of u-PA material, and the same results were obtained whether puriﬁed reagents or suramin were tested in duplicate. Suramin in the latter experiment ensures that suramin exerts its effect directly on the receptor interaction; i.e., the effect is not mediated through a foreign molecule.

The chemical cross-linking system with DSS is likely to be inert against reagents like suramin which do not contain amino groups or other nucleophilic functionalities. However, in order to completely rule out a trivial effect of the drug on the binding assay as such, it seemed worthwhile to test the inhibition in more than one system. Therefore, we studied the effect of suramin in a ligand-binding ELISA (36) based on measurement of that amount of DFP-treated u-PA which can bind to a certain amount of u-PA, the latter being ﬁxed to a microtiter well through a monoclonal anti-u-PA antibody (34). Using a ﬁxed concentration of u-PA, the effect of suramin on the u-PA:u-PA interaction could be followed directly (Fig. 2B). This experiment conﬁrmed that suramin inhibits the interaction between u-PA and u-PA directly, in the absence of foreign molecules other than antibodies. A suramin concentration of about 150 µg/ml was needed to obtain half-maximal inhibition in this case. However, a moderate difference in the inhibitor concentration dependence, relative to

1 N. Behrendt, E. Renne, and K. Dana, unpublished results.
2 E. Renne, unpublished work.
Fig. 1. Suramin inhibits the binding of ATF to U937 cells. U937 cells were washed and acid-treated as described under “Experimental Procedures.” Aliquots of 1 x 10⁶ cells were incubated in 300 μl of phosphate-buffered saline with 0.1% bovine serum albumin, including 0.3 nM [125I]-ATF and varying concentrations of suramin, for 1 h at 4 °C on a shaking table. The cell-bound radioactivity was measured after washing the cells.

The result found above with whole cells, is not unexpected when considering the change in assay conditions. Factors like, e.g. orientation effects imposed by the antibody may thus have a slight influence on the ligand-binding characteristics of u-PAR.

The Inhibitory Mechanism Includes a Noncompetitive Element—Using variants of the ATF binding assay with whole cells, we studied certain mechanistic characteristics of the inhibitory phenomenon.

First, we tested the ability of suramin to displace ATF, prebound to u-PAR on the cells (Fig. 3A). Washing steps, introduced after the binding of ATF, ensured that free ligand at subsequent steps could originate only from previously bound material and therefore would occur in negligible concentrations. This design excluded the rebinding of dissociated ligand and thus allowed the specific study of the dissociation process. The off-rate of ATF proved to be accelerated by suramin; the slopes of the log-transformed plots (Fig. 3A) obtained in the absence and presence of the drug were found to be significantly different (p = 0.001) by calculation of regression coefficients, including all duplicate determinations and t test. An exact determination of the individual dissociation rates would require prolonged incubation periods which would hamper the assay by leading to significant losses of intact cells. However, extrapolation of the plots suggests a half-life of the ATF-receptor complex of about 10 h when suramin (1 mg/ml) is present. After this period, 80–90% of the ligand would remain receptor-bound in the absence of inhibitor.

This experiment suggests a noncompetitive element in the mode of inhibition, i.e. the mechanism involving a reaction between suramin and the receptor-ligand complex. As a competitive inhibition control, we used u-PA (200 nM). Under equilibrium binding conditions, this concentration of u-PA would hinder the binding of simultaneously added, labeled ATF (0.3 nM) by more than 98%. As expected, u-PA proved unable to displace ATF when the latter was prebound to the cells (Fig. 3A); no difference was found between the slopes obtained for the buffer control and the u-PA-treated samples, respectively (p = 0.45).

In order to study whether the above effect on the receptor-ligand complex could account for the whole inhibitory mechanism, we next determined binding curves with varying concentrations of ATF in the presence of different, fixed concentrations of suramin. Double-reciprocal plots of the binding data (Fig. 3B) indicated that the mechanism is not solely noncompetitive. The point of intersection above the abscissa is consistent with a mixed competitive and noncompetitive mode of inhibition (37). The same conclusion was reached by determination of suramin inhibition curves at different, fixed concentrations of ATF, and representation of the data according to Cornish-Bowden (38) (data not shown).

**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration, yielding 50% inhibition (μg/ml)</th>
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</thead>
<tbody>
<tr>
<td>Heparan sulfate</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Pentosan polysulfate</td>
<td>≈500</td>
</tr>
<tr>
<td>Heparin</td>
<td>≈1000</td>
</tr>
<tr>
<td>Low molecular weight heparin</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Evans Blue</td>
<td>40</td>
</tr>
<tr>
<td>Trypan Blue</td>
<td>90</td>
</tr>
<tr>
<td>Suramin</td>
<td>50</td>
</tr>
</tbody>
</table>

*The best grade available was tested. Since the exact purity was not stated by the manufacturer, the actual concentration may be slightly lower.

No inhibition was found for heparan sulfate, tested in concentrations up to 1000 μg/ml.

The present work demonstrates that the interaction between u-PA and u-PAR is inhibited by low concentrations of suramin. When this drug is present at concentrations of 200–300 μg/ml, i.e. the serum levels typically found in suramin-treated cancer patients (25), the inhibition is almost complete (Fig. 1). To our knowledge, suramin (and the structurally related reagents also tested in this work) are the first low molecular weight compounds shown to possess this capability.

In a recent study (27), suramin was shown to possess a remarkable inhibitory potential in a model system measuring the matrix degradation and penetration of reconstituted basement membranes by B16 melanoma cells. It was speculated that an anti-invasive effect plays a role in the activity of the
drug against metastatic cancers. In a similar model system, it has been shown that the promoting effect of u-PA in matrix degradation is strongly enhanced by an interplay with u-PAR on the cells (21). In view of these findings, it is tempting to speculate that the inhibition of the u-PA-u-PAR interaction contributes to the anti-invasive effects of suramin.

A complete elucidation of the inhibitory mechanism is outside the scope of the present work. Despite several attempts we have been unable to directly demonstrate a binding of suramin to u-PA, to u-PAR, or both. While the strong effect against the u-PAR-ligand interaction indicates that such binding must occur, it could not be demonstrated in ELISA studies modified to analyze a blocking effect against either component alone. These studies suggested that the binding is short-lived and that the effect against the ligand-receptor interaction is to a certain degree selective; no binding to antibodies? However, the noncompetitive element in the inhibitory mechanism has an important consequence in that suramin actively displaces the bound ligand, rather than just preventing rebinding after spontaneous dissociation. This observation is relevant for a comparison to purely competitive (protein) inhibitors of the u-PA-u-PAR interaction (i.e. soluble u-PAR variants (10, 33) and recombinant ATF (39)). Such considerations are important since the microenvironment in, e.g. colon cancer tissue, including malignant as well as stroma cells, includes a complicated pattern of discrete cells producing u-PA and/or u-PA, specifically surrounding the invasive front (24, 40). Therefore, u-PAR-containing cancer cells are likely to exist in, and move

**FIG. 3.** Effect of suramin on the ligand dissociation rate and on the concentration dependence of ligand binding. Assays for binding of [125I]-ATF to U937 cells were performed as described in the legend to Fig. 1, except for the changes indicated. A, effect on off-rate. Cells were preincubated with 0.3 nM [125I]-ATF for 1 h at 4°C in the presence of buffer alone. Nonbound ATF was subsequently removed by washing the cells with buffer. To separate samples, suramin (1 mg/ml) (triangles), u-PA (200 nM) (squares), or buffer alone (circles) was added in a volume of 1 ml. After varying times of incubation at 4°C, the cells were washed again and the bound radioactivity was measured. The data are represented on a semilogarithmic plot. B, effect of suramin on the concentration dependence of ligand binding. Cells were incubated for 1 h at 4°C with varying concentrations of [125I]-ATF in the absence (circles) or presence of the following concentrations of suramin: 100 μg/ml (triangles) or 200 μg/ml (squares). The cell-bound radioactivity was measured after washing. The nonspecific binding (accounting for up to 15% of total at the highest ATF concentration) was determined in samples incubated in the simultaneous presence of a large excess (700 nM) of unlabeled u-PA and subtracted. The data are represented in the form of double-reciprocal plots.

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5 N. Behrendt, unpublished observations.
through, areas with very different local levels of u-PA concentration.

The use of suramin as an anticancer drug is complicated by severe side effects caused by high concentrations of the drug in humans (25, 26, 41). However, less toxic suramin analogues may be found, also when studying the present receptor system. When comparing suramin and structurally related compounds, large differences have been noted in, e.g. related compounds with less toxicity against cultured cells have already been found (42).

Acknowledgments—The excellent technical assistance of Marianne Valla Nielsen, Mette Villingshøj, and Jimmy E. Weng is gratefully acknowledged, as is the expert photographic assistance of John Post. Dr. Ib J. Christensen is thanked for advice on statistical calculations. Drs. A. Mazan, N. Pedersen, and M. E. Scholz are thanked for the generous gifts of ATP, Su-PAR and pentosan polysulfate, respectively.

REFERENCES