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LETTER TO THE EDITORS

Tyrphostins which Interfere with the Actin Cytoskeleton do not Increase Outflow Facility

Cytoskeletal reagents, such as cytochalasins, H-7, staurosporine and latrunculins, which affect cell–cell (C–C) contacts (e.g. adherens junctions; AJ), cell–extracellular matrix (C–ECM) attachments (e.g. focal contacts; FC) and cytoskeletal networks in cultured cells (Spector et al., 1983; Bershadsky and Vasiliev, 1988; Mobley et al., 1994; Volberg et al., 1994), increase aqueous humour outflow facility in living monkeys (Kaufman and Erickson, 1982; Tian et al., 1998; Peterson et al., 1999; Tian, Gabelt and Kaufman, 1999). Although the drug-induced changes in C–C contacts, C–ECM attachments, cytoskeletal networks, and cell shape, volume and contractility, could be major factors in reducing flow resistance in the outflow pathways, the specific target(s) in the trabecular meshwork (TM) for these compounds are still unknown. Since C–C and C–ECM junctional complexes are rich in phosphotyrosine-phosphorylated proteins, changes in the phosphorylation levels of these proteins are believed to modulate adhesive function and junctional organization in endothelial cells (Burridge, Turner and Romer, 1992; Volberg et al., 1992; Romer et al., 1994). Inhibitors of tyrosine phosphorylation interfere with the assembly of FC and induce reorganization of AJ in several types of cultured cells (Burridge et al., 1992; Volberg et al.; 1992; Romer et al., 1994; Miyamoto et al., 1995). Typical inhibitors of tyrosine phosphorylation are tyrphostins, which have specific inhibitory capacity towards different tyrosine specific protein kinases (Levitzki, 1992; Osherov et al., 1993; Levitzki and Gazit, 1995). In a recent study, 14 different tyrphostins were shown to have different effects on the actin cytoskeleton in cultured calf pulmonary artery endothelial cells and were grouped into three categories: those which have no discernible effect on C–C junctions, C–ECM attachments or stress fibres (i.e. their cellular targets are unknown); those which produce cell retraction with concomitant disruption of C–C junctions, C–ECM contacts and stress fibres (except one compound which shows greater specificity for C–C junctions); and those which completely disrupt stress fibres and C–ECM contacts without causing C–C separation (Farooki, Epstein and O'Brien, 1998). Based on this information, and in view of the involvement of the actin cytoskeleton in regulating outflow facility (Kaufman and Erickson, 1982; Tian et al., 1998, 1999; Peterson et al., 1999), the authors decided to explore the effect of different tyrphostins on outflow dynamics. Therefore, they surveyed the effects of 28 tyrphostins on the cytoskeletal architecture in cultured bovine aortic endothelial cells (BAEC), and then tested those which affected the actin system most strongly in different ways (AG1406, AG1478, AG1498 and AG1714) for their effects on outflow facility in living monkeys.

BAEC form a stable, confluent monolayer of well-differentiated cells with a well-developed actin cytoskeleton, FC and AJ, and are widely used in cytoskeleton studies (Tian et al., 1998; Peterson et al., 1999; Volberg et al., 2000). Previous studies have shown that BAEC behave similar to HTM cells in culture and to TM cells in situ in live monkeys, in response to agents such as H-7 and latrunculin A that affect cellular contractility and the actin cytoskeleton (Cai et al., 2000; Liu et al., 2001; Peterson et al., 1999; Sabanay et al., 2000; Tian et al., 1998). Thus, even though BAEC and HTM cells may not precisely represent the behaviour of the same cells in vivo, they can provide clues as to the possible mechanism of action and effective drug doses. Given the dose and effect concordance of the BAEC, HTM cell and live monkey work for H-7 and latrunculin A (Volberg et al., 1994; Tian et al., 1998; Peterson et al., 1999; Cai et al., 2000; Liu et al., 2001), the well-established system in Dr Geiger's laboratory for screening cytoskeletal and cell adhesion responses to drugs using BAEC, and the relative ease of culturing and maintaining BAEC compared to the more 'finicky' HTM cells, it seemed reasonable to choose BAEC as a cellular model to screen tyrphostin candidates for the in vivo study in monkeys.

Twenty eight tyrphostins were provided by Drs Alexander Levitzki and Aviv Gazit (The Hebrew University, Jerusalem, Israel). The tyrphostins were initially dissolved in DMSO (Sigma Chemical Co., St. Louis, MI, U.S.A.) as 0·1M stock solutions and stored at −80°C. Before use the tyrphostins were diluted in the culture medium and applied to the cells as indicated. BAEC were cultured by standard techniques (Volberg et al., 2000). In this screen cells were treated with 1–100 μM of each tyrphostin for 45 min, fixed and labelled for actin. Tyrphostins, which had a mild effect on the actin system, include AG82, AG94, AG126, AG213, AG490, AG537, AG556, AG806, AG879, AG1146, AG1296, AG1320, AG1331, AG1387, AG1489, AG1517, AG1518, AG1568,

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AG1640, AG1661 and AG1663 (data not shown). The tyrphostins which did not affect actin at all were AG18, AG30 and AG1659 (data not shown). Four of the 28 tyrphostins studied (AG1406, AG1478, AG1498 and AG1714) had a major effect on the actin system and were thus selected for further characterization based on their major effect on the actin cytoskeleton. BAEC were treated for 45 min with 1, 10 or 100 μM of AG1406, AG1478, AG1498 or AG1714, and then immunolabelled for actin, vinculin and β-catenin. The four tyrphostins affected the actin cytoskeleton in different ways dose-dependently at concentrations from 1 to 100 μM (AG1406 and 1478) or 10 to 100 μM (AG1498 and AG1714). The alterations in C–C contacts, C–ECM attachments and/or cytoskeletal networks of BAEC after 100 μM tyrphostins are shown in Fig. 1. AG1478 disrupted FC and stress fibres as shown by actin and vinculin labelling (Fig. 1(D) and (E)), but had limited effect on the organization of actin, vinculin and β-catenin in C–C junctions (Fig. 1(D)–(F)). Treatment with AG1406 disrupted stress fibres, FC and C–C junctions. After treatment with AG1406 only a few fragments of actin stress fibres were detectable (Fig. 1(G)); vinculin was reorganized into small FC but was apparently absent from residual C–C junctions (Fig. 1(H)). The disruption of C–C junctions was also apparent from β-catenin labelling (Fig. 1(I)). AG1498 had similar effects on the actin system to those of AG1406 (data not shown). As previously shown (Volberg et al., 2000), tyrphostin AG1714 disrupted microtubules, concomitantly stimulated stress fibre development (Fig. 1(J)) and increased FC size (Fig. 1(K)), and disrupted C–C junctions (Fig. 1(K), L).

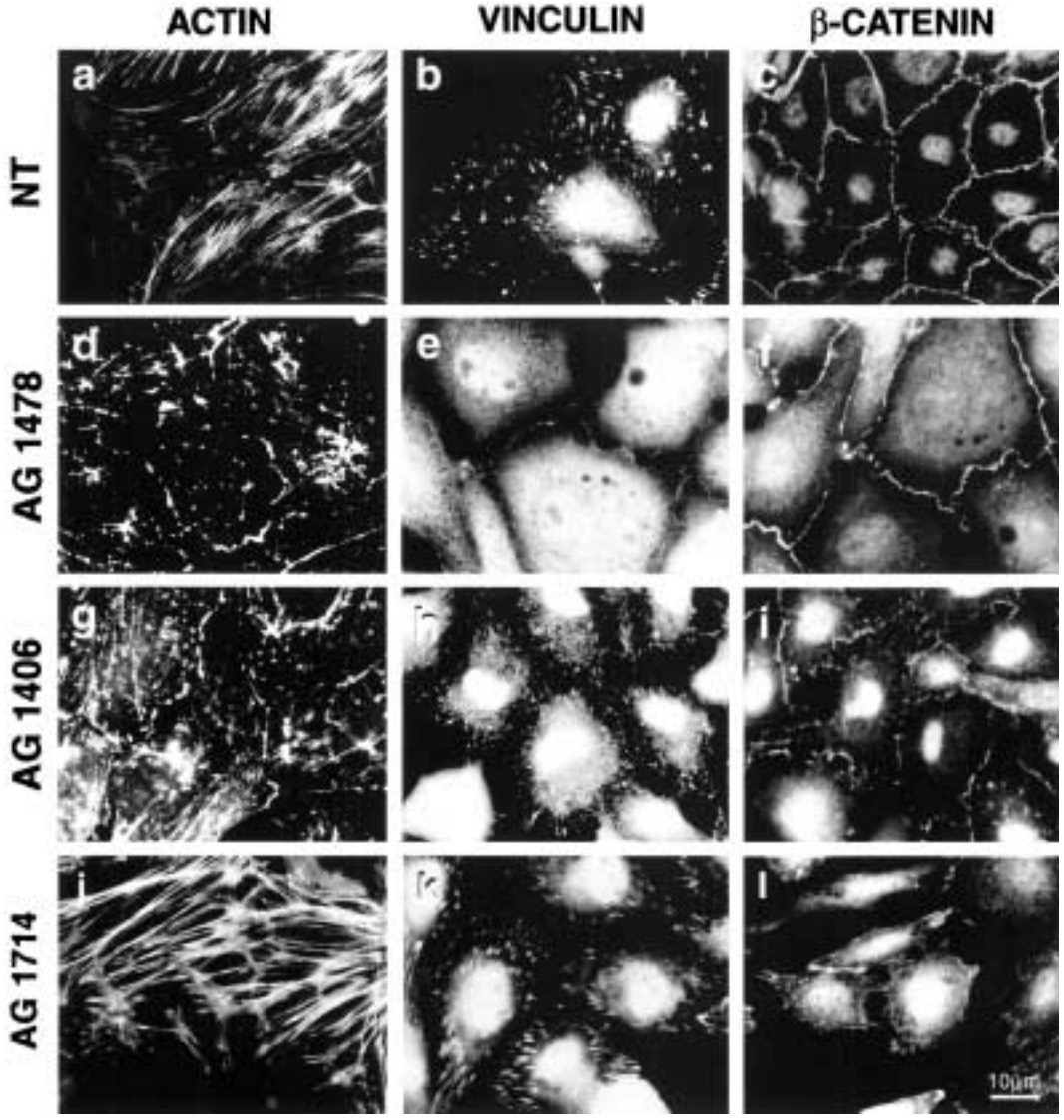

FIG. 1. The effect of tyrphostins AG1478, AG1406 and AG1714 on the organization of actin, vinculin and β-catenin in BAEC. BAEC were cultured on glass coverslips for 24 hr and then treated with 100 μM AG1478 (D–F) or AG1406 (G–I) or AG1714 (J–L) for 45 min, permeabilized/fixed and immunolabelled for actin (A, D, G), vinculin (B, E, H) and β-catenin (C, F, I). Untreated cells are shown in (A–C). AG1478 disrupts actin stress fibres and FC (D) but has limited effect on C–C adhesions (D–F). Tyrphostin AG1406 disrupts actin stress fibres, FC (G, H) and C–C junctions (H, I), while AG1714 enhances actin stress fibre bundling (J), enhances FC (K) and disrupts C–C (K, L). NT = not treated; bar = 10 μM.

To determine the effects of AG1406, AG1498, AG1714 and AG1478 on outflow facility, the total outflow facility was measured by 2-level constant pressure perfusion of the anterior chamber (AC) with Bárány's solution (Bárány, 1964), correcting for internal apparatus resistance (Bárány, 1965), in normal cynomolgus monkeys (*Macaca fascicularis*). All investigations were in accordance with the University of Wisconsin and NIH guidelines, and with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Anaesthesia for AC perfusion was induced by intramuscular (i.m.) ketamine (10 mg kg^{-1}), followed by intravenous (15 mg kg^{-1}) or i.m. (35 mg kg^{-1}) pentobarbital-Na.

For treatment of monkeys each stock solution was diluted with Bárány's solution immediately before the AC exchange perfusion to provide drug concentrations in the range of 1–100 μM in DMSO. The outflow facility variably increased after some tyrphostin dosages, but the facility increase in the tyrphostin-treated and the contralateral vehicle-treated eye was similar. Thus, the post-tyrphostin facility, adjusted for baseline and contralateral control eye resistance washout, was not significantly altered, except for a modest increase ($24 \pm 8\%$, $P < 0{\cdot}05$) following 10 μM AG1478 (Table I). However, 100 μM AG1478 insignificantly decreased the outflow facility. Similar results with tyrphostins 23, 25, 47 (Sigma, St. Louis, MO, U.S.A.) and AG 126 (10–400 μM) were obtained in the porcine whole eye perfusion system (Rowlette., L.L., Farooki, A., Epstein, D.L. and O'Brien, E.T., personal communication). These agents either had no effect on outflow facility or decreased the magnitude of the washout effect seen in paired controls. Tyrphostins 47 and 23 decreased outflow facility at higher concentrations (400 μM, $P < 0{\cdot}03$, $n = 5$ for each agent), even though tyrphostin 47 induced loss of F-actin and FC and tyrphostin 23 disrupted C–C junctions in cultured calf pulmonary artery endothelial cells (Farooki et al., 1998).

Since FC and C–C junctions may play a critical role in maintaining the permeability barrier function, theoretically the effects of tyrphostins on these junctions would enhance endothelial cell permeability or paracellular fluid flow in the TM, leading to an increase in outflow facility. However, all four tyrphostins studied in vivo, which have major effects on the actin cytoskeleton in culture, failed to produce a definite increase in outflow facility in living monkeys. It is not clear why these tyrphostins interfere with the actin cytoskeleton in culture but do not increase the outflow facility in vivo. Differential sensitivity seems to be an unlikely explanation, since similar effects to those shown here were obtained with a variety of cells including cultured human TM cells (Farooki et al., 1997). Based on previous studies of cytoskeletal drugs, the required concentration to increase outflow facility in vivo usually is much higher than that to induce initial cytoskeletal changes in vitro. For instance, 6 μM H-7 causes alterations of microfilament bundles in cultured porcine aortic endothelial cells (Yu and Gotlieb, 1992), while 100–300μM concentrations are needed for this drug to increase significantly the outflow facility in living monkeys (Tian et al., 1998). This phenomenon could be related to the presence of molecules in vivo, that modify drug action or stability, or other pharmacokinetic considerations. Thus, lower concentration of the drug in target cells in vivo than in vitro might be responsible for the dissociation between the in vitro and in vivo effects of these compounds. However, other explanations are also possible. Similar to facility-effective concentrations, 100–300 μM H-7 are also needed to inhibit significantly cellular contractility in several types of cultured cells (Volberg et al., 1994). In the TM cells of the live monkey eye, 300 μM H-7 produces similar cellular relaxation (Sabanay et al., 2000) as in the cultured cell types (Volberg et al., 1994. This may indicate that following H-7, substantial cellular relaxation in conjunction with the consequent major cytoskeletal alterations, rather than the initial slight changes in cell contractility and/or the cytoskeleton visible in cell culture, triggers the increase in outflow facility. Given this view, the required concentration to reduce outflow resistance in vivo and that to induce responsible cellular changes in vitro seem to be similar. Under this scenario, it is possible that the lack of increase in outflow facility after the tyrphostins may not be related to insufficient doses, but rather that the specific cytoskeletal changes in the TM induced by the tyrphostins may not be sufficient or relevant to reduce outflow resistance. Tyrphostins do not affect contractility in the same manner as H-7, and do not affect the cytoskeleton or actin directly in the manner of latrunculins, and thus may have physiologic effects — or lack thereof — quite distinct from such compounds. In short, if a facility increase at the concentrations used cannot be found, in all likelihood these tyrphostins cannot physiologically induce one.

Two additional reasons support this hypothesis. The first is that the in vivo concentrations were comparable or up to 10-fold higher than the consistently effective in vitro dose. From previous experience (Tian et al., 1998; Peterson et al., 1999; Tian, Brumback and Kaufman, 2000), this concentration should be sufficient to produce at least a minimal effect on outflow facility. Secondly, in previous studies of cytoskeletal drugs (Kaufman and Erickson, 1982; Tian et al., 1998, 2000; Peterson et al., 1999), the facility response to a given drug was always dose-dependent. At doses above the plateau (i.e. supramaximal), the facility would sometimes decrease relative to the maximal effect, probably related to secondary effects such as the blood–aqueous barrier breakdown with protein in the AC clogging the meshwork (Kaufman and Erickson,

TABLE 1

Effects of tyrphostins on outflow facility in monkeys

	Tyrphostin			Vehicle			Tyrphostin/vehicle		
	BL	Rx	R × BL	BL	Rx	Rx/BL	BL	Rx	Rx/BL
AG1406									
1 μM ($n = 8$)	0·35 ± 0·06	0·54 ± 0·11	1·54 ± 0·21*	0·37 ± 0·05	0·49 ± 0·06	1·38 ± 0·12*	0·97 ± 0·11	1·13 ± 0·23	1·12 ± 0·15
10 μM ($n = 4$)	0·37 ± 0·06	0·47 ± 0·08	1·29 ± 0·29*	0·35 ± 0·04	0·44 ± 0·11	1·24 ± 0·18	1·04 ± 0·10	1·13 ± 0·18	1·08 ± 0·09
100 μM ($n = 6$)	0·27 ± 0·03	0·32 ± 0·06	1·22 ± 0·21	0·31 ± 0·05	0·37 ± 0·13	1·17 ± 0·30	0·93 ± 0·10	1·17 ± 0·25	1·24 ± 0·22
AG1498									
10 μM ($n = 4$)	0·37 ± 0·07	0·78 ± 0·10	2·21 ± 0·21*	0·31 ± 0·03	0·67 ± 0·14	2·11 ± 0·19†	1·22 ± 0·22	1·26 ± 0·17	1·06 ± 0·09
100 μM ($n = 4$)	0·41 ± 0·06	0·54 ± 0·07	1·31 ± 0·05†	0·45 ± 0·12	0·58 ± 0·10	1·40 ± 0·20	1·24 ± 0·54	1·12 ± 0·38	1·03 ± 0·23
AG1714									
10 μM ($n = 8$)	0·29 ± 0·05	0·42 ± 0·07	1·46 ± 0·17*	0·32 ± 0·05	0·43 ± 0·07	1·35 ± 0·19	0·92 ± 0·09	1·08 ± 0·14	1·17 ± 0·14
100 μM ($n = 9$)	0·33 ± 0·05	0·32 ± 0·04	1·00 ± 0·07	0·27 ± 0·04	0·26 ± 0·05	0·95 ± 0·08	1·36 ± 0·20	1·61 ± 0·35	1·10 ± 0·11
AG1478									
1 μM ($n = 4$)	0·39 ± 0·06	0·59 ± 0·11	1·50 ± 0·12*	0·45 ± 0·05	0·64 ± 0·11	1·42 ± 0·11*	0·87 ± 0·11	0·93 ± 0·13	1·07 ± 0·08
10 μM ($n = 8$)	0·38 ± 0·04	0·62 ± ·07	1·63 ± 0·10‡	0·44 ± 0·04	0·61 ± 0·10	1·35 ± 0·12*	0·88 ± 0·08	1·07 ± 0·09	1·24 ± 0·08*
100 μM ($n = 4$)	0·29 ± 0·09	0·18 ± 0·04	0·83 ± 0·25	0·25 ± 0·05	0·25 ± 0·05	0·99 ± 0·06	1·16 ± 0·20	0·99 ± 0·44	0·83 ± 0·27

Outflow facility was measured before and after AC exchange with tyrphostin AG1406, AG1498, AG1714 or AG1478 in one eye and corresponding vehicle in the opposite eye. BL, baseline facility for 35 min before AC exchange; post drug facility (Rx) encompasses 90 min, beginning 45 min after AC exchange. Facility data are mean ± S.E.(M.) (μl per min per mmHg) for n animals, each contributing one eye receiving tyrphostin and one receiving vehicle; ratios are unitless.

$^{*}P < 0{\cdot}05$, $^{\dagger}P < 0{\cdot}01$, $^{\ddagger}P < 0{\cdot}001$ for ratios different from 1·0 by the 2-tailed paired t-test.

1982; Tian et al., 1999). In the current study, 10 μM AG1478 seemed to modestly increase the outflow facility in the live monkey eye, but the 100 μM dose of this drug tended to *decrease* rather than further increase the facility. This indicates that the lack of outflow facility increase after tyrphostins is unlikely to be related to insufficient drug concentrations; i.e. a dose that substantially increased facility could never be found, and in the one instance where a modest increase was seen, a higher dose tended to push the system the other way. The authors did not try still higher doses of tyrphostins to prove this hypothesis for these compounds. Since the actin cytoskeleton is an interactive system such that affecting one target may have considerable effects on the others, further elevating the tyrphostin concentrations might have attenuated their specificity, precluded identifying the effects on or of specific targets, or even decreased outflow facility, as has been seen with cytochalasins (Kaufman and Erickson, 1982), staurosporine (Tian et al., 1999) and H-7 (Tian and Kaufman, unpubl. res.) previously and perhaps even with AG1478 here and with tyrphostins 47 and 23 in O'Brien's laboratory (Rowlette et al., personal communication).

However, possible cytoskeletal effects of tyrosine kinase inhibition on outflow facility based only on the four tyrphostins studied cannot be absolutely excluded. Further studies of the relationship between the inhibition of tyrosine phosphorylation or the disruption of the actin microfilament network in cultured cells and the reduction of outflow resistance across the TM/Schlemm's canal in the live monkey eye are needed.

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TOVA VOLBERG[a]
BAOHE TIAN[b]
PAUL L. KAUFMAN[b*]
BENJAMIN GEIGER[a]
[a]*Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel*
[b]*Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison, WI, U.S.A.*

*Author for correspondence. E-mail: kaufmanp@mhub.ophth.wisc.edu

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