

The effect of tyrphostin AG-556 on intimal thickening in a mouse model of arterial injury

Jacob George^{a,*}, Iris Barshack^{b,1}, Pnina Keren^a, Aviv Gazit^c, Alexander Levitzki^c,
Gad Keren^a, Arie Roth^a

^aDepartment of Cardiology, Tel-Aviv Sourasky Medical Center, affiliated to the Sackler Faculty of Medicine, 6 Weizman Street, Tel Aviv University, Israel

^bInstitute of Pathology, Sheba Medical Center, Tel-Hashomer, Israel

^cDepartment of Biological Chemistry, The Hebrew University, Jerusalem

Received 25 October 2004

Available online 8 February 2005

Abstract

Background. Inflammation has been shown to play an important role in promoting the response to arterial injury and proinflammatory cytokines, such as tumor necrosis factor (TNF) alpha, are candidate mediators. AG-556 is a tyrosine kinase inhibitor proven to be effective in a model of multiple sclerosis-like syndrome in mice due to its immunomodulating effect. In the current study, we investigated the effect of the tyrphostin AG-556 on neointimal thickening and cytokine profile in a model of arterial injury in the mouse. **Methods.** Injury was induced by external cuff placement on the left femoral artery of wild-type C57BL/6 mice. AG-556 dissolved in DMSO was injected intraperitoneally daily to the injured mice in a dosage of 2 mg/mouse. Control mice received DMSO injections. Histological analysis was carried out to assess neointimal formation. Splenocytes were cultured in the absence and presence of a mitogen for evaluation of thymidine incorporation and cytokine production. **Results.** AG-556 treatment significantly attenuated intimal thickening ($43,000 \pm 17,000 \mu\text{m}^2$; $n = 11$) when compared to DMSO administration ($286,000 \pm 127,000 \mu\text{m}^2$; $n = 10$; $P < 0.05$). Basal interferon-gamma production by splenocytes from AG-556-treated mice was increased by approximately 20-fold in comparison with levels in DMSO-treated animals, whereas Con-A induced secretion of the cytokine was similar between both groups. Levels of TNF-alpha, IL-4 and IL-10 in the culture supernatant from treated and non-treated animals did not differ significantly. **Conclusion.** The tyrosine kinase inhibitor AG-556 may have a role in the reduction of intimal thickening. The effect could be mediated via an immune modulating effect involving a significant increase in the smooth muscle cell inhibitory cytokine IFN-gamma.

© 2004 Published by Elsevier Inc.

Introduction

Atherosclerosis and restenosis are multifactorial processes resulting from a complex cascade of events involving abnormal proliferation of vascular smooth muscle cells (VSMCs) (Fuster et al., 1992; Libby et al., 1992; Liu et al., 1989; Ross, 1993). Although the occurrence of restenosis in ~20–55% of patients after clinically successful revascularization remains a limitation to percutaneous transluminal coronary angioplasty (Kaltenbach et al., 1985), no clinical

trials of systemically delivered agents have yet demonstrated convincing effects in reducing the incidence of restenosis. Intravascular brachytherapy and drug eluting stents represent effective currently employed approaches for the prevention of restenosis. However, brachytherapy has been associated with delayed adverse events, whereas no long-term follow up is present for drug eluting stents (Kereiakes and Willerson, 2003; Marx and Marks, 2001; Morice et al., 2002; Sheppard et al., 2003; Sousa et al., 2003).

In recent years, considerable knowledge has accumulated with respect to the involvement of the immune system in atherosclerosis and restenosis (Moreno et al., 1996; Rogers et al., 1996; Ross, 1993; Tanaka et al., 1993). Experimental in vivo evidence from transgenic animal

* Corresponding author. Fax: +972 3 5469832.

E-mail address: jacobg@post.tau.ac.il (J. George).

¹ Both first authors equally contributed to the manuscript.

studies indicates that effector immune-potent cells as well as soluble mediators (i.e., cytokines, chemokines) may have a role in regulating the response to injury within the vessel wall.

Tyrosine kinase inhibitors represent a strategy for modulating inflammatory-mediated disorders by acting directly on cells rather than on mediators (Levitzki and Gazit, 1995). A family of tyrosine kinase inhibitors, tyrphostins, has proven considerable *in vitro* and *in vivo* efficacy in this respect (Levitzki and Gazit, 1995). Tyrphostin AG-126, given before endotoxin administration, lowered TNF- α secretion by murine peritoneal macrophages (Novogrodsky et al., 1994). AG-556, a similarly selected tyrphostin reduced mortality in murine models when given up to 2 h after either lipopolysaccharide administration, or a peritoneal *E. coli* O55:B5 infection (Sevransky et al., 1997). AG-556 was also capable of ameliorating the T-cell mediated disorder experimental autoimmune encephalomyelitis (EAE) and autoimmune myocarditis in rats (Banai et al., 1998; Brenner et al., 1998). The major advantage of tyrphostins is their ability to target multiple mediators of inflammation in parallel and, depending on the pharmacokinetic properties of the particular agent, have the potential to reach therapeutic concentrations in a variety of body compartments (Levitzki and Gazit, 1995). Previous studies have attributed beneficial effects to tyrphostins in the prevention of intimal thickening (George et al., 2004; Golomb et al., 1996), possibly mediated by their ability to inhibit smooth muscle cell migration. However, the current agents were selected by virtue of their immunomodulatory properties namely their ability to interfere with effector functions.

Based on the immune modulating properties of tyrphostin AG-556, we tested the hypothesis that this agent may be useful in attenuating neointimal formation in a mouse model of arterial injury employing external cuff placement. We have studied concomitantly, the effect of the tyrphostins on the cytokine secreting properties of spleen cells from the treated mice.

Materials and methods

Mice

Eight-week-old female C57BL/6J mice were purchased from Tel Aviv University and maintained at the local animal house.

Treatment with the tyrphostin AG-556

AG-556 was synthesized as previously described (Gazit et al., 1989, 1991). Mice received daily intraperitoneal injections of AG-556 (2 mg/mouse) starting from induction of injury by cuff placement until sacrifice 2 weeks later.

Arterial injury induced by femoral cuff placement

Mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). The left femoral artery was isolated from surrounding tissues, loosely sheathed with a 2.0-mm polyethylene cuff made of PE-50 tubing (inner diameter, 0.56 mm; outer diameter, 0.965 mm; Becton Dickinson, Mountain View, CA) and tied in place with an 8–0 suture as previously described (Keren et al., 2001; Moroi et al., 1998). The cuff was larger than the vessel and did not obstruct blood flow. The right femoral artery was dissected from surrounding tissues (sham-operated), but a cuff was not placed. After recovery from anesthesia, the animals were given a standard diet and water *ad libitum*.

Proliferation assays

Spleen cell suspensions were made from splenocytes of four mice in each of the groups. The assays were performed as previously described (George et al., 1999) with minor modifications. Briefly, 1×10^6 cells per ml were incubated in triplicates for 72 h in 0.2 ml of culture medium in microtiter wells in the presence or the absence of concanavalin A (2.5 μ g/ml). Proliferation was measured by the incorporation of [3 H] thymidine into DNA during the final 12 h of incubation. The results were computed as stimulation index (S.I.): the ratio of the mean cpm of the antigen to the mean background cpm obtained in the absence of the antigen.

Cytokine levels in cultured medium from splenocytes

The levels of IFN- γ , IL-4, and IL-10 were determined in the cultured medium obtained from splenocytes from all experimental groups in the absence or presence of Con-A. TNF- α levels were assayed in medium from elicited macrophages. Cytokine levels were determined employing ELISA kits (PharMingen) according to the manufacturer's instruction.

Determination of CD4/CD8 cells by FACS

In some experiments, spleen cells were evaluated for the effect of AG-556 on the percentage of CD4/CD8 lymphocytes, employing FACS analysis.

Isolation of murine peritoneal macrophages

Peritoneal macrophages from separate mice ($n = 4$) treated with AG-556 or DMSO for 2 weeks were elicited by intraperitoneal injection of 2 ml thioglycollate medium (Difco) 3 days before harvest by lavage. Peritoneal macrophages from AG-556- and DMSO-treated mice were cultured overnight with RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in a 37°C humidified CO $_2$ incubator. In some experiments, LPS (10 μ g/ml) was added

and the supernatant was collected for evaluation of TNF- α levels.

Tissue harvesting and histopathology

Vessels were fixed in situ by constant pressure fixation at 100 mm Hg with 10% formalin through a 22-gauge butterfly angiocatheter placed in the left ventricle of the heart. Both right and left femoral arteries were harvested. Each artery was embedded in paraffin, and cross-sections (10 μm) were continuously cut from one edge to the other edge of the cuffed portion, and in the corresponding segment of the contralateral control artery. Each section was mounted in order of five series of slides. Parallel sections were subjected to standard hematoxylin and eosin staining. Morphometric analyses were performed on hematoxylin and eosin-stained tissue. For each animal, cross-sections from the cuffed left femoral artery and the control right femoral artery were photographed, and the images were digitized using a Kodak RF 2035 Plus Film Scanner (Eastman Kodak Co., Rochester, NY). For each artery section, the thickness of the intima and media was measured.

Statistical analysis

Data were compared employing the unpaired Student's *t* test. $P < 0.05$ was considered as statistically significant.

Results

The effect of AG-556 on intimal thickening

Mice treated with AG-556 developed reduced intimal thickening ($n = 11$; mean \pm SEM: $43,000 \pm 17,000 \mu\text{m}^2$) and neointimal/medial ratio (1.2 ± 0.5) in comparison with control treated animals ($n = 11$; $286,000 \pm 127,000 \mu\text{m}^2$ and 0.2 ± 0.07 ; respectively; $P < 0.05$ for both comparisons) (Fig. 1). Treatment with AG-556 did not influence medial area ($368,000 \pm 91,000 \mu\text{m}^2$ for AG-556 as compared with $342,000 \pm 82,000 \mu\text{m}^2$ for control DMSO mice; $P = 0.82$) (Fig. 1). Representative H&E-stained sections from AG-556- and DMSO-treated mice are shown in Fig. 2.

AG-556 does not influence splenocyte proliferation

The response of splenocytes obtained from AG-556 to the mitogen Con-A as demonstrated by thymidine uptake did not differ from that of DMSO-treated animals (Fig. 3).

AG-556 increases basal IFN- γ secretion by splenocytes

IFN- γ levels in the supernatant obtained from splenocytes of AG-556-treated mice, 48 h after incubation with

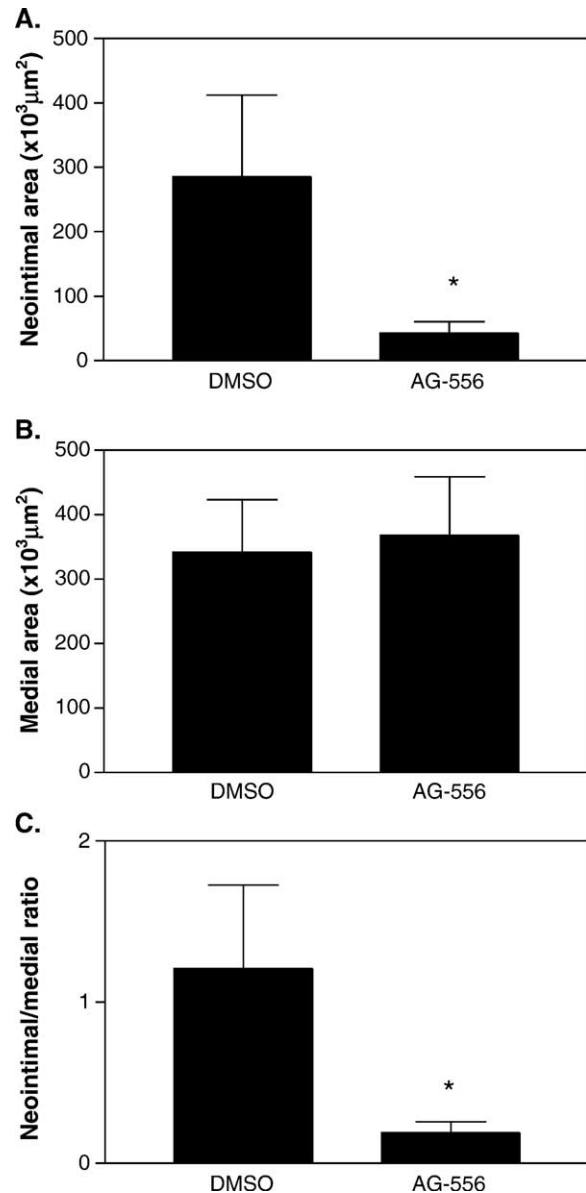


Fig. 1. The effect of treatment with tyrphostin AG-556 on intimal thickening in the mouse. (A) The effect on Neointimal area, (B) medial area (C) neointima/media ratio. $P < 0.05$.

medium were significantly higher than levels found in supernatant from DMSO-treated animals (Fig. 3A). AG-556 did not alter IL-4 or IL-10 secretion by splenocytes.

No differences were evident between AG-556- and DMSO-treated animals with regard to secretion of IL-4 and IL-10 by spleen cells in absence or presence of Con-A (Figs. 3C and 4B).

AG-556 (2 mg/dose) does not inhibit TNF- α production by peritoneal macrophages

These experiments were carried out to assess whether the tyrphostin doses employed for 2 weeks were capable of inhibiting TNF- α secretion by peritoneal macrophages

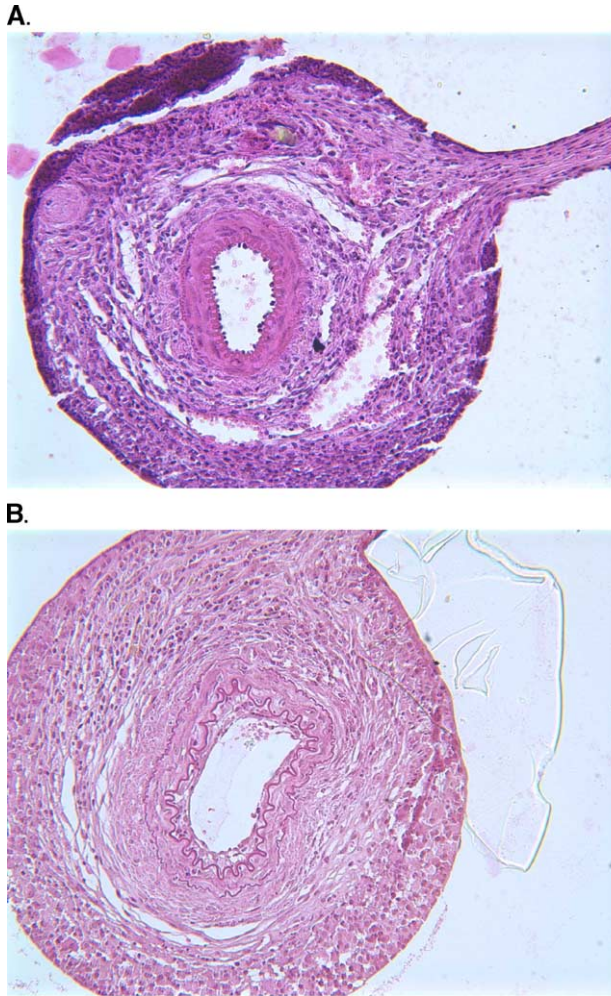


Fig. 2. Intimal hyperplasia in a femoral artery from AG-556- and DMSO-treated mice. Representative H&E-stained sections from AG-556 (A) or DMSO (B) treated mice, 2 weeks following cuff placement.

obtained from treated mice. Non-stimulated levels of TNF- α in the supernatant from AG-556- and DMSO-treated mice were below the detection threshold. No differences were

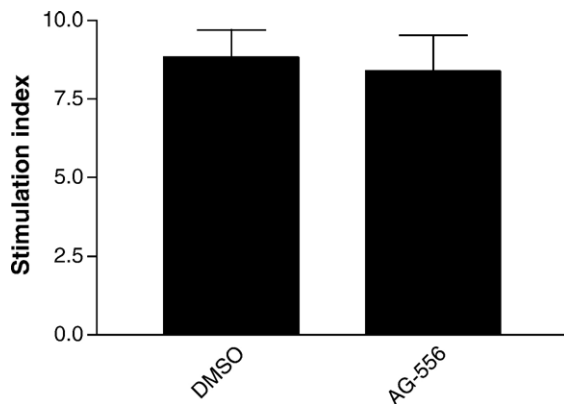


Fig. 3. Con A stimulated proliferation of spleen cells from mice treated with tyrphostins or DMSO. Spleen cells were isolated from AG-556 or DMSO treated animals, 2 weeks after femoral cuff placement. Isolated cells were incubated in the presence or absence of Con-A and proliferative response was determined by assessing thymidine incorporation.

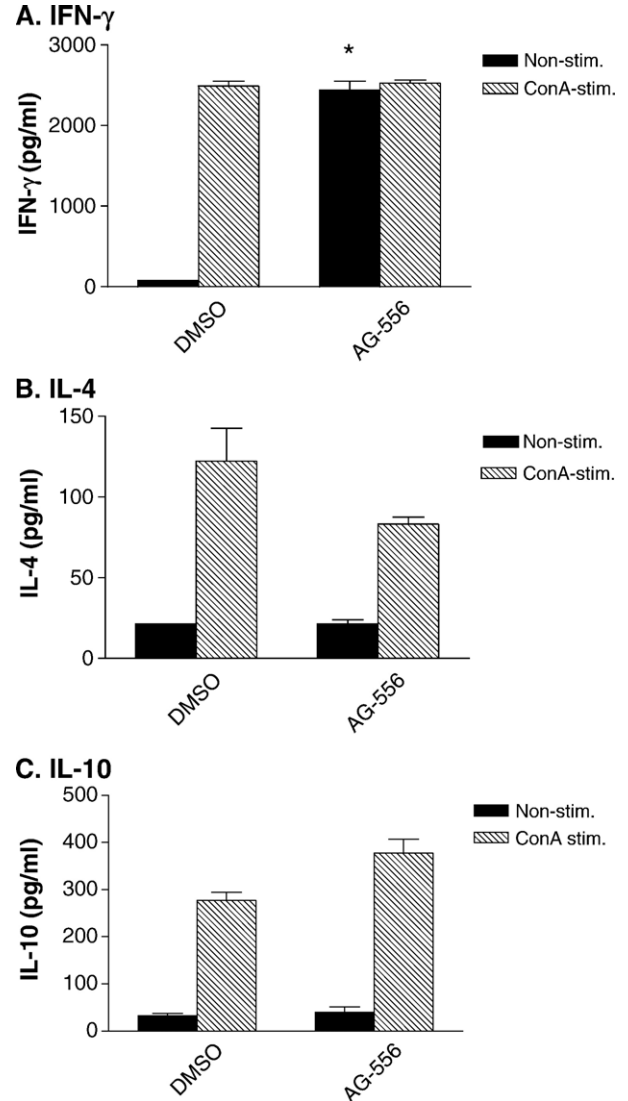


Fig. 4. Cytokine secretion in tyrphostin or DMSO treated mice. Supernatants were collected from AG-556 or DMSO treated mice following incubation with or without Con-A. IFN- γ (A), IL-4 (B), and IL-10 (C) were evaluated employing a capture ELISA as described in Materials and methods. * $P < 0.05$.

found between TNF- α levels in the supernatant following LPS treatment between tyrphostin-treated and DMSO-treated mice (Fig. 5).

The effect of AG-556 on the ratio of CD4/CD8 in the spleen

No effect of AG-556 treatment was evident on spleen cell CD4 or CD8 numbers or on CD4/CD8 ratio in comparison with DMSO treatment (data not shown).

Discussion

In the current study, we explored the possibility that the immune-modulating compound AG-556 will be effective

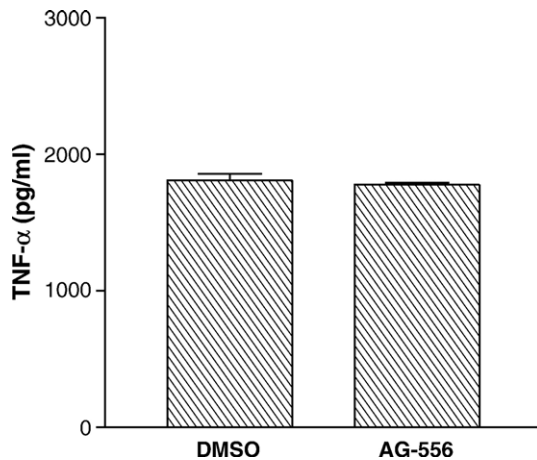


Fig. 5. TNF- α production by peritoneal macrophage from tyrphostin of DMSO treated mice. Peritoneal macrophages were elicited by thioglycolate injection to 3 mice from each of the treatment groups ($n = 4$, each) and incubated with LPS for 24 h. Supernatants were collected and evaluated for the presence of TNF- α using a capture ELISA.

in attenuating intimal thickening in a mouse model of arterial injury. We have chosen a model of external cuff placement and not the conventional internal injury as inflammation plays a prominent role in the pathogenesis of neointimal growth evident in this model (Moro et al., 1998). Moreover, our previous observations suggest that immune modulation with intravenous gammaglobulins in this model has proven successful (Keren et al., 2001).

We have found that treatment with the immunomodulating tyrphostin AG-556 significantly reduced neointimal growth as well as neointimal/medial ratio in the cuff injured femoral arteries. Importantly, treatment with AG-556 was not associated with a significant alteration in the size of the stabilizing medial layer (Fig. 1).

AG-556 was synthesized based on its ability to inhibit TNF- α secretion as well as cell cytotoxicity (Levitzi and Gazit, 1995). Indeed, subsequent studies have demonstrated their beneficial role in models of sepsis in which tissue damage is mediated by excessive TNF- α production. Our recent observations suggest that the ameliorating effect on TNF- α was associated with a protective effect of this agent in a model of induced myocardial infarction (George et al., 2003) and in experimental myocarditis (George et al., 2004) in the rat.

We have found that the concentrations of tyrphostins administered to the mice throughout the study did not appear to influence LPS-induced TNF- α secretion by peritoneal macrophages obtained from the treated animals. This observation is, however, not surprising, as the inhibitory effects on TNF- α , described previously with these cytokines, were achieved by employing a significantly higher amount of the tyrphostins given in a short-term model of sepsis.

In view of these findings, we have chosen to study the levels of additional cytokines that are produced and secreted by lymphocytes infiltrating the adventitia of cuff placed femoral arteries. IFN- γ is a cytokine produced by T-lymphocytes that plays a pivotal role in mediating the cellular immune response (Farrar and Schreiber, 1993). Conflicting results have been presented with respect to the role of IFN- γ in atherosclerosis and intimal thickening. IFN- γ stimulates the expression of the adhesion molecule VCAM-1 on endothelial cells thus possibly enhancing recruitment of immunopotent cells (Li et al., 1993). Moreover, IFN- γ increases major histocompatibility complex (MHC) II expression on macrophages and smooth muscle cells thus potentially promoting a local immune reaction within the arterial wall (Jonasson et al., 1985). In vivo, it has been shown that atherosclerosis-prone apoE knockout mice crossed against an IFN- γ receptor-deficient background animal results in diminished lesion progression suggesting that IFN- γ has a proatherogenic role (Gupta et al., 1997). On the other hand, IFN- γ appears to block smooth muscle cell (SMC) proliferation and to decrease collagen synthesis by SMC, thus possibly ameliorating neointimal growth (Hansson et al., 1989). Herein, the secretion of IFN- γ by splenocytes treated with AG-556 was significantly enhanced in the absence of any form of stimulation in comparison to control cells. This finding may aid in explaining the favorable effect of AG-556 on the number of arteries affected by neointimal proliferation.

Unlike IFN- γ secretion, we have found that IL-4 as well as IL-10, which are both considered as counter-regulatory cytokines were not significantly influenced by tyrphostin administration. Moreover, splenocyte CD4/CD8 ratios and proliferation to a non-specific mitogen were not altered by treatment with AG-556 despite its immune modulating effects thus excluding a significant suppressive effect on cellular immunity. The current study cannot determine with certainty the mechanism of the protective effect of AG-556. However, considering the dominant role of inflammation in this model of injury and the impressive effect of the agent on basal splenocyte IFN-gamma secretion, it may be assumed that the antiproliferative effect is mediated by a reduced activity of this cytokine.

In conclusion, AG-556 treatment was found to reduce significantly neointimal and neointimal/medial ratio. The effect was associated with a markedly enhanced IFN- γ secretion by non-stimulated spleen-derived lymphoid cells. These observations may represent an interesting approach by which to design modulatory agents for the prevention of restenosis.

Acknowledgment

Supported in part by a grant from the Israeli Chief Scientist (JG).

References

- Banai, S., Wolf, Y., Golomb, G., Pearle, A., Waltenberger, J., Fishbein, I., Schneider, A., Gazit, A., Perez, L., Huber, R., Lazarovichi, G., Rabinovich, L., Levitzki, A., Gertz, S.D., 1998. PDGF-receptor tyrosine kinase blocker AG1295 selectively attenuates smooth muscle cell growth in vitro and reduces neointimal formation after balloon angioplasty in swine. *Circulation* 97, 1960–1969.
- Brenner, T., Poradosu, E., Soffer, D., Sicsic, C., Gazit, A., Levitzki, A., 1998. Suppression of experimental autoimmune encephalomyelitis by Tyrphostin AG-556. *Exp. Neurol.* 154, 489–498.
- Farrar, M.A., Schreiber, R.D., 1993. The molecular cell biology of interferon γ and its receptor. *Annu. Rev. Immunol.* 11, 571–611.
- Fuster, V., Badimon, L., Badimon, J.J., Chesebro, J.H., 1992. The pathogenesis of coronary artery disease and the acute coronary syndromes. *N. Engl. J. Med.* 236, 242–250.
- Gazit, A., Yaish, P., Gilon, C., Levitzki, A., 1989. Tyrphostin I: synthesis and biological activity of protein tyrosine kinase inhibitors. *J. Med. Chem.* 32, 2344–2352.
- Gazit, A., Osherov, N., Posner, P., Yaish, E., Poradosu, E., Gilon, C., Levitzki, A., 1991. Tyrphostin II: heterocyclic and α -substituted benzonomaloronitrile Tyrphostins as protein inhibitors of EGF receptor and ErbB2/neu tyrosine kinases. *J. Med. Chem.* 34, 1897–1907.
- George, J., Shoenfeld, Y., Afek, A., Gilburd, B., Keren, P., Shaish, A., Kopolovic, Y., Wick, G., Harats, D., 1999. Enhanced fatty streak formation in C57BL/6J mice by immunization with heat shock protein 65. *Arterioscler. Thromb. Vasc. Biol.* 19, 505–510.
- George, J., Biner, S., Keren, P., Barshack, I., Goldberg, I., Sherez, J., Keren, G., Roth, A., 2003. The tyrphostin AG-556 reduces myocardial infarct size and improves cardiac performance in the rat. *Exp. Mol. Pathol.* 74, 314–318.
- George, J., Barshack, I., Goldberg, I., Keren, P., Gazit, A., Levitzki, A., Keren, G., Roth, A., 2004. The effect of early and late treatment with the Tyrphostin AG-556 on the progression of experimental autoimmune myocarditis. *Exp. Mol. Pathol.* 76, 234–241.
- Golomb, G., Fishbein, I., Banai, S., Mishaly, D., Moscovitz, D., Gertz, S.D., Gazit, A., Poradosu, E., Levitzki, A., 1996. Controlled delivery of a tyrphostin inhibits intimal hyperplasia in a rat carotid artery injury model. *Atherosclerosis* 125, 171–182.
- Gupta, S., Pablo, A.M., Jiang, X., Wang, N., Tall, A.R., Schindler, C., 1997. IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J. Clin. Invest.* 99, 2752–2761.
- Hansson, G.K., Hellstrand, M., Rymo, L., Rubbia, L., Gabbiani, G., 1989. Interferon- γ inhibits smooth muscle cell proliferation and expression of differentiation specific α -smooth muscle cell actin in arterial smooth muscle cells. *J. Exp. Med.* 170, 1595–1608.
- Jonasson, G.K., Holm, J., Skalli, O., Gabbiani, G., Hansson, G.K., 1985. Expression of class II transplantation antigen on smooth muscle cells in human atherosclerosis. *J. Clin. Invest.* 76, 125–131.
- Kaltenbach, N., Kober, G., Scherer, D., Vallbracht, C., 1985. Recurrence rate after successful coronary angioplasty. *Eur. Heart J.* 6, 276–281.
- Kereiakes, D.J., Willerson, J.T., 2003. Vascular brachytherapy boon or bust? *Circulation* 108, 389–390.
- Keren, G., Pri-chen, S., Keren, P., Barshack, I., Goldberg, I., George, J., 2001. The effect of intravenous gammaglobulins on intimal thickening in a mouse model of arterial injury. *Atherosclerosis* 159, 77–83.
- Levitzki, A., Gazit, A., 1995. Tyrosine kinase inhibition: an approach to drug development. *Science* 267, 1782–1788.
- Li, H., Cybulsky, M.I., Gimbrone, M.A., Libby, P., 1993. An atherogenic diet rapidly induces VCAM-1, a cytokine regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. *Arterioscler. Thromb.* 13, 197–204.
- Libby, P., Schwartz, D., Brogi, E., Tanaka, H., Clinton, S.K., 1992. A cascade model for restenosis: a special case of atherosclerosis progression. *Circulation* 86, III47–III52 (Suppl.).
- Liu, M.W., Roubin, G.S., King, S.B., 1989. Restenosis after coronary angioplasty. Potential biologic determinants and role of intimal hyperplasia. *Circulation* 79, 1374–1387.
- Marx, S.O., Marks, A.R., 2001. The development of rapamycin and its application to stent restenosis. *Circulation* 104, 852–855.
- Moreno, P.R., et al., 1996. Macrophage infiltration predicts restenosis after coronary intervention in patients with unstable angina. *Circulation* 94, 3098–3102.
- Morice, M.C., Serruys, P.W., Sousa, J.E., et al., 2002. A randomized comparison of sirolimus-eluting stent with a standard stent for coronary revascularization. *N. Engl. J. Med.* 346, 1773–1780.
- Moroi, M., Zhang, L., Ysuda, T., et al., 1998. Interaction of genetic deficiency of endothelial nitric oxide, gender and pregnancy in vascular response to injury in mice. *J. Clin. Invest.* 101, 1225–1232.
- Novogrodsky, A., Vanichkin, A., Patya, M., Gazit, A., Osherov, N., Levitzki, A., 1994. Prevention of lipopolysaccharide induced lethal toxicity by tyrosine kinase inhibitors. *Science (Washington, DC)* 264, 1319–1322.
- Rogers, C., Welt, F.G., Karnovsky, M.J., Edelman, E.R., 1996. Monocyte recruitment and neointimal hyperplasia in rabbits: coupled inhibitory effects of heparin. *Arterioscler. Thromb. Vasc. Biol.* 16, 1312–1318.
- Ross, R., 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature (Lond.)* 362, 801–809.
- Sevransky, J.E., Shaked, G., Novogrodsky, A., et al., 1997. Tyrphostin AG 556 improves survival and reduces multiorgan failure in canine *Escherichia coli* peritonitis. *J. Clin. Invest.* 99, 1966–1973.
- Sheppard, R., Eisenberg, M.J., Donath, D., Meerkin, D., 2003. Intra-coronary brachytherapy for the prevention of restenosis after percutaneous coronary revascularization. *Am. Heart J.* 146, 775–786.
- Sousa, J.E., Serruys, P.W., Costa, M.A., 2003. New frontiers in cardiology: drug-eluting stents: part II. *Circulation* 107, 2383–2389.
- Tanaka, H., et al., 1993. Sustained activation of vascular cells and leukocytes in the rabbit aorta after balloon injury. *Circulation* 88, 1788–1803.