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Research article

IN VITRO EVALUATION OF THE CYTOTOXIC AND ANTI-PROLIFERATIVE PROPERTIES OF RESVERATROL AND SEVERAL OF ITS ANALOGS

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Abstract: Resveratrol (RES), a component of red wine, possesses anti-inflammatory properties. The studies described in the present work were aimed at evaluating the potential for RES and related stilbene analogs (piceatannol, PIC; pterostilbene, TPS; *trans*-stilbene, TS; and *trans*-stilbene oxide, TSO) to exhibit toxicity towards RAW 264.7 mouse macrophages. The effect of TS, TSO, RES and TPS on RAW 264.7 macrophage viability was determined by two standard methods: (a) the MTT assay and (b) the trypan blue dye exclusion test. Whereas macrophages were more sensitive to PIC ($LC_{50\ trypan}$ ~ 1.3 μM) and to TPS ($LC_{50\ trypan}$ ~ 4.0 μM and $LC_{50\ MTT}$ ~ 8.3 μM) than to RES ($LC_{50\ trypan}$ ~ 8.9 μM and $LC_{50\ MTT}$ ~ 29.0 μM), they were relatively resistant to TSO ($LC_{50\ trypan}$ ~ 61.0 μM and $LC_{50\ MTT}$ > 100 μM) and to TS ($LC_{50\ trypan} \geq 5.0$ μM and $LC_{50\ MTT} \geq 5.0$ μM). The ability of selected stilbenes (RES, TPS and PIC) to exhibit growth inhibitory effects was also examined. Although RES and TPS were observed to inhibit cell proliferation in macrophages ($IC_{50} \leq 25$ μM), these cells were resistant to growth inhibition by PIC ($IC_{50} \geq 50$ μM). The data obtained in the present analysis demonstrate that substituted stilbene compounds such as RES have the capacity to exhibit cytotoxic and anti-proliferative activities in macrophages.

Key words: Resveratrol, Piceatannol, Pterostilbene, Stilbenes, Cell viability, Cell proliferation, Macrophages, *TLR4* (-/-), Antioxidants

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Abbreviations used: MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PIC – *trans*-piceatannol; RES – *trans*-resveratrol; TPS – *trans*-pterostilbene; TS – *trans*-stilbene; TSO – *trans*-stilbene oxide

INTRODUCTION

Red wine contains numerous phenolic ingredients including a wide range of polyphenols such as flavonols (quercetin and myricetin), flavanols (catechin and epi(gallo)catechin), phenolics (caffeic acid and gallic acid), stilbenes (*trans* and *cis* isomers of resveratrol), and anthocyanins [1]. Among these agents, *trans*-resveratrol (RES) may contribute to the protective effect of red wine believed to underlie the French Paradox [2-5]. Whereas definitive human clinical trials would be required to confirm a role for RES in the French Paradox, many other types of studies have shown that RES exhibits potent anti-inflammatory, antioxidant, anti-proliferative, cardioprotective and chemopreventive properties [6-9]. RES also exhibits antimicrobial activity [10].

Other stilbene compounds, namely *trans*-piceatannol (PIC) and *trans*-pterostilbene (TPS), have also been reported to exhibit chemopreventive, anti-inflammatory and antioxidant properties [11-14]. Growth inhibition or apoptosis has been observed in diverse cell types such as lymphocytes, melanocytes, epidermoid cells, and colon cells treated in vitro with stilbenes such as PIC, RES, or TPS [15-17]. However, with the exception of RES, the anti-proliferative potential of these and other stilbenes on cells of the myeloid lineage, such as macrophages, is less studied [18].

In addition to their antioxidant properties, it is worthy to note that cytotoxic effects of RES, PIC, and TPS have also been reported. In this regard, RES has demonstrated pro-oxidant, genitourinary, immunotoxic, hepatotoxic and genotoxic properties [18-23]. PIC, a metabolite of RES, has been reported to induce apoptosis of transformed and primary lymphocytes [17] and to also exhibit cytotoxic actions towards macrophages in vitro [24]. TPS has been found to possess strong apoptosis-inducing properties in leukemia cell lines but not in normal hematopoietic stem cells [14]. Despite these interesting studies, the cytotoxicity of stilbenes such as RES, TPS, and PIC on cells of the immune system, particularly the macrophages, remains to be fully defined.

Compared to RES, PIC, and TPS, much less is known concerning the health effects of other stilbenes such as *trans*-stilbene (TS) and *trans*-stilbene oxide (TSO), particularly with regard to cells of the immune system. TS is a commercially available raw material used in the production of stilbene dyes. It is also utilized as a fluorescent brightening agent [25] and may exhibit estrogenic activity in humans [25, 26]. Unlike the related compound RES [10], Grohs and Kunz found TS to exhibit poor fungitoxic properties [27]. TSO, an oxide analog of TS, is also commercially-available and is a potent inducer of epoxide hydrolase, DT-diaphorase and phenobarbital-inducible CYP-450 enzymes [28-30]. Like other stilbenes, the cytotoxic and anti-proliferative effects of TS and TSO on macrophages are not well established.

The studies described in the present work utilized RAW 264.7 mouse macrophages to evaluate the cytotoxicity and anti-proliferative effect of the stilbene compounds described above (Fig. 1). These mouse-derived macrophages

represent a valuable tool to study the effects of toxicants in cells of the myeloid lineage. For comparison purposes, and because it was found to be the most toxic to macrophages at 24 hr, the anti-proliferative activity of PIC was also assessed in 10ScNCr/23 mouse macrophages as well as in non-myeloid cells such as skin cells and T cells.

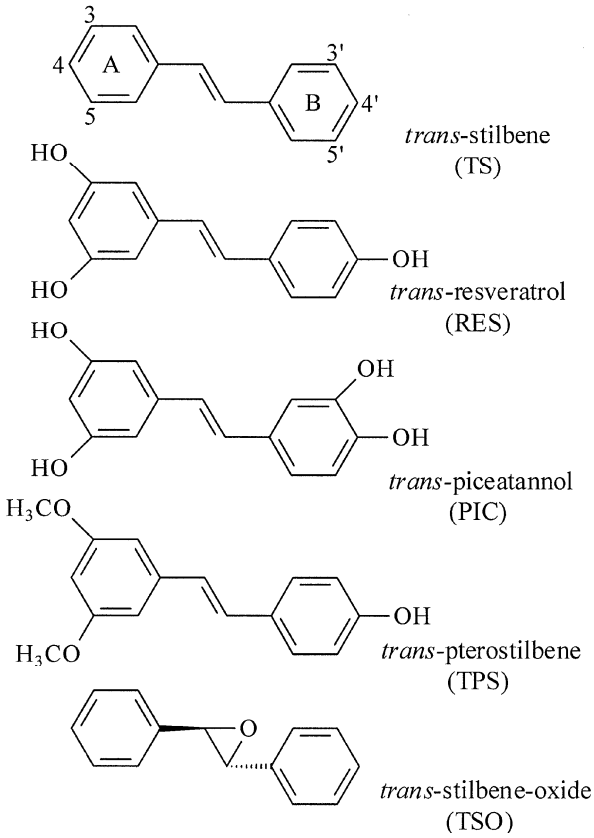

Fig. 1. Structures of the stilbene derivatives. Numbering scheme is as per Ovesna *et. al.*, 2006 [38].

MATERIALS AND METHODS

Chemicals and reagents

Unless otherwise indicated, all chemicals were obtained from local suppliers and used as received. Piceatannol (PIC, 3,3',4',5-tetrahydroxystilbene), pterostilbene (TPS, 3,5-dimethoxy-4′-hydroxy-*trans*-stilbene), resveratrol (RES, 3,4',5-trihydroxy-*trans*-stilbene), *trans*-stilbene (TS), and *trans*-stilbene oxide (TSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO). The declared purities of PIC and RES was > 99%, of TS was 96%, of TSO was 98% and of TPS was > 97%. Stilbene stock solutions (100 mM) were prepared in 100% dimethylsulfoxide (DMSO). The concentration of DMSO vehicle in all studies did not exceed 0.1%.

Previous studies in our lab have shown that 0.1% DMSO does not affect macrophage viability (unpublished observation).
DMSO was purchased from EMD Chemicals Inc. (Gibbstown, NJ). The Dulbecco's Modified Eagle's Medium (DMEM), containing phenol-red, glutamine and 4.5 mg glucose/L, was purchased from Cellgro (Herndon, VA). DMEM (500 ml) was supplemented with 50 ml of heat-inactivated fetal bovine serum, 550 µl of gentamicin (Invitrogen, Carlsbad, California), and collectively designated as complete DMEM. Complete DMEM was mixed thoroughly and stored at 4°C. DMEM/F12 medium was obtained from Invitrogen (Carlsbad, CA) and contained L-glutamine and glucose, but no serum, gentamicin, or phenol-red. This medium was used for the treatment of all cell types.

Cell lines
RAW 264.7 mouse macrophages [31], A-431 human epidermoid carcinoma cells [32] and 10ScNCr/23 mouse macrophages [33], a macrophage line deficient in the toll-like receptor 4 (TLR4), were obtained from the American Type Culture Collection (Manassas, VA). CCRF-CEM tumor-derived human T cells [34] were kindly provided by Dr. Jason Chen (St. John's University, Jamaica, NY). Macrophage colony-stimulating factor-1 (MCSF-1) was purchased from Sigma-Aldrich Co. (St. Louis, MO). MCSF-1 was required for efficient growth of the 10ScNCr/23 macrophages. Therefore, to grow and use these cells for experiments, complete DMEM and DMEM/F12 was supplemented with MCSF-1 to give a final concentration of 1 ng/ml.

MTT viability assay
Macrophages were grown in 24 well plates to 90% confluency (~ 250,000 cells/well) in complete DMEM. All medium was removed from the wells and replaced with 0.5 ml of DMEM/F12 containing increasing concentrations of RES (0-50 µM), TPS (0-50 µM), TS (0-5 µM), or TSO (0-100 µM). Cells were then cultured for 24 hr, after which the MTT viability assay was performed as described by Mosmann [35]. The incubation time with MTT reagent was modified as carried out by Shah and colleagues [36]. In brief, 50 µl of MTT reagent was added to each well and the RAW 264.7 cells were cultured at 37°C for 30 min. After culturing, 550 µl of MTT solubilizing solution was added to each well, and mixed thoroughly. Plates were incubated at 37°C for another 10 min, after which the reduction of MTT was assessed. Cells were considered to be viable if they were able to convert MTT to a crystal of formazan, whose concentration is measured spectrophotometrically at 570 nm after dissolving it in a suitable solvent. Hence, the intensity of the color in the solution is directly proportional to cell viability [35]. Absorbance values were converted to number of viable cells by reference to a MTT standard curve for the cell line under investigation.

Trypan blue dye exclusion method
RAW 264.7 mouse macrophages were grown to 90% confluency (~ 1,000,000 cells/well) in 6 well plates in complete DMEM. Prior to treatment, all medium

was removed and replaced with DMEM/F12 containing increasing concentrations of PIC (0-50 μM), RES (0-50 μM), TPS (0-50 μM), TS (0-5 μM), or TSO (0-100 μM). After culturing for 24 hr, the medium was removed, and the remaining cells were washed once with 1 ml of PBS. To each well, 500 μl of trypsin solution was added, and the mixture incubated at 37ºC for approximately 15 min. Cells were transferred to microfuge tubes with the aid of scraping, and then centrifuged at 5000 rpm for 10 min. The supernatant was removed and cells were resuspended in 1 ml of DMEM/F12, which was mixed thoroughly and appropriate dilutions were performed. Then, equal volumes of resuspended cells and trypan blue solution (0.4% wt/vol) were mixed in a ratio of 1:2. Only cells that excluded trypan blue dye were included in the analysis. A hemocytometer was used to count cells.

Cell proliferation studies

RAW 264.7 and 10ScNCr/23 macrophages, T cells, or epidermoid carcinoma cells were inoculated into 6 well plates (1×10^4 cells/well) and cultured in complete DMEM containing RES (0-50 μM), PIC (0-50 μM) or TPS (0-30 μM). Cells were harvested at specified intervals and the number of cells per well was determined by cell counting with a hemocytometer. Only cells that excluded trypan blue dye were included in the analysis.

Statistical analysis

Unless otherwise indicated, all experiments were carried out in triplicate, and their results are reported as the mean ± standard error of the mean from at least three representative experiments. Statistical comparisons were made using either Student's t-test (for comparisons between two groups), or one-way ANOVA with Tukey's post hoc test (for comparisons across multiple groups) using GraphPad Prism 4.0® software (GraphPad Software, Inc., San Diego, CA). Differences were considered to be significant at $p < 0.05$.

RESULTS

To first determine if the MTT viability assay could be used to assess the cytotoxic potential of stilbenes, the MTT-reducing potential of each of the respective stilbene analogs depicted in Fig. 1 was assessed in the absence of cells. To this end, 0.5 ml samples of serum-and phenol red-free DMEM/F12 containing either RES, PIC, TPS or TSO (each at a final concentration of 10, 50, or 100 μM) were co-incubated with MTT reagent at 37ºC for 90 min, after which an equal volume of solubilizing buffer was added. Then, the absorbance of the sample at 570 nm was measured on a spectrophotometer. Whereas, RES, TSO and TPS were found to exhibit insignificant reducing activity, PIC induced a concentration-dependent increase in MTT reducing activity (Fig. 2). TS, at the highest concentration tested (5 μM) did not interfere with the MTT assay (data not shown).

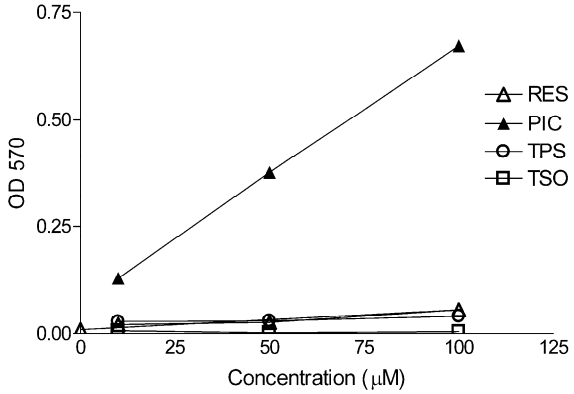

Fig. 2. The MTT-reducing potential of the stilbene analogs after a 90 min exposure in a cell-free environment. Each data point is the mean of two samples.

The effect of TS, TSO, RES and TPS on RAW 264.7 macrophage viability was determined by two standard methods: (a) the MTT assay and (b) the trypan blue dye exclusion test (Tab. 1). Due to the ability of PIC to reduce MTT, all cell viability studies with this compound were based on the trypan blue dye exclusion method rather than on the MTT method. Cells were cultured in serum-and-phenol-free DMEM/F12 containing increasing concentrations of the stilbene analog for 24 hr. It is noteworthy that both methods yielded similar results with respect to the patterns of cytotoxicity exhibited by the different stilbene analogs (Tab. 1). Whereas macrophages were more sensitive to PIC ($LC_{50\ trypan}$ ~ 1.3 μM)

Tab. 1. The effect of stilbene compounds on the viability of RAW 264.7 cells and estimated LogP values[a,b,c].

Stilbene	LC_{50} (MTT)	LC_{50} (trypan blue dye exclusion)	Estimated LogP
TS	> 5.0 (n=2)	> 5.0 (n=2)	4.52[e]
TSO	> 100 (n=2)	60.67 ± 5.93 (n=3)	3.22[e]
RES	29.20 ± 0.65 (n=3)	8.93 ± 0.61 (n=3)	3.08[e] , 4.01[f]
TPS	8.33 ± 0.88 (n=3)	4.03 ± 0.12 (n=3)	4.21[e] , 4.23[f]
PIC	ND[d]	1.30 ± 0.12 (n=3)	2.60[e] , 3.42[f]

[a]LC_{50} values are reported as the mean ± standard error of the mean. [b]LogP represents the octanol:water partition coefficient. [c]All cell treatments were for a 24 hr duration. [d]ND, not determined. [e]Determined using the Interactive LogKow Online Program for LogP determination available at: http://www.syrres.com/esc/est_kowdemo.htm. [f]Reported by Murias *et. al*, 2004 [8].

and to TPS ($LC_{50\ trypan}$ ~ 4.0 μM and $LC_{50\ MTT}$ ~ 8.3 μM) than to RES ($LC_{50\ trypan}$ ~8.9 μM and $LC_{50\ MTT}$ ~ 29.0 μM), they were relatively resistant to TSO ($LC_{50\ trypan}$ ~ 61.0 μM and $LC_{50\ MTT}$ > 100 μM). The macrophages were also found to be resistant to TS at concentrations ≤ 5.0 μM. An assessment of the cytotoxicity of TS at higher concentrations was not possible because of the

propensity of the TS to form crystals in the aqueous culture medium at concentrations above 5.0 μM (data not shown).

The anti-proliferative activity of RES and its analogs was next evaluated. The growth of 10ScNCr/23 macrophages was inhibited by RES in a concentration-dependent manner, with maximum inhibition occurring in the range of 25 to 50 μM (Fig. 3A).

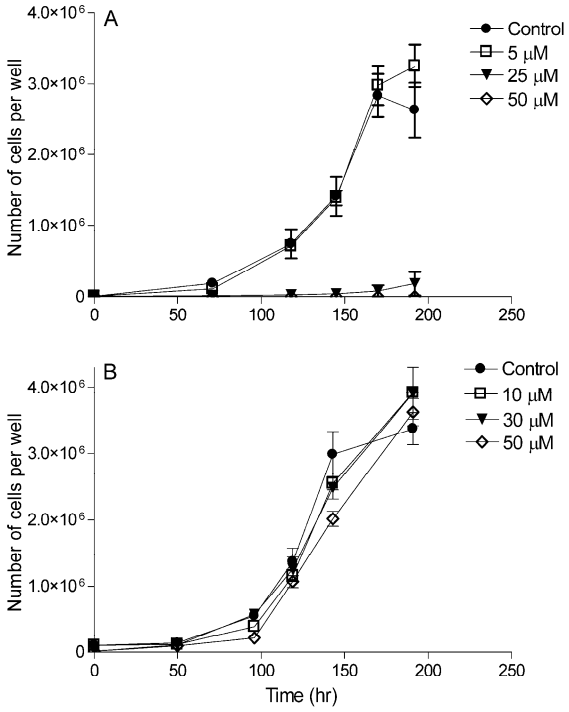

Fig. 3. The effect of RES (A) and PIC (B) on the proliferation of 10ScNCr/23 macrophages. Each data point represents the mean and each vertical line is the standard error for the results of three representative experiments. In Panel A, cells cultured for ≥ 100 hr with ≥ 25 μM of RES were significantly different from untreated control cells ($p < 0.001$).

The anti-proliferative capacity of PIC was assessed in RAW 264.7 and 10ScNCr/23 macrophages, as well as in non-myeloid cells such as skin cells (A-431) and T cells (CCRF-CEM). A diverse range of growth inhibition was observed amongst these different types of cells. Whereas PIC did not affect the proliferation of 10ScNCr/23 cells at concentrations ≤ 50 μM (Fig. 3B), it was found to exert an inhibitory effect toward the growth of RAW 264.7 macrophages and A431 skin cells at the 50 μM concentration (Fig. 4A and Fig. 4C). In contrast, PIC was found to inhibit growth of T cells at concentrations ≥ 10 μM (Fig. 4B). Moreover, PIC was not able to fully suppress proliferation of any cell type tested.

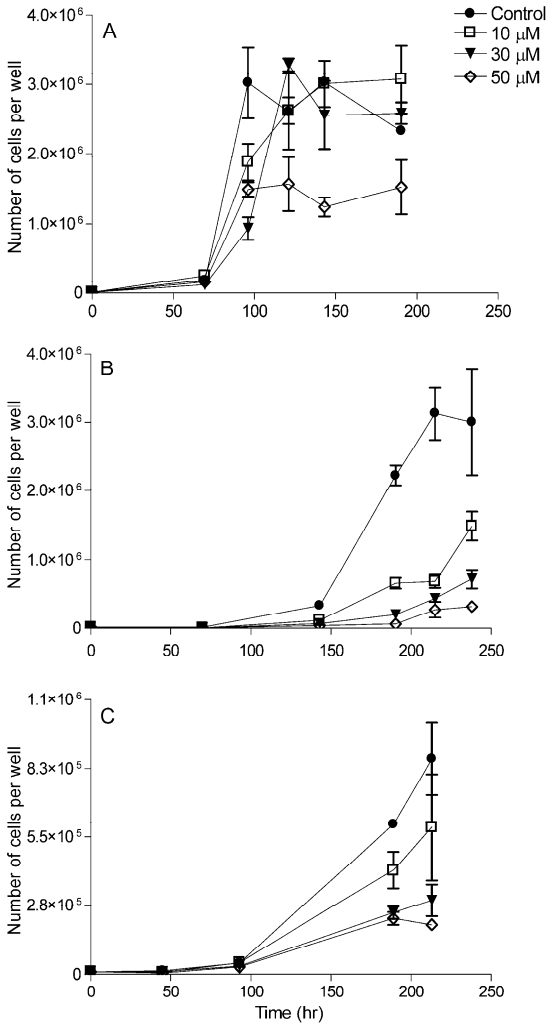

Fig. 4. The effect of PIC on the proliferation of (A), transformed mouse macrophages (RAW 264.7); (B), transformed human T cells (CEM); and (C) tumor-derived skin cells (A-431). Each point represents the mean and each vertical line is the standard error for the results from three representative experiments. In Panel A, cells cultured for $\geq$ 144 hr with 50 μM of PIC were significantly different from untreated control cells ($p < 0.05$). In Panel B, cells cultured for $\geq$ 168 hr with $\geq$ 10 μM of PIC were significantly different from untreated control cells ($p < 0.01$). In Panel C, cells cultured for > 200 hr with 50 μM of PIC were significantly different from untreated control cells ($p < 0.05$).

The other stilbenes that were evaluated for growth inhibition varied in this capacity. Whereas TS and TSO failed to inhibit the proliferation of macrophages at concentrations as high as 5 μM (data not shown), TPS suppressed the growth of RAW 264.7 cells in a concentration-dependent manner, with maximum inhibition occurring in the range of 20 to 30 μM (Fig. 5).

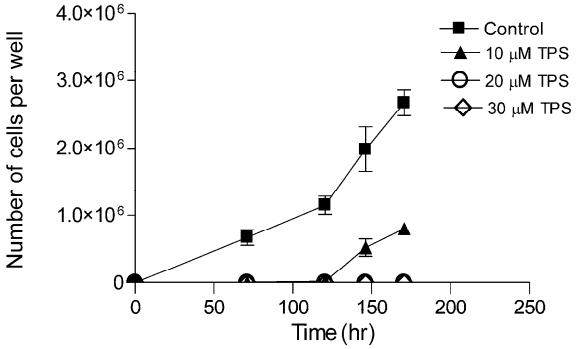

Fig. 5. The effect of TPS on the proliferation of transformed mouse macrophages (RAW 264.7). Each data point represents the mean and each vertical line is the standard error for the results from three representative experiments. All cells cultured for ≥ 72 hr with ≥ 10 μM of TPS were significantly different from untreated control cells ($p < 0.001$).

DISCUSSION

In order to determine the cytotoxic properties and structure-function relationship of stilbene analogs, viability studies were performed in RAW 264.7 macrophages. Cytotoxicity for TS, TSO, RES and TPS was evaluated by the MTT viability assay (Tab. 1). With regard to PIC, the trypan blue dye exclusion assay was used to assess cell viability due to the MTT-reducing activity of the compound in a cell-free environment (Fig. 2), which prevented accurate readings using the MTT assay. In particular, PIC has been reported to be highly unstable in culture medium due to its *ortho*-dihydroxy substituents on phenyl ring B of the stilbene backbone (catechol moiety; Fig. 1) which can have a direct reductive potential in a cell-free system [16, 37]. These previous studies are in line with the results seen here (Fig. 2) and, taken together, indicate that use of the MTT viability assay with PIC may lead to skewed results. Indeed, in a prior study we used the MTT assay to assess the LC_{50} value of PIC-treated RAW 264.7 macrophages and found it to be ~ 40 μM [18], which is considerably higher than that seen here using the trypan blue dye exclusion test. It is therefore advisable to clarify results concerning PIC that have been obtained using the MTT assay and to use an alternate measure of cell viability such as trypan blue dye exclusion. Moreover, in the present work, the respective LC_{50} values for RES and TPS that were obtained using the trypan blue dye exclusion method were lower than those obtained using the MTT assay, suggesting that the trypan blue dye exclusion test was the more sensitive method of the two for determining stilbene cytotoxicity.

The effect of stilbene treatments on macrophage cell viability was observed to vary according to the analog used. The greatest toxicity at 24 hr was exhibited with PIC, followed by TPS and RES. Macrophages were found to be resistant to stilbene analogs TS and TSO (Tab. 1). To our knowledge, the present study is

among the first to examine the structure-activity relationship of stilbene analogs for toxicity in RAW 264.7 macrophages.

PIC is a metabolite of RES (Fig. 1), and differs from its parent compound due to the extra hydroxyl group on the 3'- position of phenyl ring B [38-40]. In the present analysis, RAW 264.7 macrophages demonstrated greatest sensitivity to PIC. Djoko and colleagues also observed that PIC exhibited the greatest cytotoxicity amongst RES and other stilbene analogs in RAW 264.7 macrophages [24]. However, in light of our observation concerning the reducing power of PIC, their data may need to be interpreted with caution, since cell viability in that report was assessed using the MTT assay. The toxicity of PIC seems to arise from the *ortho*-dihydroxyl groups, also known as the catechol moiety, on phenyl ring B. To this end, Murias and colleagues found that the catechol group formed by hydroxyl groups at the 3'- and 4'- positions of phenyl ring B increases the cytotoxic potential of stilbenes [11]. Similarly, another group observed stilbene compounds bearing *ortho*-dihydroxyl groups induce greatest DNA damage in the presence of Cu (II) [41]. The greater DNA-damaging actions of compounds bearing *ortho*-dihydroxyl groups is likely due to a stable oxidation intermediate, known as the *ortho*-hydroxyl phenoxyl radical [41].

The second most potent stilbene agent found to exhibit cytotoxicity in macrophages at 24 hr was TPS. TPS is another naturally occurring stilbene derivative and differs from RES due to the presence of methoxy groups, rather than hydroxyl groups, on the 3- and 5-positions of phenyl ring A (Fig. 1). Huang and colleagues found that compared to RES, methylation of the hydroxyl groups on the 3-, 5- and 4'-positions remarkably increased the cytotoxic actions in human nasopharyngeal epidermoid tumor cells [42]. Roberti and colleagues observed that the introduction of 3,5-dimethoxy motifs on phenyl ring A of *cis* stilbenes may be responsible for the pro-apoptotic activity of such compounds [43]. The 3,5-dimethoxy substituents on ring A of TPS confer lipophilicity to this analog compared to RES as evident from the slightly higher estimated octanol-water partition coefficient (LogP) for TPS (LogP = 4.23) than for RES (LogP = 4.01) [8]. Therefore, the more lipophilic TPS should be able to permeate the cell membrane more readily than RES, thereby increasing its potential cytotoxic actions [14]. Taken together, our results are in line with those reported by groups who have demonstrated that the dimethoxy motif significantly increases the cytotoxic action in stilbenes [14, 42].

RES was found to exhibit intermediate toxicity when compared to its other stilbene analogs in macrophages. It is one of the most extensively studied stilbenes, with hydroxyl groups on the 3- and 5- positions of phenyl ring A, and a hydroxyl group on the 4'-position of phenyl ring B (Fig. 1). The toxicity of this compound may be due to its hydroxyl group on the 4'- position. Matsuoka and colleagues found that the 4'-hydroxyl group enacts an essential role in the genotoxicity of stilbenes [44]. Similarly, the 4'-hydroxyl group was observed to confer effective DNA-damaging properties amongst many hydroxylated stilbenes [45]. Larrossa and colleagues also found RES to be a potent inducer of

apoptosis in melanoma cells compared to other analogs, due to this 4'-hydroxyl group [16]. Therefore, the toxicity of stilbenes is likely dependent upon the presence of hydroxyl groups, specifically the 4'-hydroxyl group in RES, which greatly contributes to its cytotoxic actions in vitro. It should be noted that the most toxic stilbenes in the present study (PIC, TPS and RES) all contain a 4'-hydroxyl group.

The toxic effects of stilbenes such as TS and TSO on macrophages are not well understood. Therefore, in the present work, the cytotoxic potential of these compounds was evaluated in macrophages. Whereas TS is an unsubstituted stilbene, TSO is composed of two phenyl rings, joined together by an epoxide bridge (Fig. 1). Compared to RES, macrophages were found to be resistant to both of these stilbene compounds. This is not surprising, since the presence of hydroxyl groups on the 3- and 5- positions on phenyl ring A and the 4'-position on phenyl ring B seem to have an important role in the cytotoxic effects of stilbenes [41, 44, 45]. To our knowledge, no groups have evaluated the structure-activity relationship of TSO in macrophages. It should be noted that TSO is an inducer of epoxide hydrolase, DT-diaphorase and certain P-450 enzymes [28-30]. Although these enzymes are generally capable of detoxifying xenobiotics, the induction of DT-diaphorase, in particular, can result in pro-oxidant effects [46, 47]. However, we found the cytotoxicity of TSO in the RAW 264.7 macrophages to be notably less than that of the hydroxylated stilbenes.

Although the present study did not determine the type of death incurred by macrophages treated by PIC, RES, or TPS, previous reports have shown that these stilbenes, in particular, can kill other types of cells by apoptosis [14, 17, 20]. Currently, our lab is exploring the exact mechanism through which the macrophages are dying and this subject will be communicated in a future manuscript relating to this topic. Nevertheless, it is clear that RES, PIC, and TPS do exhibit cytotoxic activity towards these cells which may, in part, contribute to their respective anti-inflammatory effects.

In the present work, the effect of stilbene exposure on cell proliferation was also evaluated. To this end, RES was found to exhibit a concentration-dependent inhibition of the proliferation of TLR4-deficient 10ScNCr/23 mouse macrophages ($IC_{50} \leq 25$ μM) (Fig. 3A). A previous study from our lab also reported that similar concentrations of RES (25 and 50 μM) were inhibitory to the growth of RAW 264.7 mouse macrophages, a TLR4-proficient cell line [18]. To our knowledge, the present study represents the first report of the inhibitory effects of RES on the proliferation of macrophages deficient in the TLR4 receptor and demonstrates that the anti-proliferative activity of RES in macrophages is independent of TLR4 receptor status. Furthermore, the inhibition of growth resulting from RES treatment in these cells is in line with a number of studies that find RES to exert an anti-proliferative effect [48-51]. However, it should be noted that there are at least two reports in the literature showing a stimulatory effect of RES on the proliferation of osteoblasts in vitro at concentrations of

RES comparable to the ones used here [52, 53]. Therefore, it is possible that the anti-proliferative potential of RES depends on the type of cell that is being studied.

The effect of PIC on cell proliferation was also evaluated in both 10ScNCr/23 macrophages (Fig. 3B) and RAW 264.7 macrophages (Fig. 4A), as well as in non-myeloid cell types such as T-cells (Fig. 4B) and skin cells (Fig. 4C). Although PIC was found to exhibit varying degrees of anti-proliferative activity in almost all the cell types studied here, the 10ScNCr/23 macrophages were surprisingly resistant to this stilbene. PIC was observed to exert less pronounced anti-proliferative activity in macrophages (IC_{50} ~ 50 μM) than either RES (Fig. 3A; $IC_{50} \leq 25$ μM) or TPS (Fig. 5; $IC_{50} \leq 10$ μM). In addition, unlike RES and TPS, PIC was unable to fully suppress proliferation in any cell type (Fig. 4).

A discrepancy appears to exist between the cell viability data presented in Tab. 1 (with PIC being the most toxic of the stilbenes) and the data obtained in proliferation studies (with RES being a stronger inhibitor of cell growth than PIC). The reason for this is not presently known but may be due to the presence of metabolic enzymes such as catechol-O-methyltransferase (COMT) in macrophages [54]. COMT specifically catalyzes the methylation of meta-OH functional groups. Whereas PIC contains a meta-OH functional group at the 3' position, which could serve as a substrate for methylation by COMT, both RES and TPS lack such meta-OH functional groups and are not potential substrates for this enzyme. In this manner, cells that survive the initial treatment with PIC would be able to metabolize it to a less active meta-OCH3 form and therein survive and proliferate. Alternately, the instability of PIC in cell culture medium [16] could potentially result in its spontaneous degradation, allowing the surviving cells to proliferate. Moreover, the lack of anti-proliferative effect of PIC on 10ScNCr/23 macrophages may be linked to deficiency in the TLR4. These possibilities are currently under investigation and will be commented upon in a future publication.

While the anti-proliferative effects of PIC or TPS have been observed in colon carcinoma, melanoma and lymphoma cell lines [15-17], the present study is among the first to evaluate the anti-proliferative potential of RES, PIC and TPS in cultured macrophages. Our findings suggest that the presence of a 4'-hydroxyl group either alone or in combination with 3,5-dimethoxy groups on an intact stilbene backbone increases the likelihood of anti-proliferative activity in these cells.

In summary, the anti-proliferative of RES and its analogs were assessed in two macrophage cell lines. The anti-proliferative activity of PIC in T cells and skin cells was also evaluated. Whereas RES was observed to inhibit cell proliferation in 10ScNCr/23 macrophages (TLR4-deficient), these cells were completely resistant to PIC. However, PIC was able to partially suppress proliferation of RAW 264.7 cells and non-myeloid cells, such as T cells and skin cells, at a high concentration (50 μM).

The cytotoxic effects of PIC, TS, TSO and TPS were also evaluated in RAW 264.7 macrophages. The presence of hydroxyl groups on the stilbenes was found to correlate with cytotoxicity. To this end, PIC exhibited the greatest toxicity in macrophages at 24 hr, possessing four hydroxyl groups within its stilbene structure. The stilbene with the second highest cytotoxic potency at 24 hr was TPS. The potency of TPS, with respect to toxicity, likely arises from its methoxy groups, which in turn, increases its lipophilic properties compared to the less toxic and less lipophilic RES. RES also demonstrated intermediate cytotoxicity in macrophages, most probably arising from its 4'- hydroxyl group found on phenyl ring B. Moreover, the nonhydroxylated stilbene analogs TS and TSO demonstrated no considerable toxicity toward macrophages. Taken together, it appears likely that the 4'-hydroxyl group confers cytotoxicity to stilbene analogs which can be increased by the presence of *ortho*-hydroxyl groups on the B ring or methoxy groups on the A ring of the stilbene backbone.

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