Cancer Chemother Pharmacol (2011) 67:723–728
DOI 10.1007/s00280-010-1457-z

SHORT COMMUNICATION

Suramab, a novel antiangiogenic agent, reduces tumor growth and corneal neovascularization

Emiliano S. Lopez · Manglio M. Rizzo · J. Oscar Croxatto · Guillermo Mazzolini · Juan E. Gallo

Received: 3 June 2010 / Accepted: 1 September 2010 / Published online: 21 September 2010

Abstract
Purpose Oncological and ophthalmological diseases are increasingly treated with antiangiogenic agents. These agents have different intensities and duration of effects that should be considered to choose the most suitable therapy. Our purpose was to evaluate the synergistic effect of two drugs, jointly administered as a pharmaceutical compound, in two animal models.
Methods Corneal neovascularization was induced in three groups of nine white New Zealand rabbits, applying a filter paper disk soaked in 1 M NaOH on the central cornea (Ormerod et al., Invest Ophthalmol Vis Sci 30:2148–2153, 1989). Group one was treated immediately after injury with intravenous Suramab, compound of Bevacizumab + Suramin, and group two with intravenous Bevacizumab. A third group of non-treated rabbits was included as control group. Digital photographs were taken at days 9, 15, 21, and 35. Neovessel index (NVI) was calculated using the Image J Program. Neovessels formation was quantified and given a score from 0 to 4 to each quadrant according to the centripetal growth of the longest vessel. *Colorectal animal model*: 6- to 8-week-old male BALB/c mice were inoculated with cancer cells. Seven days after tumor inoculation, four groups of BALB/c mice were treated with intravenous Bevacizumab ($n = 9$); intravenous Suramin ($n = 10$); intravenous Suramab ($n = 10$); and intravenous saline solution ($n = 4$). Tumor growth was assessed twice weekly by caliper measurement.
Results The NVI was remarkably inferior in the group of rabbits treated with intravenous Suramab compared with controls after 35 days of follow-up. A greater inhibitory effect was obtained with Suramab compared to that obtained with Bevacizumab. Suramab significantly reduced tumor volume and prolonged survival of mice compared to controls.
Conclusions Suramab strongly reduced neovascularization in a rabbit model of corneal angiogenesis and induced a potent antitumoral effect in mice.

Keywords Angiogenesis · Cancer · Suramin · Bevacizumab · Cornea

Background

Antiangiogenic agents are of great value for the treatment of cancer and, recently, for eye disorders. It is known that neovascularization is a key factor for tumor growth [1] and corneal graft failure [2]. Most types of cancer cells and corneal tissue with neovessels express vascular endothelial growth factor (VEGF), which is strongly implicated in neovascularization [2]. There are a number of studies that showed that VEGF inhibition has the ability to reduce corneal neovascularization [3, 4]. Bevacizumab, a recombinant humanized monoclonal antibody (Ig1) that binds to soluble VEGF, prevents the binding of this growth factor to VEGF receptors (Flt-1 and KDR), inhibiting neovessel formation. This drug is currently being used as an important neoadjuvant for colorectal adenocarcinoma, breast cancer, and

E. S. Lopez · M. M. Rizzo · G. Mazzolini · J. E. Gallo (✉)
Departments of Ophthalmology and Internal Medicine, Austral University Medical School and Austral University Hospital, Av. Juan Perón 1500, Pilar, Buenos Aires, Argentina
e-mail: jgallo06@gmail.com; jgallo@cas.austral.edu.ar

J. O. Croxatto
Ocular Pathology Department, Fundación Oftalmológica Argentina "Jorge Malbran", Buenos Aires, Argentina

non-small-cell lung cancer [5]. Systemic application of Bevacizumab was well tolerated and effective in patients with age-related macular degeneration [6]. Besides, it has been topically and subconjunctivally administered in animals and humans with promising results [3, 4]. Several antiangiogenic agents have been tested as monotherapy. Early or late resistance to antiangiogenic treatment is frequently observed. It should be mentioned that the mechanism of neovascularization involved different pathways, and so a combination of agents aiming to interfere with several mechanisms might be useful to improve outcomes [5, 7].

The purinergic pathway could play a role in the pathogenesis of neovascularization [8, 9]. Previous investigations identified the effect of P1 and P2 purinergic receptors on the promotion of angiogenesis [8]. It was observed that the blockade of these receptors in an alkali burn model of corneal neovascularization diminished neovascularization [10]. Suramin is a non-specific, non-competitive antagonist of P2 purinergic receptors with antiangiogenic effect on several tissues. Suramin also inhibits the binding of several growth factors (FGF-2, PDGF, insulin-like growth factor, TGF-β), the GTPase activity of certain G-proteins, the ecto-nucleotidase as well as DNA and RNA polymerases that are known to be involved in angiogenesis [9, 11]. In addition, it has been observed that Suramin antiangiogenic effect may be mediated, at least in part, by the inhibition of VEGF activity [12]. The P2X2 is an ion channel purinergic receptor found upregulated in the retina of a mouse model of oxygen-induced retinopathy [8]. Blockade of this receptor diminished neovessels formation [8, 10, 13]. In previous studies, we showed that intravenous Suramin (20 mg/kg) and Bevacizumab (5 mg/kg), separately, diminished corneal neovascularization [14]. The present study aims at evaluating a possible synergistic effect of the two drugs as a new pharmaceutical compound in the two animal models of angiogenesis.

Materials and methods

Corneal neovascularization

Animal model

Eighteen white New Zealand rabbits weighing on average 3 kg each were used in this study. Animals were anesthetized with 70 mg/kg IM ketamine (Fada Pharma, Buenos Aires, Argentina) and 1 mg/kg IM midazolam (Roche, Buenos Aires, Argentina), and topical anesthesia was applied using 0.5% proparacaine (Alcon, Sao Paulo, Brazil). Corneal neovascularization was induced by applying a Whatman filter paper disk (7-mm diameter) soaked in 1 M NaOH in the central cornea of the left eye for one minute. Immediately after injury, the eye was washed with saline solution [15]. Two groups made up of nine rabbits each were treated immediately after injury with intravenous Suramab, a compound of 3 mg/kg of Bevacizumab (Avastin, Roche, Buenos Aires) + 10 mg\kg of Suramin (Biomol International, LP, USA), and intravenous Bevacizumab (5 mg/kg), respectively. A third group of non-treated rabbits was included as control group ($n = 9$). Animals were kept in individual cages with free access to food and water. All animals were handled according to ARVO Statement for the Use of Animals in Ophthalmic Research.

Quantification of neovascularization

Digital photographs using a digital camera (Canon 5D, USA) were taken at days 9, 15, 21, and 35. Neovascularization index (NVI) was calculated using the Image J program (Image Processing and Analysis in Java, NIH, USA). Neovessel formation was quantified and given a score from 0 to 4 to each quadrant according to the centripetal growth of the longest vessel in increments of 1 mm. Scores of the four quadrants were summed to obtain the NVI.

Colorectal carcinoma animal model

Six- to 8-week-old male BALB/c mice were purchased from Biofucal S.A., Buenos Aires, Argentina. The initial body weight of the animals at the time of arrival was between 18 and 20 g. Mice were allowed to acclimatize to local conditions for 1 week before receiving injections of cancer cells. Logarithmically growing CT26, an undifferentiated murine CRC cell line established from an *N*-nitroso-*N*-methylurethan-induced transplantable tumor in BALB/c (H-2d) mouse cells (kindly provided by Prof. Prieto, University of Navarra, Spain), were harvested by trypsinization, and each mouse was given injections of 5×10^5 cells subcutaneously into the right flank. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL streptomycin, and 100 mg/mL penicillin and incubated at 37°C in a 5% CO2 humidified atmosphere. Seven days after tumor inoculation, four groups of BALB/c mice were treated with intravenous Bevacizumab 5 mg/kg (GROUP 1, $n = 10$); intravenous Suramin 20 mg/kg (GROUP 2, $n = 10$); intravenous Suramab, compound of 3 mg/kg of Bevacizumab + 10 mg/kg of Suramin (GROUP 3, $n = 10$); and IV. saline solution (GROUP 4, $n = 7$). Tumor growth was assessed twice weekly by caliper measurement. Tumor volume (mm³) was calculated by the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. Results are presented as mean $\pm$ SEM. One-way Anova was used to compare tumor sizes, and LogRank test was used to

compare survival among different groups at the end of treatment. Mice were maintained and treated at our Animal Resources Facilities (School of Biomedical Sciences, Austral University) in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the experimental ethical committee guidelines.

Results

Suramab inhibits corneal neovascularization

The NVI was remarkably inferior in the group of animals treated with intravenous Suramab compared with controls after 35 days of follow-up. Nine days after injury, the NVI was much higher in the control group (mean NVI = 4.77 ± 0.79) than in the treated groups (mean NVI of Suramab = 0.77 ± 0.46 and Bevacizumab = 1.11 ± 0.61). The centripetal growth of neovessels was threefold diminished in Suramab group compared to control group ($P < 0.0005$). Fifteen days after injury, the inhibitory effect of IV Suramab was even greater (NVI = 1.55 ± 0.67) compared to controls (NVI = 10 ± 1.01) and Bevacizumab (NVI = 2.55 ± 0.94). From day 22 onward, Bevacizumab antiangiogenic effect began to diminish. A greater inhibitory effect was obtained with Suramab compared to that obtained with Bevacizumab. The difference between Suramab and the other groups was observed along the 35 days of follow-up and was statistically significant (Student's *t*-test, $P < 0.02$). No serious ocular or systemic adverse event was detected. See Fig. 1.

Suramab has a potent antitumoral effect in mice

All treatments were well tolerated, and no signs of toxicity were observed during therapy (not shown). To examine the antitumor effects of Suramab (Bevacizumab and Suramin), animals with well-established CT26 tumors (approximately 85 mm^3 in size) were treated with a compound of 3 mg/kg of Bevacizumab and 10 mg/kg of Suramin intravenously. In addition, mice treated with each single agent, Bevacizumab 5 mg/kg, Suramin 20 mg/kg, and saline solution were included. Antitumor effects obtained with the administration of both Suramin and Bevacizumab alone were similar. However, Suramab administration resulted in a significant tumor volume reduction compared with control and Bevacizumab groups, as shown in Fig. 2. Survival of mice receiving Suramab therapy was prolonged compared to other groups of animals, but the difference was only significant with regard to control mice.

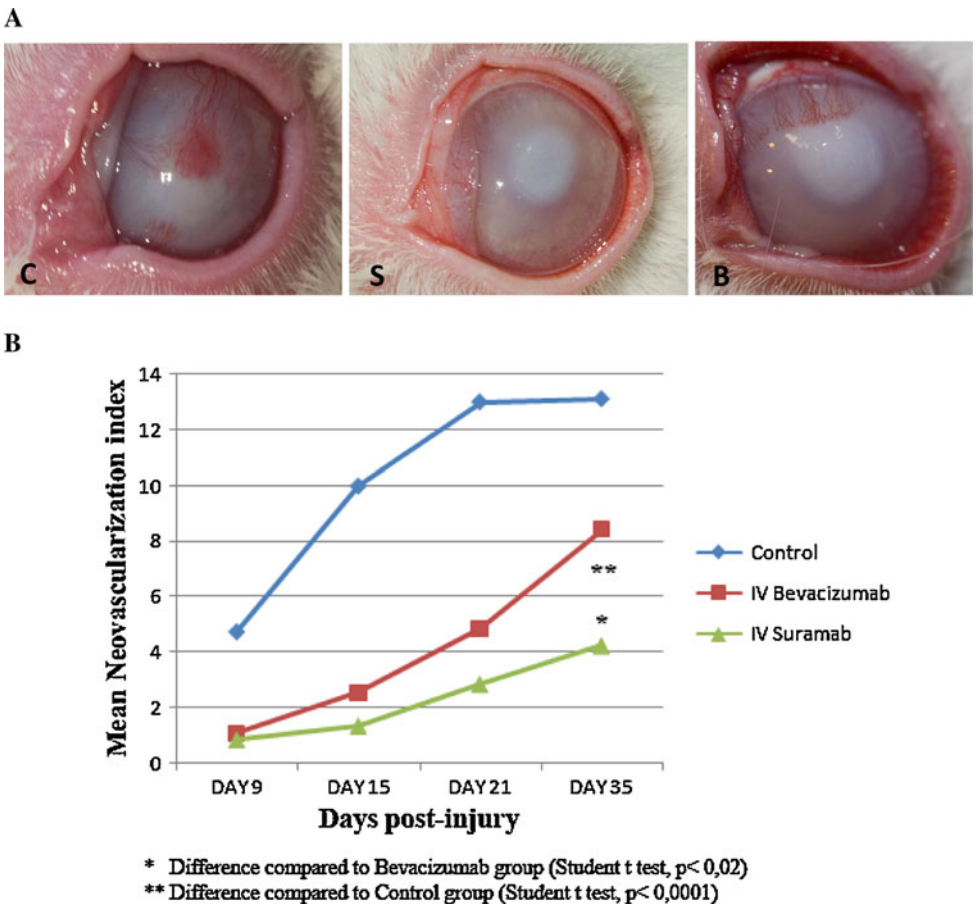

Fig. 1 **a** Clinical evolution. Corneal photographs showing neovessel ingrowth in control (C) and treated (*S* Suramab, *B* Bevacizumab) groups at 35 days after injury. Corneal neovascularization is remarkably inferior in treated animals, especially in the Suramab group. **b** Graph showing mean corneal neovascularization index in Suramab, Bevacizumab, and control groups, through 35 days of follow-up. Controls showed much more neovascularization than treated groups. The differences at 35 days postinjury, between control and treated groups, and between Suramab and Bevacizumab, were statistically significant (Student's *t*-test, $P < 0.02$)

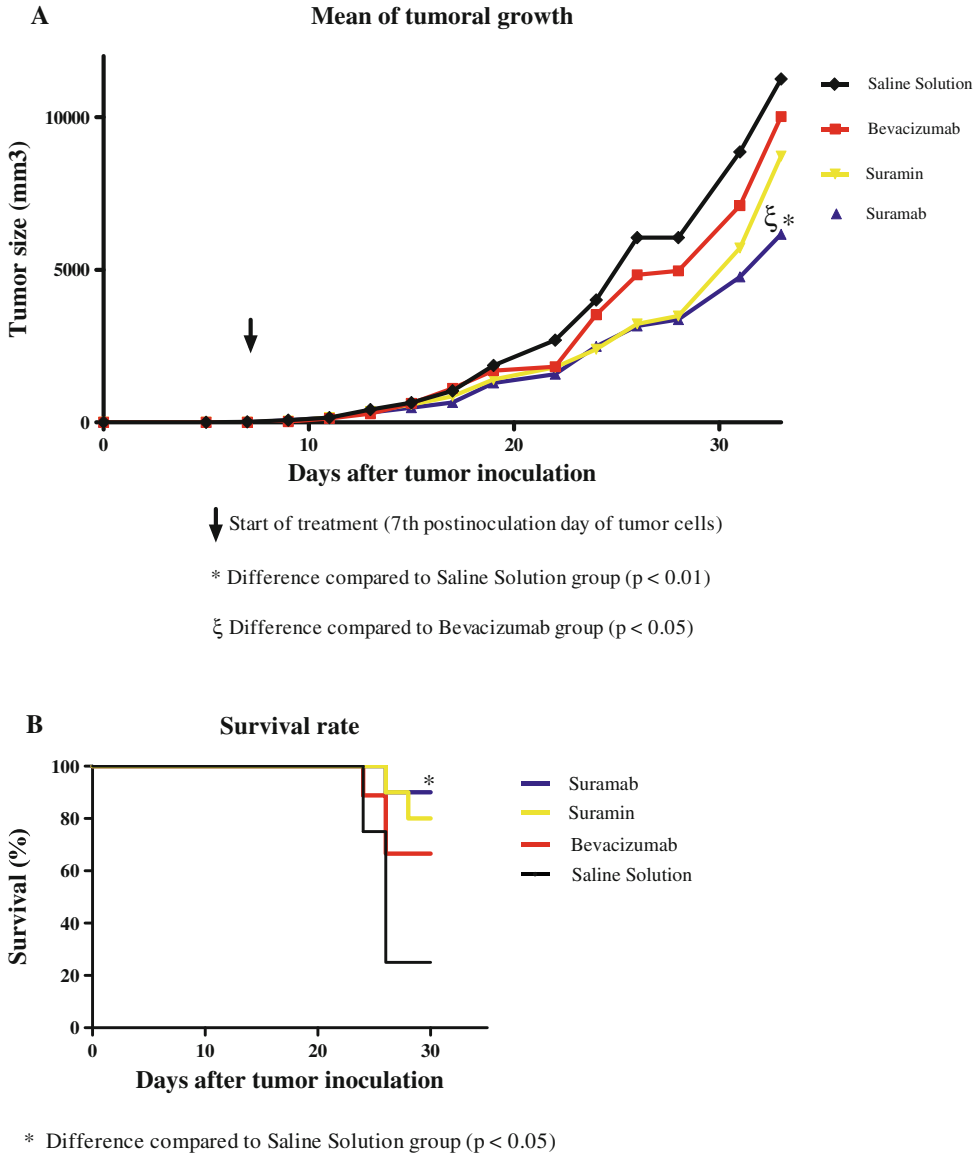

Fig. 2 **a** Longitudinal analysis of tumor growth in a mice model of colorectal cancer. Suramab significantly reduced tumor volume compared to controls and Bevacizumab groups. (* $P < 0.01$; ζ $P < 0.05$) **b** Survival rate in a mice with colorectal cancer. Suramab significantly prolonged survival of mice compared to controls

Discussion

In this work, we have demonstrated that Suramab, a pharmaceutical compound of Bevacizumab and Suramin, strongly reduced neovascularization in a rabbit model of corneal angiogenesis and induced a potent antitumoral effect in mice. In both models, an important biological effect was obtained, even greater than that achieved with Bevacizumab or Suramin alone. In previous studies, we showed that IV Bevacizumab had higher angiostatic effect in the first 2 weeks than that seen using IV Suramin. However, Suramin had a longer duration of that effect after 35 days [14]. Similar results were recently reported by other researchers, by injecting subconjunctival Suramin and Bevacizumb, separately, in an animal model of corneal neovascularization [13].

Targeting a molecule or a receptor involved in angiogenesis is a known strategy to arrest neovascularization in several cancer and ophthalmological disorders [6, 8, 16]. Unfortunately, these therapies have a rather brief effect because of the short half-life of most antiangiogenic drugs. This makes frequent repeat treatments necessary [6, 17]. To tackle this problem, investigators have combined therapies targeting two or three molecules involved in different angiogenic pathways [5, 7]. In spite of this, it is difficult to obtain a permanent inhibition of neovessel growth.

The combination of drugs looking for synergy is a common strategy to treat several diseases including cancer. In our work, we have found a novel and synergistic effect of Suramin and Bevacizumab to reduce angiogenesis in rabbits. After 1 month of therapy, Suramab-treated rabbits disclosed a 50 and 75% decrease in corneal neovascularization

compared to Bevacizumab and control animals, respectively. In addition, Suramab showed a potent antitumoral effect on mice with subcutaneous implantation of colorectal cancer cells compared to controls and Bevacizumab-treated mice, as well as a higher survival rate than that found in controls.

Suramab has a potent antiangiogenic effect because it inhibits not only VEGF (through Bevacizumab) but also other pro-angiogenic growth factors such as TGF-β, PDGF, bFGF, and postreceptor-inducible tyrosine phosphorylation of KDR [9, 11, 12]. In addition, Suramin has the ability of interfering in the interaction between mural cells (particularly pericytes) and endothelial cells [7, 12]. It should be noted that our experiments tested the effect of Suramab on different animal models. Side effects of Bevacizumab and Suramin have been reported [10, 18]. Nevertheless, the concentration and doses of these compounds used in Suramab are much lower than those pointed out as toxic levels of Suramin and Bevacizumab [18]. Accordingly, no significant side effects were seen with the therapeutic combination.

The antiangiogenic effects of Suramab might be applied to several corneal disorders associated with neovascularization such as alkali burns and infectious keratitis (herpes, acanthamoeba) as well as diabetic retinopathy and wet age-related-macular degeneration. In addition, Suramab will be used at our laboratory to prevent neovessel ingrowth during the postoperative period of corneal stem cell transplantation [19].

The inhibition of neovessel formation is of paramount importance to control tumur growth [2]. Suramab could be employed in adjuvancy with chemotherapeutic agents in colorectal carcinoma, as well as in other types of cancer. Interestingly, one of the components of Suramab, Suramin, was reported to increase the susceptibility of cancer cells to chemotherapy [18]. It was reported that Suramin could interfere with the binding of IL-6 to its receptor decreasing cancer-associated wasting (cachexia) [20]. In addition, Suramin inhibits the binding of transferrin to its receptor, and this may result in decreased cell growth, particularly in prostate cancer metastasis to the bone, which seems to be highly dependent on transferrin for growth [11]. These effects of Suramab mediated by Suramin will be further investigated.

Suramab seems to be a potent antiangiogenic agent with multiple applications. A local route of administration, either periocular or intratumoral, using a drug delivery system is under study.

Acknowledgments We are grateful to Guillermo Gastón, Norma Montalbetti, and Soledad Arregui for their skillful technical assistance. This work was supported by a grant from Universidad Austral.

Conflict of interest An international patent application has been submitted about Suramab (patent pending). *Title of the patent*: Synergistic pharmaceutical composition useful for inhibiting corneal and retinal neovascularization (angiogenesis), and in other organs, in a human being or animal. *(10) Publication number*: WO2009/022897;*(21) Application Number*: PCT/MX2008/000104;*(51) International Patent Classification*: *A61 K 45/06* (2006.01); *A61P 27/02* (2006.01); *A61P 35/00* (2006.01) *(71) Applicant(s)*: Asociación Civil De Estudios Superiores [AR/AR]; Av. Juan de Garay No. 125 Ciudad de Buenos Aires C1063ABB; (AR) *(for all designated states except US)* RIVAS CUEVAS, Claudio Manuel [MX/MX]; Ruiseñor No. 51-B Col. El Rosedal México, D.F.04330 (MX) *(for all designated states except US)*GALLO BARRACO, Juan Eduardo María [AR/AR]; Paraná 945, 3er Piso Ciudad de Buenos Aires C1017AAS (AR) *(for US only)*.

References

1. Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. Nature 438:7070
2. Williams KA, Lowe M, Bartlett C, Kelly TL, Coster DJ (2008) Risk factors for human corneal graft failure within the Australian corneal graft registry. Transplantation 86(12):1720–1724
3. Manzano R, Peyman GA, Khan P, Carvounis PE, Kivilcim M, Ren M, Lake JC, Chévez-Barrios P (2007) Inhibition of experimental corneal neovascularisation by Bevacizumab (Avastin). Br J Ophthalmol 91:804–807
4. Erdurmus M, Totan Y (2007) Subconjunctival bevacizumab for corneal neovascularization. Graefe's Arch Clin Exp Ophthalmol 245:1577–1579
5. Greil R, Moik M, Reitsamer R, Ressler S, Stoll M, Namberger K, Menzel C, Mlineritsch B (2009) Neoadjuvant bevacizumab, docetaxel and capecitabine combination therapy for HER2/neu-negative invasive breast cancer: efficacy and safety in a phase II pilot study. Eur J Surg Oncol 35:1048–1054
6. Moshfeghi AA, Rosenfeld PJ, Puliafito CA, Michels S, Marcus EN, Lenchus JD, Venkatraman AS (2006) Systemic Bevacizumab (Avastin) therapy for neovascular age-related macular degeneration: twenty-four-week results of an uncontrolled open-label clinical study. Ophthalmology 113:2002–2011
7. Jo N, Mailhos C, Ju M, Cheung E, Bradley J, Nishijima K, Robinson GS, Adamis AP, Shima DT (2006) Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular endothelial growth factor therapy in multiple models of ocular neovascularization. Am J Pathol 168:2036–2053
8. Sarman S, Mancini J, van der Ploeg I, Croxatto JO, Kvanta A, Gallo JE (2008) Involvement of purinergic p2 receptors in experimental retinal neovascularization. Curr Eye Res 33:285–291
9. Abbracchio MP, Burnstock G (1994) Purinuceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64:445–475
10. Bocci G, Danesi R, Benelli U, Innocenti F, Di Paolo A, Fogli S, Del Tacca M (1999) Inhibitory effect of suramin in rat models of angiogenesis in vitro and in vivo. Cancer Chemother Pharmacol 43:205–212
11. Stein CA (1993) Suramin: a novel antineoplastic agent with multiple potential mechanisms of action. Cancer Res 53(Suppl):2239–2248
12. Waltenberger J, Mayr U, Frank H, Hombach V (1996) Suramin is a potent inhibitor of vascular endothelial growth factor. A contribution to the molecular basis of its antiangiogenic action. J Mol Cell Cardiol 28:1523–1529
13. Lee HS, Chung SK (2010) The effect of subconjunctival suramin on corneal neovascularization in rabbits. Cornea 29(1):86–92
14. Lopez ES Croxatto JO, Kvanta A, and Gallo JE(2007). Effect of Bevacizumab (Avastin) and Suramin in a rabbit model of corneal neovascularization. Invest Ophthalmol Vis Sci 48: E-Abstract 1717

15. Ormerod LD, Abelson MB, Kenyon KR (1989) Standard models of corneal injury using Alcali-Inmersed filter discs. Invest Ophthalmol Vis Sci 30:2148–2153
16. Chang JH, Gabison EE, Kato T, Azar DT (2001) Corneal neovascularization. Curr Opin Ophthalmol 12:242–249
17. Jyothi S, Chowdhury H, Elagouz M, Sivaprasad S (2010) Intravitreal bevacizumab (Avastin) for age-related macular degeneration: a critical analysis of literature. Eye (Lond) 24(5):816–824
18. Villalona-Calero MA, Otterson GA, Wientjes MG, Weber F, Bekaii-Saab T, Young D, Murgo AJ, Jensen R, Yeh TK, Wei Y, Zhang Y, Eng C, Grever M, Au JL(2008) Noncytotoxic suramin as a chemosensitizer in patients with advanced non-small-cell lung cancer: a phase II study 19:1903–1909
19. Luengo Gimeno F, Lavigne V, Gatto S, Croxatto JO, Correa L, Gallo JE (2007) Advances in corneal stem-cell transplantation in rabbits with severe ocular alkali burns. J Cataract Refract Surg 33:1958–1965
20. Strassman G, Fong M, Freter CE, Windsor S, D`Alessandro F, Nordan RP (1993) Suramin interferes with interleukin-6 receptor binding in vitro and inhibits colon-26-mediated experimental cancer cachexia in vivo. J Clin Invest 92:2152–2159