Suramin Promotes Proliferation and Scattering of Renal Epithelial Cells

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ABSTRACT

Primary cultures of renal proximal tubules are known to recapitulate several early events in the process of renal regeneration following injury. In this study, we show that suramin, a polysulfonated naphthylurea, stimulates outgrowth, scattering, and proliferation of primary cultures of renal proximal tubule cells (RPTC). These responses were comparable to those produced by epidermal growth factor (EGF). However, AG-1478 [4-(3-chloroanilino)-6,7-dimethoxyquinazoline], a specific inhibitor of the EGF receptor, blocked EGF but not suramin-induced RPTC outgrowth, scattering, and proliferation. Suramin stimulated phosphorylation of Akt, a downstream kinase of phosphoinositide 3-kinase (PI3K), extracellular signaling-regulated kinase 1/2 (ERK1/2), and Src, but not the EGF receptor. Blockade of Src, but not the EGF receptor, inhibited Akt and ERK1/2 phosphorylation. Furthermore, inactivation of PI3K with LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] blocked suramin-induced RPTC outgrowth, scattering, and proliferation, whereas blockade of ERK1/2 had no effect. These data identify novel effects of suramin in RPTC outgrowth, scattering, and proliferation. Furthermore, suramin-induced outgrowth, scattering, and proliferation of RPTC are through Src-mediated activation of the PI3K pathway but not ERK1/2 or the EGF receptor.

Suramin is a hexasulfonated, polyaromatic compound that has been used in the prevention and treatment of early stages of human trypanosomiasis (Barrett et al., 2003). It also has antitumor activity in numerous types of cancer cells in culture and in cancer xenograft models (Stein, 1993). Although the molecular basis of the antitumor effects of suramin is not completely understood, interruption of autocrine growth factor loops may be responsible. For example, suramin can inhibit the binding of multiple growth factors to their receptors. These growth factors include epidermal growth factor (EGF), platelet-derived growth factor, fibroblast growth factor, and insulin growth factor II (Wang and Williams, 1984; Hosang, 1985; Betsholtz et al., 1986; Coffey et al., 1987). In addition, suramin blocks the interaction of transforming growth factor (TGF)-β with its receptor; TGF-β exerts a growth-inhibitory effect in many cell types, including renal epithelial cells (Wade et al., 1992; Sponsel et al., 1994; Nowak and Schnellmann, 1997).

Although suramin is effective when it is used as a chemotherapeutic agent for treatment of some tumors (e.g., prostate cancer) (Konetty and Getzenberg, 1997), its antitumor activity has not proven successful in all the tumors tested (Eisenberger and Reyno, 1994; Kaur et al., 2002). One explanation for this finding is that suramin may induce other biological actions that are in conflict with its antitumor effect. Indeed, suramin can induce proliferation in certain cancer cell lines, in particular, epithelial tumor cell lines with high expression of the EGF receptor (e.g., A431 cells) (Cardinali et al., 1992) and nonsmall cell lung cancer cells (Lokshin et al., 1999). Furthermore, suramin stimulates proliferation of several nontumor cell lines, such as Chinese hamster ovary (CHO) cells (Nakata, 2003). Study of the mechanisms of such proliferation has suggested that suramin may activate the EGF receptor by stimulating the release of TGF-α, an EGF receptor ligand (Cardinali et al., 1992), or directly induce EGF receptor dimerization (Lokshin et al., 1999).

Activation of the EGF receptor leads to multiple intracellular signaling events (Herbst, 2004). Among the signaling enzymes that mediate proliferation, phosphoinositide 3-kinase (PI3K) and extracellular signaling-regulated kinase (ERK) 1/2 pathways have been reported to be activated by...
suramin and required for the mitogenic response in CHO cells (Nakata, 2004).

Src is a nonreceptor tyrosine kinase that plays an important role in mediating a variety of cellular functions by delivering a signal to downstream effectors, including the PI3K and ERK1/2 pathways. Src activity is tightly controlled by phosphorylation of two tyrosine sites: activation by phosphorylation of Tyr416 and inactivation by phosphorylation of Tyr527 (Roskoski, 2004). A recent study showed that suramin could also stimulate Src phosphorylation in keratinocytes (Brown et al., 2004). The role of Src in suramin-mediated biological responses remains to be determined.

Our recent studies showed that renal proximal tubular cells (RPTC) proliferate and migrate in an EGF receptor-dependent manner in the absence of exogenous growth factors (Zhuang et al., 2004), suggesting the involvement of autocrine mechanisms in this process. Because suramin has been reported to block the interaction of growth factors with their receptors and thereby inhibit cell proliferation (Wang and Williams, 1984; Hosang, 1985; Betsholtz et al., 1986; Coffey et al., 1987; Anderson and Ray, 1998), we initially attempted to assess the autocrine mechanism of RPTC proliferation and migration using suramin. Unexpectedly, suramin stimulated RPTC outgrowth, scattering, and proliferation, and the signaling pathways for these actions were investigated.

**Materials and Methods**

**Chemicals and Reagents.** Human recombinant EGF was obtained from R&D Systems (Minneapolis, MN). LY294002 and U0126 were purchased from Cell Signaling Technology Inc. (Beverly, MA). AG-1478 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to phospho-EGF Tyr1068 receptor, phospho-Akt (Tyr473), Akt, phospho-ERK1/2 (Thr202/Tyr204), phospho-Src (Tyr416 or Tyr527), and Src were obtained from Cell Signaling Technology Inc. Antibodies to ERK1/2 and EGF receptor were purchased from BD Biosciences (San Jose, CA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Fluorescein-labeled phalloidin was obtained from Molecular Probes (Eugene, OR).

**Isolation and Culture of Renal Proximal Tubules.** Isolation and culture of renal proximal tubules were performed as described previously (Rodeheaver et al., 1990; Nowak and Schnellmann, 1995, 1996). Female New Zealand White rabbits (1.5–2.5 kg) were purchased from Myrtle’s Rabbitry (Thompson Station, TN). RPTC were isolated using the iron oxide perfusion method and grown in six-well tissue culture dishes under improved conditions. The culture medium was a 1:1 mixture of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (without glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM l-glutamine, 1 mM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 μM), selenium (5 ng/ml), human transferrin (5 μg/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μM) were added daily to fresh culture medium.

Isolated tubules were plated at 1 mg of protein/well in a six-well plate. On day 2, RPTC were incubated in the presence and absence of various pharmacological inhibitors for different time periods as indicated in the figure legends. For some experiments, suramin was added to RPTC on day 3 and then incubated for 24 h in the presence or absence of various inhibitors before samples were taken.

**MTT Assay.** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to assess cell proliferation (Kooistra et al., 1997; Maeshima et al., 2002). Following a 48-h exposure to various inhibitors or diluents, MTT was added (final concentration, 0.5 mg/ml) to individual cultures for an additional 30 min. Tetrazolium was released by dimethyl sulfoxide, and the optical density was determined with a spectrophotometer (570 nm). Data were normalized to diluent-treated cultures.

**Cell Cycle Analysis.** Cell cycle phase was determined using flow cytometry as previously described (Zhuang et al., 2004). Cells were harvested and stained with propidium iodide, and the number of cells in S-phase of the cell cycle was determined.

**Preparation of Cell Lysates and Immunoblot Analysis.** Following different treatments, RPTC were washed twice with phosphate-buffered saline and harvested in lysis buffer (0.25 M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 1 mg/ml bromophenol blue, and 0.5% 2-mercaptoethanol). Cells were disrupted by sonication for 15 s, and lysates were stored at −20°C. Equal amounts of cellular protein lysate were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. After treatment with 5% skim milk at 4°C overnight, membranes were incubated with various antibodies for 1 h and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences Inc., Piscataway, NJ). Bound antibodies were visualized following chemiluminescence detection on autoradiographic film.

**Immunocytochemistry.** After various treatments, RPTC were fixed with 10% buffered formalin, washed, permeabilized, and blocked with bovine serum albumin. Fluorescein-conjugated phalloidin was added, and RPTC were visualized by fluorescent microscopy.

**Statistical Analysis.** Renal proximal tubules isolated from an individual rabbit represent a single experiment (n = 1) consisting of data obtained from three wells. Data are presented as means ± S.E.M. and were subjected to one-way analysis of variance. Multiple means were compared using Tukey’s test. P < 0.05 was considered a statistically significant difference between mean values.

**Results**

**Suramin Induces RPTC Outgrowth, Scattering, and Proliferation.** We previously developed primary cultures of RPTC in which isolated tubules are plated and RPTC grow out from the tubules and proliferate to form a monolayer in 6 days in the absence of exogenous growth factor stimulation (Nowak and Schnellmann, 1995). Outgrowth, scattering, and proliferation of RPTC from the tubule fragments were observed within 3 days of plating and reached confluence at 6 days (Fig. 1A and data not shown). Using this system, we recently showed that the EGF receptor is activated and required for RPTC proliferation and migration following plating (Zhuang et al., 2004), suggesting that autocrine production of EGF receptor ligands is involved in the activation of the EGF receptor and RPTC proliferation.

Because suramin has been reported to inhibit the growth-promoting effects of conditioned media following injury of a renal epithelial cell line (Anderson and Ray, 1998), we examined whether suramin exerted the same effect on RPTC proliferation following plating. Surprisingly, the addition of suramin stimulated RPTC outgrowth (Fig. 1A). Consistent with our previous observations (Zhuang et al., 2004), exogenous EGF promoted RPTC outgrowth. However, coincubation of suramin with EGF did not further stimulate RPTC outgrowth. Thus, suramin potentiated outgrowth of RPTC from isolated renal proximal tubules and had no effect on EGF-induced RPTC outgrowth.

The two major factors that contribute to the movement of cells away from the edge of the explants are cell proliferation and migration (Boland et al., 1996). To investigate the effect of suramin on cell proliferation, we measured the number of
cells in S-phase of the cell cycle. Approximately 20% of RPTC were in S-phase 4 days following plating and increased to 29% following a 24-h suramin (50 μM) treatment (Fig. 1B). Incubation with exogenous EGF resulted in 32% of RPTC in S-phase. The combination of suramin and EGF did not significantly increase the number of RPTC in S-phase. Suramin promotes RPTC scattering and formation of lamellipodia. RPTC were cultured for 3 days and then treated with diluent, 50 μM suramin, 10 ng/ml EGF, or suramin + EGF. After 24 h, bright field photographs were taken (original magnification, 40×) (A), or RPTC were fixed with methanol and then stained with fluorescein-conjugated phalloidin (B). Original magnification, 80×. Arrows show lamellipodia.
concentrations lower than 50 μM did not statistically increase the number of RPTC in S-phase, whereas 100 μM suramin did not further increase RPTC proliferation (Fig. 1C). Using the MTT assay, a 48-h exposure of suramin also increased the number of RPTC by approximately 40% (Fig. 5A). Thus, suramin has the ability to induce RPTC proliferation.

Motility requires the dissociation of some cells from cohesive epithelial sheets and their transformation into elongated, fibroblast-like cells (Casanova, 2002). As shown in Fig. 2A, most RPTC exhibited cobble stone morphology, with few RPTC exhibiting fibroblast-like cell morphology during their growth. In response to suramin, RPTC at the edge of the cell island lost contact with their neighbors and became elongated, fibroblast-like cells. These morphological changes resemble RPTC at the edge of the cell island when treated with exogenous EGF.

A prominent feature of migrating cells is the formation of lamellipodia and the concomitant reorganization of the actin cytoskeleton (Fukata et al., 2003). Therefore, we examined the effect of suramin on RPTC scattering and motility using immunofluorescence staining of the actin cytoskeleton. Lamellipodia were seen at the edge RPTC in control cultures (Fig. 2B). Incubation of RPTC with suramin or EGF increased the formation of lamellipodia and decreased intercellular contacts, indicating cell scattering. Thus, RPTC scattering and motility increase following treatment with suramin and EGF.

Suramin-Induced RPTC Proliferation and Scattering Are Not Mediated by the EGF Receptor. Our previous studies showed that the EGF receptor is critical for RPTC proliferation (Zhuang et al., 2004). Because it was reported that suramin can induce the release of TGF-α, an EGF receptor ligand, and stimulate proliferation in tumor cells (Cardinali et al., 1992), we examined the role of the EGF receptor in suramin-induced RPTC proliferation and scattering using AG-1478, a selective EGF receptor inhibitor. AG-1478 treatment did not affect suramin-induced RPTC proliferation, as measured by MTT assay (Fig. 3A). Similarly, AG-1478 did not affect suramin-induced formation of the fibroblast-like phenotype and scattering in RPTC (Fig. 3B). In contrast, AG-1478 blocked EGF-induced formation of the fibroblast-like phenotype and scattering.

To determine whether suramin caused EGF receptor activation, we performed immunoblot analysis using an antibody against phosphorylated Tyr1068 of the EGF receptor. Total EGF receptor content was measured using immunoblot analysis and an antibody that recognizes the EGF receptor independent of its phosphorylation state to control for gel loading. We did not detect phosphorylated EGF receptor after treatment with suramin but did observe phosphorylated EGF receptor in response to exogenous EGF (Fig. 4A). The combination of suramin and EGF had no additional effect on EGF receptor phosphorylation. Furthermore, AG-1478 inhibited growth. In response to suramin, RPTC at the edge of the cell island lost contact with their neighbors and became elongated, fibroblast-like cells. These morphological changes resemble RPTC at the edge of the cell island when treated with exogenous EGF.

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EGF-induced EGF receptor phosphorylation (Fig. 4B). Thus, suramin-induced RPTC proliferation and scattering do not occur through the EGF receptor.

Activation of PI3K/Akt, but Not ERK1/2, Is Required for Suramin-Induced RPTC Proliferation and Scattering. The PI3K/Akt and ERK1/2 pathways mediate cell proliferation and migration in different cell types and can be activated in response to different stimuli (Kyosseva, 2004; Richardson et al., 2004). In RPTC, PI3K/Akt, but not ERK1/2, mediates RPTC proliferation (Zhuang et al., 2004). To determine whether these two signaling pathways play a role in suramin-induced proliferation and scattering, RPTC were treated with the PI3K inhibitor LY294002 or the mitogen-activated protein kinase kinase inhibitor U0126 before suramin exposure. Inhibition of PI3K decreased suramin-induced proliferation, whereas inhibition of mitogen-activated protein kinase kinase had no effect on proliferation, as measured by the MTT assay and the number of RPTC in the S-phase of the cell cycle (Fig. 5, A and B). With respect to suramin-stimulated formation of fibroblast-like phenotype in the presence and absence of 20 μM LY294002 (LY) and 10 μM U0126. Cell proliferation was determined using the MTT assay. Data are expressed as means ± S.E.M. of the percentage of MTT activity compared with controls grown with diluent (n = 3). B and C, RPTC were cultured for 3 days and then incubated with 50 μM suramin for 24 h in the presence and absence of 20 μM LY294002 (LY) and 10 μM U0126. Cell proliferation was determined by measuring the number of cells in S-phase of the cell cycle. Data are expressed as means ± S.E.M., n = 3. Bars with different superscripts are significantly different from each other (P < 0.05). Bright field photographs were taken at the edges of the cell islands. Original magnification, 40×.

Fig. 5. Effects of PI3K and ERK1/2 pathway inhibitors on RPTC proliferation and scattering following plating and suramin exposure. A, RPTC were cultured for 2 days and then incubated with 50 μM suramin for 48 h.
and RPTC scattering, LY294002 treatment partially inhibited them, whereas U0126 had no effect (Fig. 5C).

We evaluated whether the PI3K/Akt and/or ERK1/2 pathways are activated in RPTC in response to suramin. The activation of the PI3K and ERK1/2 pathways was measured using immunoblot analysis and antibodies that recognize phosphorylated Akt at Ser473 (a target of PI3K) and ERK1/2, respectively. Total Akt and ERK1/2 content was measured using immunoblot analysis and antibodies that recognize Akt and ERK1/2 independent of their phosphorylation state to control for gel loading. Suramin induced Akt phosphorylation within 10 min, reached a maximum at 30 min, and was sustained through 120 min of treatment (Fig. 6A). ERK1/2 phosphorylation was also induced within 10 min and maximal at 30 min but decreased to the control levels at 120 min.

To confirm that LY294002 and U0126 selectively inhibited their respective kinases, we monitored Akt and ERK activation using immunoblot analysis and antibodies that recognized phosphorylated Akt and ERK1/2 as described above. LY294002 blocked Akt phosphorylation and U0126 blocked ERK1/2 phosphorylation of RPTC following plating in the presence and absence of suramin (Fig. 6, B and C). Thus, whereas suramin activates PI3K/Akt and ERK1/2, PI3K/Akt, but not ERK1/2, is critical for suramin-induced RPTC proliferation and scattering. However, suramin-induced RPTC scattering is not completely dependent on PI3K.

**Src Is Required for RPTC Proliferation and Scattering Induced by Suramin.** It has been reported that Src activation mediates proliferation of renal epithelial cells in response to arachidonic acid metabolites and that suramin can activate Src phosphorylation in keratinocytes (Chen et al., 1998; Brown et al., 2004). Thus, we examined whether Src is required for RPTC proliferation and scattering using PP1, a specific inhibitor of Src (Liu et al., 1999). Treatment of RPTCs with PP1 inhibited RPTC proliferation in control and suramin-treated cells (Fig. 7, A and B). Suramin-stimulated formation of fibroblast-like phenotype and scattering in RPTC also was blocked by PP1 (Fig. 7C).

To determine whether suramin activates Src and whether Src mediates Akt activation, we examined the effect of suramin on Src phosphorylation and the effect of PP1 on suramin-induced Akt phosphorylation. Src activation requires dephosphorylation of Tyr527 (Zheng et al., 2000) and phosphorylation of Tyr416 in the catalytic domain (Leu and Maa, 2003). Src phosphorylation at Tyr416 was detected in control samples. Following suramin treatment, Src phosphorylation increased within 10 min and returned to the basal level at 60 min (Fig. 8A). In contrast, suramin treatment reduced phosphorylation of Src at Tyr527 within 5 min and was sustained through 120 min (Fig. 8B). Pretreatment with PP1 blocked suramin-induced phosphorylation of Akt (Fig. 8C). Thus, suramin-induced RPTC proliferation and scattering depend on Src activity, and activation of the PI3K/Akt signaling pathway requires Src.

**Discussion**

In this study, we found that suramin stimulates RPTC outgrowth, scattering, and proliferation. Consistent with our observations, the stimulatory effect of suramin on cell proliferation has been reported in several tumor cell lines, including breast cancer cells (Foekens et al., 1992), nonsmall cell lung cancer cell lines (Lokshin et al., 1999), and esophageal and epidermoid carcinoma cell lines (Cardinali et al., 1992), as well as PC12 cells and dorsal root ganglion neurons (Gill et al., 1996). Thus, in addition to its antiproliferative activity in some models, suramin induces cellular proliferation in...
certain cell types. Furthermore, the effect of suramin on formation of motile RPTC phenotype and scattering suggests that it is also a stimulator of RPTC migration. Our results are different from a previous report that showed that suramin inhibits \(^{3}H\)thymidine uptake induced by conditioned media in an injured renal epithelial cell line (LLC-PK1) (Anderson and Ray, 1998). The reasons for the difference of these two observations are currently unknown; it may be because of different cell types and models being used.

The mechanisms underlying suramin stimulation of cell proliferation and scattering are not clear. Because an early study showed that suramin induces the release of TGF-\(\alpha\) (a EGF receptor ligand), activates the EGF receptor, and induces proliferation of the carcinoma cell line KEsC-II (Cardinali et al., 1992), we initially thought that an EGF receptor-mediated mechanism might account for RPTC proliferation and scattering. However, our data do not support this hypothesis. First, suramin did not induce phosphorylation of the EGF receptor. Second, suramin did not affect exogenous EGF-induced EGF receptor phosphorylation. Finally, treatment of cells with AG-1478, a specific EGF receptor inhibitor, did not affect suramin-induced phosphorylation of Akt, proliferation, or scattering. Consistent with our observation, inhibition of the EGF receptor with AG-1478 did not attenuate suramin-induced ERK activation and proliferation in CHO cells (Nakata, 2004). Although it is possible that suramin may stimulate activation of other growth factor receptors through ligand-dependent mechanisms, and platelet-derived growth factor and fibroblast growth factor recep-tors are found on RPTC, the ligands for these receptors are not expressed in RPTC (Toback, 1992). Thus, we conclude that suramin-stimulated RPTC proliferation and scattering are not through the production of autocrine growth factors.

Another potential mechanism for suramin-induced RPTC proliferation and scattering may involve interference with growth inhibitory factors. In this regard, it has been reported that suramin can block the interaction of TGF-\(\beta\) with the TGF receptor and block the inhibitory effect of TGF-\(\beta\) in renal cancer cells (Wade et al., 1992). Previous results from our laboratory showed that RPTC can produce TGF-\(\beta\) and that oxidant-induced autocrine production of TGF-\(\beta\) has an inhibitory effect on RPTC proliferation (Nowak and Schnellmann, 1997). Furthermore, the addition of exogenous TGF-\(\beta\) to RPTC inhibited proliferation following plating and oxidant injury (Counts et al., 1995; Kays et al., 1996; Nowak and Schnellmann, 1997) and inhibited wound healing of renal epithelial cells following mechanical injury (Sponsel et al., 1994). Thus, it is possible that inhibition of TGF-\(\beta\) binding to its receptor may result in the enhancement of RPTC proliferation and scattering after suramin treatment. This possibility is currently under investigation in our laboratory.

Our results revealed that Src mediates suramin-induced proliferation and scattering of RPTC. Evidence for this statement is the activation of Src and Akt by suramin, the inhibition of suramin-induced Src and Akt phosphorylation by the Src inhibitor PP1, and the inhibition of RPTC proliferation and scattering by PP1. The mechanism by which suramin induces Src activation is currently unclear. One possibility is that suramin induces activation of protein tyrosine phosphatases (PTPs), and as a result Src is activated. Previous studies have shown that Src activation results from dephosphorylation of Tyr527 and subsequent Src autophosphorylation (Roskoski, 2004), and PTP-\(\alpha\) has been reported to positively regulate Src activity via dephosphorylation (Roskoski, 2004). Furthermore, suramin induces activation of PTP-\(\alpha\) in vitro (McCain et al., 2004). Alternatively, suramin may induce Src activation by blocking TGF-\(\beta\) binding to its receptor. In this context, it has been reported that TGF-\(\beta\) treatment decreases Src activity and cell growth in the prostate carcinoma cell line PC3 and hepatoma cell line HepG2 (Atfi et al., 1994; Fukuda et al., 1998) and prevents hepatocyte growth factor-induced tyrosine phosphorylation of Src and migration in endothelial cells (Mangamini and Maier, 2000).

To identify the intracellular signaling mechanism responsible for suramin-induced proliferation and scattering of RPTC, we found that Akt, a downstream target of PI3K, is phosphorylated after suramin treatment, and LY294002, a specific inhibitor of the PI3K signaling pathway, attenuated proliferation and scattering. These data illustrate that PI3K signaling and scattering in response to suramin stimulation. However, it should be noted that the PI3K/Akt signaling pathway might not be the sole pathway responsible for RPTC scattering because blockade of PI3K does not result in complete inhibition of RPTC scattering in response to suramin stimulation. Although suramin also induced phosphorylation of ERK1/2, inhibition of this signaling pathway did not affect either proliferation or scattering, suggesting that the ERK pathway does not mediate this suramin-induced biological process.
In summary, our studies have shown that suramin stimulates proliferation and scattering of RPTC, which were mediated by Src-dependent activation of the PI3K/Akt pathway. Furthermore, suramin-stimulated biological responses do not involve EGF receptor or ERK1/2 activation. Because proliferation and migration of renal epithelial cells are two crucial processes needed for structural regeneration of nephron following injury, it will be of interest to determine whether suramin has a therapeutic potential in promoting renal recovery after injury.

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