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(Z)-1,1-Dichloro-2-(4-methoxyphenyl)-3-phenylcyclopropane induces concentration-dependent growth inhibition, apoptosis, and coordinates regulation of apoptotic genes in TRAMP cells

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Abstract

(Z)-1,1-Dichloro-2,3-diphenylcyclopropane (A_{II}) and (Z)-1,1-dichloro-2-(4-methoxyphenyl)-3-phenylcyclopropane [2-(4-methoxyphenyl)-A_{II}] inhibit tubulin polymerization, PSA production, and the proliferation of human prostate cancer cells. The actions of the agents were studied in three transgenic adenocarcinomas of the mouse prostate (TRAMP) cell lines. Antiproliferative potencies were determined and cells treated with the more potent 2-(4-methoxyphenyl)-A_{II} were examined for induction of apoptosis. Microarray analyses were conducted to determine the apoptosis-related genes up- and down-regulated by the agent. 2-(4-Methoxyphenyl)-A_{II} concentration-dependently inhibited growth of all three cell lines. Fifty percent and 100% growth inhibitory and 50% lethal concentrations were determined to be 0.3, 1.5, and 5 μ M, respectively. Minimum detectable apoptosis-inducing concentrations by ELISA were 0.10 to 0.14 μ M. PARP cleavage and two-color flow cytometry assays verified apoptosis induction. Microarray analyses showed *Bok* and *Siva-pending* to be up-regulated and that *Birc*, *Dad1*, and *Atf5* were down-regulated. 2-(4-methoxyphenyl)-A_{II} inhibits proliferation and induces apoptosis in the in vivo-adaptable TRAMP cells, suggesting the compound should be further examined in preclinical models.

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Keywords

Prostate cancer; Microtubule inhibitor; Microarray; TRAMP cells; Apoptosis

1. Introduction

Prostate cancer remains the second leading cause of cancer deaths in American men. Reports of prostate cancer have risen rapidly but mortality rates have declined, possibly due to increased public knowledge, as well as early screening and detection [1]. Early detection of prostate cancer has improved; for example, the implementation of widespread PSA-based screening programs have helped to enhance the ability to diagnose prostate cancer at an early stage [2]. While organ-confined prostate cancer is readily curable, no satisfactory treatment exists for advanced disease. Hormonal therapy is currently the only effective treatment available to patients with advanced prostate cancer. Androgen ablation is not curative, and the majority of patients will eventually develop hormone-resistant cancer. Standard chemotherapeutic agents have primarily not been effective against advanced prostate cancer. Exploration of novel therapeutic agents will provide additional means of combating the disease.

The implementation and utilization of newly developing cancer drugs and models is key to arresting or curing this too commonly diagnosed disease. The use of an animal model that mimics some aspects of prostate cancer development in humans is an ideal way of studying initiation, progression, and metastasis. The transgenic adenocarcinoma of the mouse prostate (TRAMP) model was developed to study a wide variety of issues in prostate cancer [3,4]. The TRAMP model was genetically engineered so that mice develop prostate cancer with age. Many other mouse models use human prostate cancer cell grafts injected subcutaneously or directly onto the prostate to produce the disease model. However, in such xenograft models, the mice usually have a defective immune system that prevents rejection of the graft. This results in an unnatural situation in which the normal interplay between cancer, its native environment, and the immune system cannot be examined [5]. The progression of the disease in the TRAMP model mimics to some extent what is seen in humans, and since the cancer is developed within the TRAMP mouse, preventative studies can also be conducted.

The TRAMP mouse model was developed by employing a minimal probasin promoter to target expression of SV40 large T-antigen to the epithelium of the mouse prostate. In the TRAMP model, prostatic disease progresses from mild to severe intraepithelial neoplasia, to focal adenocarcinoma that metastasizes to the lymph nodes and lungs, and occasionally to the bone, kidney, and adrenal glands with neuroendocrine differentiation [3–10]. Three cell lines (TRAMP-C1, -C2, and -C3) were derived from the prostatic adenocarcinoma of a 32-week old C57BL/6 TRAMP mouse. C1 and C2 are tumorigenic and C3 is nontumorigenic. From C1 and C2, 6 clonal cell lines were created by three rounds of limiting dilutions. The C1 line produced the C1A and C1D lines. The C2 line produced C2D, C2G, and C2N lines. C1A, C2G, C2H, and C2N cells are tumorigenic when grafted into syngenic C57BL/6 male

hosts. The clonal cell lines express cytokeratin, androgen receptor (AR), fibroblast growth factor receptor 1 (FGFR1), and FGFR2 [6–10].

We have previously shown the easily accessible synthetic agents (*Z*)-1,1-dichloro-2-(4-methoxyphenyl)-3-phenylcyclopropane [2-(4-methoxyphenyl)-A_{II}] and (*Z*)-1-1-dichloro-2,3-diphenylcyclopropane (A_{II}) (Fig. 1) to be antiproliferative. These agents inhibit tubulin polymerization by binding at the colchicine site of the protein [11]. Proper tubulin/microtubule dynamics are critical for the segregation of sister chromatids in mitosis, and is a proven antitumor target. A_{II} is a cyclopropanated *cis*-stilbene derivative [12] first found to be antiestrogenic in the mouse [13]. Several in vitro and in vivo studies have shown A_{II} to have activity against both estrogen receptor-positive and -negative breast cancer cells [14–19]. Notably, the lethal dose of A_{II} is >3,000 mg/kg in rodents. In breast cancer cell lines, A_{II} disrupts microtubules and causes apoptosis [11,19]. Most recently, we have shown A_{II} at low concentrations to block PSA production and cell proliferation in the LNCaP human androgen-responsive prostate cancer cell line [20].

In that most recent study, we found 2-(4-methoxyphenyl)-A_{II} to be more potent than A_{II} in tubulin perturbation and inhibition of human prostate cancer cell proliferation. The goal of the present study was, in preparation for in vivo studies, to utilize the TRAMP cell lines to determine the antiproliferative effects of A_{II} and 2-(4-methoxyphenyl)-A_{II}, to choose the more potent of the two, and then study that agent's induction of apoptosis.

2. Materials and methods

2.1. Chemicals

The test agents were synthesized by previously reported methods [12,18,21] and were of >99% purity as determined by ¹H and ¹³C NMR, capillary GC-MS, HPLC-UV and HPLC-MS.

2.2. Cell culture

TRAMP cell lines were obtained from Dr. Barbara Foster (Roswell Park Cancer Institute). C1A cells were isolated and characterized from the TRAMP-C1 cell line and C2H and C2N lines were isolated from TRAMP-C2 cells previously [6]. Cells were grown in Phenol red-free, high glucose Dulbecco's modified Eagle medium (DMEM) with L-glutamine and without sodium pyruvate obtained from Gibco BRL (Grand Island, NY). The medium was supplemented with 10% fetal bovine serum obtained from Hyclone, 5 µg/ml insulin obtained from Sigma (St. Louis, MO), 25 U/ml penicillin-streptomycin obtained from Gibco BRL, and 1 mM dihydrotestosterone from Sigma. Medium was refreshed every 3 days. Cultures were split when they reached confluence by rinsing in Ca⁺⁺/Mg⁺⁺-free Hanks balanced salts solution obtained from Gibco BRL. The cells were then detached with 0.25% trypsin.

2.3. Growth inhibition studies

Cells were seeded at 1,200 cells/well in 96-well plates in 100 µl/well of complete culture media. After 24 hours, the cells were treated with test agents predissolved in DMSO (final concentration 0.2%). The concentrations of test agents used for the first screening were at 5-

fold dilutions to generate seven concentrations (640 pM to 10 μ M). The test agent concentrations used for the second screening were 2-fold dilutions centered around the 50% growth inhibitory concentration (GI_{50}) estimated from the first screen. The control for each cell line contained DMEM media and 0.2% DMSO. Time points of continuous agent exposure considered were 0, 24, 48, and 72 hours. At the end of each incubation, MTT was added to each well and incubated at 37°C for an additional 3 hours. After incubation, 100 μ l of 20% SDS was added to each well and the plate was placed back in the incubator overnight. The following morning, formazan formation was determined at 595 nm on a microplate reader (Bio-Rad, Hercules, CA). The number of determinations per treatment was performed in triplicate. The GI_{50} , total growth inhibitory (TGI) concentration (i.e., GI_{100}) and, where applicable, the concentration at which the agent caused a decrease in cell number to 50% that of the time zero control culture (LC_{50}) were calculated.

2.4. Apoptosis ELISA

Cells were plated in 6-well plates at 20,000 cells/well and allowed to attach for 24 hours. Wells were then treated with 2-(4-methoxyphenyl)- A_{II} at the indicated concentrations, or with vehicle (0.2% DMSO) only. Twenty-four hours after the test agent was added, an ELISA to measure small DNA fragments and histones (Roche Diagnostics, Indianapolis, IN) was performed. The ELISA assay makes use of antihistone and anti-DNA-POD antibodies; the antihistone binds to the histone-component of the nucleosomes and simultaneously captures the immunocomplex to the coated microtiter plate, whereafter the anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes. The unbound antibodies were removed by washing. Quantitative determination of the amount of nucleosomes by the POD retained in the immunocomplex and the amount of nucleosomes was determined spectrophotometrically at 405 nm using a Bio-Rad Benchmark microplate reader and analysis software. Data was analyzed and plotted using Graph Pad Prism v. 4.0 (Graph Pad Software, San Diego, CA).

2.5. Protein extraction and Western blot analysis

C1A cells were plated and cultured in complete medium and allowed to attach for 24 hours, followed by the addition of the TGI concentration of 2-(4-methoxyphenyl)- A_{II} (1.5 μ M) and incubation for 0, 24, 48, and 72 hours. Control cells were incubated in the medium and were treated with 0.2% DMSO for the same time periods. After incubation, the cells were harvested by scraping from the culture dishes and collected by centrifugation. Cell number was then determined with a Coulter counter. Cells were resuspended in 100 μ l of a protease inhibitor cocktail mixed with 1 ml of lysis buffer and 20 μ l of 50 $\times$ protease inhibitors (Pharmingen, San Diego, CA), then lysed by one freeze (dry ice in ethanol)-thaw cycle. The protein concentration in the lysate was determined with the Bio-Rad Protein Assay kit. For each sample, 50 μ g of total protein was loaded into and resolved in a 10% SDS polyacrylamide gel. The electrophoretically-resolved lysate was transferred to a nitrocellulose membrane. The membrane was incubated with primary monoclonal PARP rabbit antibody (1:1,000) purchased from Cell Signaling Technologies (Danvers, MA) for 1 hour and washed twice with PBS supplemented with 0.1% Tween. The membrane was incubated for 1 hour with a secondary anti-rabbit antibody from the ECL Western blotting kit (Amersham Biosciences, Piscataway, NJ), then washed three times with Tween/PBS. The

substrate from the ECL kit was then added and immunorecognized protein bands were detected via luminescence using Kodak X-ray film (Rochester, NY). Films were developed using a Kodak X-OMAT 2000 processor system.

2.6. Flow cytometry

Two-color annexin V (Pharmingen)/propidium iodide staining and flow cytometry were conducted to detect externalized phosphatidylserine-containing but membrane-intact, or apoptotic, cells. C1A cells were treated with 1.5 μ M 2-(4-methoxyphenyl)-A_{II} for 0, 12, 24, 48 and 72 h, or with 0.2% DMSO for the same time periods. Controls used to establish the flow cytometric parameters included: blank plus 100 μ l of annexin buffer, 5 μ l of annexin V and 100 μ l of annexin buffer, 5 μ l of propidium iodide (Sigma), and 100 μ L of annexin buffer. Samples were prepared with annexin V and propidium iodide and divided into two groups: stained versus unstained. The samples were then analyzed on an EPICS-XL benchtop cytometer (Beckman Coulter, Fullerton, CA) and results were analyzed using EXPO 32 software.

2.7. RNA isolation

The TRIZOL reagent (an aqueous solution of phenol and guanidine isothiocyanate) was used to isolate total RNA from cells [22,23]. Cells were lysed directly in 3.5 cm culture dishes with 1 ml of TRIZOL reagent and passing the cell lysate several times through a pipette. Total RNA was extracted using a Qiagen RNeasy kit (Qiagen Inc., Valencia, CA) per the manufacturer's recommendations. The amount of RNA was determined from the spectrophotometric absorbance at 260 nm.

2.8. Expression microarray analysis

Affymetrix (Santa Clara, CA) chip analysis was performed as described earlier [24] in the University of Pittsburgh Genomics and Proteomics Core Laboratory. Briefly, total RNA (approximately 8 μ g) extracted from TRAMP-C1A cells was used for the synthesis of first-strand cDNA with Invitrogen Superscript II system (Carlsbad, CA) and T7-(dT)₂₄ primer. The second-strand cDNA synthesis was carried out at 16°C by adding *Escherichia coli* DNA ligase, *E. coli* DNA polymerase I, and RNaseH in the reaction. The cDNA was purified through phenol/chloroform and ethanol precipitation. Double-stranded DNA equivalent to 5 to 7 μ g of starting RNA was then converted into cRNA using in vitro transcription (IVT) and biotinylated nucleotides as per the ENZO BioArray high efficiency RNA transcript labeling kit (Farmingdale, NY). The IVT product was cleaned using Affymetrix RNA clean up columns, and 15 μ g of cRNA was fragmented at 95°C for 35 min. A 1 μ l aliquot of the sample was examined on an Agilent Bioanalyzer (Foster City, CA) to verify that RNA of the desired size distribution was generated. The fragmented cRNA was hybridized with pre-equilibrated Affymetrix chips containing the mouse MOE430A gene and expression tag set at 45°C for 14 to 16 hours. The hybridizations were then washed and stained with streptavidin-phycoerythrin (SAPE) according to the manufacturer's (Affymetrix) recommendation. The hybridized gene chips were scanned using Agilent ChipScanner to detect hybridization signals. Basic absolute analysis was performed using the Microarray Analysis Suite (MAS) v. 5.0 with each chip scaled to a median signal intensity of 150. The signal from each probe set was calculated from the intensity levels measured for each PM

and MM probe pair in that set. Signal levels reflected the abundance of expression of a given gene in the sample. In addition, MAS 5.0 calculated a detection *P* value. This parameter provided a measure of the probability that the gene is present in the transcriptome of the sample and therefore a measure of the reliability of the calculated signal value.

3. Results

3.1. Growth inhibition studies

The growth inhibitory properties of A_{II} and 2-(4-methoxyphenyl)-A_{II} were determined in the TRAMP cell lines C1A, C2H, and C2N. Colchicine was used as a positive control. The structures of the agents are shown in Fig. 1. The highest concentration tested for any of the three test agents was 10 μ M. Unlike our previous results in human prostate carcinoma cells [20], A_{II} was inactive at concentrations of 10 μ M. Colchicine yielded 50% growth inhibitory (GI₅₀) values in the mid-nanomolar range but no detectable total growth inhibitory (TGI; the GI₁₀₀) nor 50% lethality (LC₅₀; reduction of cell number to half that originally plated) concentrations. 2-(4-Methoxyphenyl)-A_{II} had high nanomolar GI₅₀ values in all three of the three cell lines, but also yielded TGI and LC₅₀ concentrations in the low micromolar range, and was therefore carried forward for further examination. Results are given in Table 1.

3.2. Apoptosis ELISA assay

We have previously noted by flow cytometry that by 2-(4-methoxyphenyl)-A_{II} causes slight increases in the hypodiploid population, an indicator of apoptosis, in human prostate cancer cells [20]. We therefore postulated that the concentration-dependent TRAMP cell growth inhibition exerted by this agent was due to induction of the cell's apoptosis machinery. In order to test this hypothesis, an ELISA assay was conducted to quantify apoptosis and determine the apoptosis-inducing properties of 2-(4-methoxyphenyl)-A_{II} on each cell line. The results are shown in Fig. 2A–C. In each of the three cell lines, 2-(4-methoxyphenyl)-A_{II} caused a concentration-dependent increase in apoptotic cells. In the C1A cell line, 2-(4-methoxyphenyl)-A_{II} caused an increase in apoptosis up to the GI₇₀ concentration. Beyond the GI₇₀, cytotoxicity increased to the extent that the apoptosis was limited likely due to loss of apoptotic cells, but also possibly due to competing cell death mechanisms like necrosis.

3.3. PARP cleavage assay

Poly(ADP-ribose) polymerase-1 (PARP) is a nuclear enzyme involved in the repair of DNA damage. During apoptosis, certain caspase family members cleave PARP. The full size PARP protein is 116 kDa; when apoptosis occurs, PARP is cleaved by caspases into inactive fragments of 89 and 24 kDa [25], which can be detected by Western blotting. The PARP cleavage assay was conducted on C1A cells treated with the TGI of 2-(4-methoxyphenyl)-A_{II}, 1.5 μ M, for 0, 24, 48 and 72 h. Fig. 3 shows representative results. The TGI was chosen because it was expected that apoptosis would be most detectable at this concentration. No 89 kDa PARP cleavage fragment was detectable in control cells, whereas the 48 hour treatment group showed this band. Due to the cell-killing actions of 2-(4-methoxyphenyl)-A_{II}, too many cells were lost for a meaningful PARP cleavage analysis at the 72 hour timepoint.

3.4. Flow cytometry

Table 2 shows the results for flow cytometrically-detected apoptosis after 2-(4-methoxyphenyl)-A_{II} treatment. Cells were counted as apoptotic when they stained with annexin V, which recognizes externalized phosphatidylserine, but did not stain with propidium iodide (PI), an indicator of cell membrane integrity. Necrotic cells stained with both PI and annexin V, and unaffected cells stained with neither reporter. After 48 hours, there was an 85% increase in apoptotic C1A cells in the 2-(4-methoxyphenyl)-A_{II} treated cultures compared with the DMSO only control cultures. A 64% increase in apoptosis was evident after 72 hours treatment, with 30% of the cells treated with 2-(4-methoxyphenyl)-A_{II} showing evidence of apoptosis compared with 18% in the control culture. No difference in apoptosis was evident in control and treated C1A cells at the 12 and 24 hour time points.

3.5. Expression microarray analysis

Microarray analysis for mRNA levels was conducted using the Affymetrix mouse MOE430A gene chip (22,600 probe sets representing transcripts and variants from over 14,000 mouse genes) on C1A cells treated with 1.5 μ M 2-(4-methoxyphenyl)-A_{II} in comparison to a control population of untreated C1A cells. Basic absolute analysis was used to scale each microarray, and signal levels were converted to the abundance of expression of each gene in the sample. The raw data obtained was reduced using a two-tailed *P* value of 0.005 and 0.995 to examine for statistically significant changes in gene expression between the two groups. The complete file of the probe IDs were then entered into the Onto-Express algorithms to identify the genes associated with apoptosis. From the probe identities, 10 distinct genes associated with apoptosis, both inhibitors and activators of the process, with significant altered expression levels were identified. Table 3 lists the apoptosis-related genes identified, along with the changes in their expression levels due to treatment with 2-(4-methoxyphenyl)-A_{II}. The message levels for the apoptosis activating proteins Bok and Siva-pending were up-regulated due to treatment of the C1A cells with 2-(4-methoxyphenyl)-A_{II}. In addition, expression levels of the apoptosis inhibitor genes *Birc 4*, *Dad1*, and *Atf5* were down-regulated due to treatment.

4. Discussion

Our previous studies of the actions of A_{II} and 2-(4-methoxyphenyl)-A_{II} against the human prostate cancer cell lines PC-3 and LNCaP showed each agent to be antiproliferative against these cells at low micromolar concentrations. 2-(4-methoxyphenyl)-A_{II} induces G₂/M accumulation, but only weakly induces apoptosis in both LNCaP and PC-3 cell lines [20].

In this study, we examined the effects of these agents against cells from the more useful TRAMP disease model. Growth inhibition studies revealed that 2-(4-methoxyphenyl)-A_{II} inhibited proliferation of TRAMP cell lines in the high nanomolar concentrations. 2-(4-methoxyphenyl)-A_{II} was clearly found to induce apoptosis in the C1A, C2H, and C2N cell lines. The C1A cell line was the most susceptible to 2-(4-methoxyphenyl)-A_{II}. Three methods were used to detect induction of apoptosis by the agent. The ELISA assay was conducted on all three of the cell lines to quantify apoptosis and determine the minimum detectable apoptosis inducing concentrations of 2-(4-methoxyphenyl)-A_{II}. Apoptosis was

verified with the PARP cleavage assay on the C1A cell line. Two-color flow cytometric evaluation for phosphatidylserine externalization further verified the induction of apoptosis.

Microarray analyses were then used to determine molecular mechanisms associated with the 2-(4-methoxyphenyl)-A_{II}-induced apoptosis. Results from expression of genes for apoptosis-controlling proteins were examined for coordinate up- and down-regulation in the C1A cell line due to treatment with 2-(4-methoxyphenyl)-A_{II}. The apoptosis activator genes *Bok* and *Siva-pending* were found to be up-regulated, and the apoptosis inhibitor genes *Birc 4*, *Dad1*, and *Atf5* were found to be down-regulated. The Bcl-2-related ovarian killer (Bok) protein is a tissue mediator of cell death that promotes p53-mediated apoptosis by interacting with selective antiapoptotic proteins [26,27]. Overexpression of the Siva protein in various cell lines induces apoptosis, and it is suggested this protein is the cytoplasmic facilitator of the CD27 transduced apoptotic pathway. It is a direct transcriptional target of p53 [28–30]. The apoptosis inhibitor defender against cell death (Dad1) has been shown to bind Bok and prevent the latter from generating apoptosis. Its human homologue has also been found at high levels in tissue samples of perineurally invasive human prostate cancer [31]. Birc 4, so named as it contains the baculoviral inhibitor of apoptosis repeat (BIR)-containing domain, is also the rat inhibitor of apoptosis protein 3 (rIAP3) [32]. All of these proteins have been found to be important for apoptosis in sex steroid hormone-sensitive tissues. There have been few studies performed to characterize Atf5 and its biological function, but the present data show that Atf5 is an embryonically-expressed transcription factor whose expression levels decrease during differentiation [33–37].

The initiating mechanism by which 2-(4-methoxyphenyl)-A_{II} induces the apoptosis cascade in TRAMP cell lines is currently unknown, but it is likely due to perturbation of microtubule dynamics and the induction of a prolonged mitotic block. Based on these experimental results, future work will examine the specific mechanisms at different stages of the cell cycle by which the genes identified by microarray analysis produce the apoptotic results in the C1A cell line. Studies in vivo wherein TRAMP C57BL/6 mice are treated with various doses of 2-(4-methoxyphenyl)-A_{II} to yield plasma concentrations centered around the LC₅₀ would provide additional insight into the effectiveness of this structurally simple but effective in vitro agent as a potential in vivo therapeutic for human prostate cancer. Furthermore, the low toxicity of this class of agents suggests they should be examined in combination therapies. Recent clinical studies suggest that combination of a tubulin dynamics inhibitory agent with prednisone, vitamin D receptor ligands, or mechanistically dissimilar antiangiogenic agents such as thalidomide may hold promise for the treatment of prostate cancer [38].

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**(Z)-1,1-Dichloro-2,3-diphenylcyclopropane
(a.k.a. Analog II, A_{II})**

Colchicine

**(Z)-1,1-Dichloro-2-(4-methoxyphenyl)-3-phenylcyclopropane
[2-(4-methoxyphenyl)-A_{II}]**

Fig. 1.
Structures of test agents.

Fig. 2. Apoptosis due to 2-(4-methoxyphenyl)-A_{II} in the C1A, C2H, and C2N cell lines. (A) C1A cells. (B) C2H cells. (C) C2N cells. Cells were plated in 6-well plates at 20,000 cells/well. Cells were treated 24 hours after plating (time 0) with 2-(4-methoxyphenyl)-A_{II} at the GI₂₅, GI₅₀, GI₇₀, GI₇₅, and TGI concentrations of the agent. An ELISA assay for internucleosomal chromatin fragmentation was performed 24 hours after the test agent was added. The control cultures were treated with 0.2% DMSO. The minimum apoptosis-inducing concentrations of 2-(4-methoxyphenyl)-A_{II} determined by regression analysis of

the concentration-response curves were $0.14 \pm 0.05 \mu\text{M}$ for C1A cells, $0.10 \pm 0.03 \mu\text{M}$ for C2H cells, and $0.12 \pm 0.01 \mu\text{M}$ for C2N cells.

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Fig. 3.

Representative Western blot analysis of PARP cleavage in C1A cells treated with 1.5 μ M 2-(4-methoxyphenyl)-A_{II}. Lysates from test agent- (T) and DMSO only-treated (C) cells at times of 0, 24, 48, and 72 hours were separated on 10% SDS-PAGE resolving gels, transferred to membrane and probed with PARP antibody. β -Actin was probed as a loading control. Three independent experiments were performed and variation was less than 10%.

Table 1

GI₅₀, TGI, and LC₅₀ of test agents against the C1A, C2H, and C2N TRAMP cell lines

Compound	TRAMP cell line									
	C1A					C2A				
	GI ₅₀ (μM)	TGI (μM)	LC ₅₀ (μM)	GI ₅₀ (μM)	TGI (μM)	LC ₅₀ (μM)	GI ₅₀ (μM)	TGI (μM)	LC ₅₀ (μM)	
2-(4-Methoxyphenyl)-A ₁₁	0.29 ± 0.06	1.5 ± 0.7	5.3 ± 1.1	0.32 ± 0.06	1.7 ± 0.68	4.0 ± 3.5	0.36 ± 0.06	1.6 ± 0.90	3.7 ± 1.1	
Colchicine	0.04 ± 0.01	>10	>10	0.04 ± 0.01	>10	>10	0.08 ± 0.02	>10	>10	

Cells were seeded at 1,200 cells/well in 96 well plates and in 100 μl of complete culture media. After 24 hours the cells were treated with a range of concentrations of agents dissolved in DMSO (final concentration 0.2%). Cell numbers were determined using the MTT assay after 72 hours of continuous test agent exposure. The effect of the agent on culture growth and viability of the cells was determined by comparison to the time 0 and time 72 hours control cultures.

Table 2

Flow cytometric analysis of percent TRAMP-C2H apoptotic cells after treatment with 1.5 μ M 2-(4-methoxyphenyl)-A_{II}

Time (hour)	Control (0.2% DMSO)	2-(4-Methoxyphenyl)-A _{II}
48 h	8.7%	16.1%
72 h	18.1%	29.7%

Results of mRNA microarray analyses for coordinate changes in expression of apoptosis-related genes in C1A cells treated with 1.5 μ M 2-(4-methoxyphenyl)-Analog II in comparison to a control C1A cells treated only with DMSO

Table 3

Gene	Description	Apoptosis activator or inhibitor	Change in expression (treated vs. control)	Control signal	Treated signal	Change <i>P</i> value (two-tailed)
<i>Bcl2</i>	Bcl-2 related ovarian killer protein	Activator	Increase	173.6	314.6	0.00002
<i>Siva-pending</i>	CD27 binding protein	Activator	Increase	441.2	534.1	0.00003
<i>Birc 4</i>	Baculoviral IAP repeat 4	Inhibitor	Decrease	25.4	17.3	0.99997
<i>Dad1</i>	Defender against cell death 1	Inhibitor	Decrease	784.3	620.9	0.99998
<i>Atf5</i>	Activating transcription factor 5	Inhibitor	Decrease	594.3	186.7	0.99998