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ORIGINAL ARTICLE

Disruption of Golgi processing by 2-phenyl benzimidazole analogs blocks cell proliferation and slows tumor growth

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Abstract
Purpose Cancer chemotherapy continues to be challenged by the emergence of resistant tumors, and one organelle entwined in the development of drug resistance is the Golgi apparatus. Recently, we discovered a group of 2-(substituted phenyl)-benzimidazole (2-PB) compounds that displace resident Golgi proteins from the juxtanuclear region resulting in their degradation. These compounds are also potent anti-proliferative agents, which together with their action on the Golgi made a compelling case for testing them against cancer.
Methods The anti-tumor activity of a group of 2-PB compounds was examined both in vitro and in vivo. The role of the Golgi in the anti-proliferative effect was assessed by comparing the proliferation of individual cell lines with the distribution and total cellular expression of selected resident Golgi proteins.
Results The anti-proliferative activity of 2-PB compounds is partially reversible (time- and concentration-dependent), non-cell-cycle-specific, and translates to tumor growth inhibition in vivo. While 2-PB compounds displace resident Golgi proteins from the juxtanuclear region in all cells, those that are resistant to the anti-proliferative effects differ from sensitive cells in that they have the capacity to protect these Golgi proteins from degradation.
Conclusions These results illustrate the utility of targeting the Golgi for cancer drug development. They also reveal a cellular strategy for resisting 2-PB drug effects through protection of displaced Golgi proteins from degradation thus allowing their continued function.

Keywords Anti-proliferative · Anti-tumor · Benzimidazole · Golgi · Resistance

Abbreviations

2-PB	2-(substituted phenyl)-benzimidazole
MDR	Multiple drug resistant
PMA	Phorbol myristate acetate
SNARE	Soluble *N*-ethylamide-sensitive factor attachment protein receptor
GS15	Golgi SNARE protein 15 kDa
GS28	Golgi SNARE protein 28 kDa
GPP130	Golgi phosphoprotein 130 kDa
SRB	Sulforhodamine-B
GAME	Goat anti-mouse IgE
PPMP	Phenolphthalein monophosphate
HRP	Horseradish peroxidase
COG	Conserved oligomeric Golgi

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Introduction

Largely because of the expanding usage of statin drugs to control the manifestations of arteriosclerosis, cancer is supplanting cardiovascular disease as the leading cause of death and hospitalization in the industrialized world [1]. Advancements in the treatment of cancer, however, are hampered by the development of drug-resistant tumors. Although targeted agents such as inhibitors of tyrosine kinase receptors (e.g., receptors for epithelial growth factor and vascular endothelial growth factor) have lessened the toxicity and modestly improved the efficacy of chemotherapeutic regimens [2–7], the traditional non-specific inhibitors of cell growth remain the most effective anti-tumor drugs. Indeed, regimens utilizing anti-metabolite and DNA-targeting drugs dominate treatment paradigms that continue to be useful today.

Tumor resistance is largely due to the emergence of cells with a multi-drug resistant (MDR) phenotype, a phenomenon that has been shown to limit the effectiveness of both cytotoxic drugs and targeted agents [6–8]. A number of mechanisms have been proposed for MDR, including enhanced DNA repair, altered cell cycle regulation, evasion of apoptosis, and modulations in how a drug is handled by the cell [9]. Cells expressing the MDR phenotype have shown the capacity to actively isolate drugs from their site of action through the aberrations in the activity of the Golgi, lysosomes, and other organelles involved in post-translational pathway [8–13]. Major mechanisms implicated in drug sequestration in MDR cancer cells include the ATP binding cassette family of transporter proteins such as *P*-glycoprotein, the multiple drug resistance-related protein-1, and the multiple drug resistance pump-1 [9, 14, 15]. The latter is a glucosyl ceramide translocase found within the Golgi complex, as many of the MDR defects appear to require a functioning Golgi.

No cancer drugs have been developed that specifically act on processes within the Golgi body, but the central role of this organelle in maintaining the growth and survivability of cancers make the Golgi a compelling target. This report describes the anti-tumor activity of a group of novel 2-(substituted phenyl)-benzimidazole (2-PB) compounds that were originally investigated in an anti-allergy context [16, 17]. These agents disrupt Golgi cisternae and block the recycling of resident Golgi proteins in primary lymphoid cells resulting in an acceleration of their degradation.[1] The discovery that proliferation of mitogen-activated spleen cells is also potently inhibited by 2-PB compounds prompted further exploration of this activity in cancer. Herein we show that 2-PB compounds have a novel anti-proliferative activity profile in vitro and suppress tumor growth in vivo following oral or i.p. administration. Moreover, while Golgi proteins are displaced from the juxtanuclear region of non-transformed and tumor cells in the presence of drug, resistant cells are distinguished by their ability to protect resident Golgi proteins from degradation. These results thus support the development of these agents for cancer, and expose a potential strategy of targeting these essential Golgi proteins to block the growth of resistant tumor cells.

[1] Ludwig JW, Soneff RY, Banie H, Galang C, Marcantonio D, Scholz W, Johnson J, Sircar JC, Chatterjee A, Richards ML (2006) Novel 2-phenyl-benzimidazole inhibitors of cytokines and cellular proliferation foster Golgi enzyme depletion through accelerated degradation (submitted).

Materials and methods

Cells and reagents

All cell lines were obtained from ATCC, Manassas, VA. Mice were purchased from Jackson Laboratories, Bar Harbor, ME. SW-480, H460, HCC1806, OVCAR-3, and MOLT-4 cells were cultured in RPMI media (Invitrogen, Carlsbad, CA). HT-29 and CAKI-1 cells were maintained in McCoy's 5a media (Invitrogen). B16-F1, B16-F10, Hs578t, and mouse spleen cells were cultured in high glucose DMEM media (Invitrogen). All media formulations contained 10% FBS (HyClone: Logan, UT), 2 mM L-glutamine and 1% penicillin-streptomycin solution (final 50 U/ml and 50 μg/ml, respectively) (Invitrogen), and maintained at 37°C in a 10% CO_2 (DMEM and McCoy's 5a) or 5% CO_2 (RPMI) atmosphere. Spleen cell cultures also contained 10 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Ionomycin and anti-mouse CD40 antibody were obtained from BD-Pharmingen, Franklin Lakes, NJ. Phorbol myristate acetate (PMA), doxorubicin, paclitaxel, LPS (*Escherichia coli* serotype 055:B5), and ConA were obtained from Sigma-Aldrich. Mouse IL-4 was obtained from Peprotech, Rocky Hill, NJ. Mouse monoclonal antibodies to Golgi SNARE protein 15 kDa (GS15) and Golgi SNARE protein 28 kDa (GS28), and polyclonal anti-CD3, -CD4, and -CD8 antibodies were obtained from BD-Pharmingen. The antibody to mannosidase II and the rabbit polyclonal antibody to Golgi phosphoprotein 130 kDa (GPP130) were purchased from Covance, Gaithersburg, MD. All other goat and rabbit polyclonal antibodies, and all secondary antibodies conjugated with horseradish peroxidase (HRP) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA.

Spleen cell proliferation

Spleen cell were prepared from naive BALB/c mice as described [16], and cultures were established at 2×10^6

lymphocytes/ml in the presence and absence of either PMA (10 ng/ml) plus ionomycin (100 nM), ConA (5 μg/ml), LPS (1 μg/ml), anti-CD3 Ab (100 ng/ml), or anti-CD40 antibody (100 ng/ml) plus IL-4 (10 ng/ml). Lymphoid cells were added together with AVP-13358 at the initiation of each culture, and incubated for 4 days. Cells were then pulsed with ^{3}H-thymidine (Perkin-Elmer, Boston, MA) for 6 h, and harvested using a 96-well plate harvester (Molecular Devices, Sunnyvale, CA) onto glass filter papers (Whatman, Brentford, UK). These filters were subsequently immersed in scintillation fluid (Fisher, Springfield, NJ) and the disintegrations per minute measured using a β-counter (Beckman-Coulter, Fullerton, CA).

Tumor cell line proliferation

Tumor cell proliferation was assessed both in-house and at the National Cancer Institute (NCI). Compounds AVP-893, AVP-25752, and AVP-13358 were submitted to the NCI for evaluation in their 60-cell anti-cancer drug screen. Compounds were tested at concentrations between 10 ng/ml and 10 μg/ml, and proliferation was determined following 2 and 6 days in culture using the NCI sulforhodamine-B (SRB) assay [18]. Results provided by the NCI were modified to reflect the IC_{50} of each compound against the proliferation of each cell line.

Tumor cell proliferation was assessed in-house by the SRB, Alamar blue, and thymidine incorporation assays. Thymidine incorporation in B16-F10 cells was performed essentially as described above for spleen cells except that the cells were detached prior to harvesting by incubating them in trypsin-EDTA (Invitrogen) for 5 min at 37°C. For quantifying proliferation by incorporation of SRB and Alamar blue dyes, 4-day cultures were established in 96-well plates at between 1,000 and 3,000 cells/well in the presence of a range of concentrations of the respective compounds. For CAKI-1, H460, B16-F1, B16-F10, SW480, HCC1806, and OVCAR-3 cells the SRB assay was carried out by cooling the culture plates at 4°C, adding 50 μl/well cold 50% trichloroacetic Acid (Sigma-Aldrich), and incubating for 1 h. Plates were then washed in water, loaded with 100 μl/well of 4% SRB (Molecular probes, Eugene, OR) and allowed to set for 10 min. Several washes were then performed using 1% acetic acid. Plates were dried for 3–5 h, bound SRB was dissolved in 100 μl/well of 10 mM Trizma base, and plates were scanned at 515 nm using a microplate reader (Molecular Devices). An assay employing Alamar blue dye (Biosource, Camarillo, CA) was used to quantify proliferation of MOLT-4 and Hs578t cells, and to independently confirm SRB results for other cell lines. On day 4, 20 μl of Alamar blue dye was added to each culture well, and plates were scanned 4 and 24 h later at 570 nm. Plates were kept at 37°C between readings.

In vitro IgE assay

This procedure was previously described [16]. In brief, spleen cells were obtained from naïve female BALB/c mice and cultured at 3×10^6 per ml in quadruplicate at 37°C, 10% CO_2 in the presence of IL-4 (10 ng/ml) and anti-CD40 antibody (100 ng/ml). After 5–7 days of culture, 2 μl of supernatant was removed for quantifying IgE by ELISA. The ELISA plates were prepared by coating with 1 μg/ml of polyclonal goat anti-mouse IgE (GAME, prepared as described in [19]) overnight at 4°C. After washing and blocking the plates with 200 μl 1% FBS in PBS, an aliquot (1:4 dilution in PBS with 1% BSA, 0.1% azide, and 0.5% tween-20) of each culture supernatant was transferred directly to the ELISA plates and incubated overnight in a humidified box at 4°C. IgE was quantified following successive 90 min incubations with biotinylated GAME (prepared in-house), alkaline phosphatase-streptavidin (Invitrogen), and 100 μl of phenolphthalein monophosphate (PPMP), DCHA salt, 40 mg/ml). Absorption was measured at 540 nm.

In vivo tumor studies

Female C57BL/6 mice were inoculated in the flank with 1 million B16-F10 or B16-F1 cells in 100 μl of Hanks basic salt solution (HBSS). Female Nu/Nu mice were similarly inoculated with the following cell lines: Hs294t at 10^7 cells, CAKI-1 at 2×10^6 cells, and HCC1569 at 7×10^6 cells. Daily administration of 2-PB compounds, chemotherapeutic agents, or formulation alone was commenced as soon as the tumors attained a volume of at least 200 mm^3. All mice were removed from the study when tumor volume exceeded 3,000 mm^3. Except where noted, the formulation used for solubilizing the 2-PB compounds was 38% Gelucire 44/14 (Gattefossé, Cedex, France), 38% polyethylene glycol 400, 10% tween 80, and 10% 1-methyl-2-pyrrolidinone (Sigma-Aldrich). Tumors were measured using calipers, and mice were weighed every 4–7 days to assess toxicity from the administered compounds. All animal protocols have been reviewed by the institutional Animal Care Committee and are in compliance with NIH guidelines.

Cell cycle analysis

B16-F10 cells were seeded at 350,000 per six-well plate, allowed to attach at 37°C for 1 h, and then cultured for 24 h in the presence and absence of AVP-25752. In a second protocol, paclitaxel was added to cultures 1 h prior to AVP-25752 and incubated overnight. Cultures were harvested in PBS/EDTA/trypsin, centrifuged, washed, and 10^5 cells of each sample were re-suspended in 0.3 ml PBS. Cells were fixed in 0.7 ml ice-cold ethanol while vortexing slowly. The

suspension was kept on ice for 1 h, washed, and re-suspended in 0.25 ml PBS with 50 μg RNAse-A. Following a 1 h culture at 37°C, propidium iodide (Sigma-Aldrich) was then added at 10 μg/ml final concentration and the suspension kept on ice until analysis. Flow cytometry was carried out on a FACSVantage SE (Becton-Dickinson, San Diego, CA) at 488 nm.

Western blotting

Cells were seeded (10^6 cells/ml) in 100 mm dishes and harvested by aspirating the supernatant and adding 50–100 μl of a cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA pH 8.0, and 0.5% Triton X-100) containing a protease inhibitor cocktail (Sigma-Aldrich). The pellet was re-suspended and placed on ice while vortexing occasionally over 30 min before centrifuging the debris. Protein concentrations in each lysate were determined with BCA protein reagent kit (Pierce-Endogen, Woburn, MA). Appropriate aliquots of each lysate were separated on a NuPage 10% bis-Tris gel, and transferred onto PVDF membranes (Invitrogen). Membranes were blocked overnight at 4°C in TBS-T (150 mM NaCl, 10 mM Tris pH 7.4, 0.1% tween-20) and 5% blotting-grade milk. Membranes were then incubated for 1 h at room temperature with antibodies directed to GS15, GS28, or GPP130 diluted in TBS-T + 5% milk (Bio-Rad, Hercules, CA). After four washes in TBS-T, goat anti-rabbit or rabbit anti-goat HRP Ab in TBS-T + 5% milk was applied for 1 h at room temperature, followed by another four washes in TBS-T. HRP staining was visualized with the ECL Plus kit (Amersham Biosciences/GE Healthcare, Buckinghamshire, UK).

Immunofluorescence

H460 and HT-29 cells were cultured on 12 mm coverslips in 24-well plates. Following overnight culture with drug, media was aspirated and cells were fixed for 30 min with cold 4% paraformaldehyde in PBSCM (0.1 M PBS, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$, pH 7.3). All processing steps were performed on ice in a volume of 500 μl per well. Fixative was removed and the cells washed twice for 10 min each in PBSCM followed by one wash with 0.1 M glycine in PBSCM. Cells were blocked and permeated for 30 min in blocking buffer (3% normal donkey serum, 1% fish gelatin, 1% BSA, and 0.1% Triton-X), and washed for 5 min in PBSCM. Urea (6 M in PBSCM) was added for 5 min, the cells washed four times in PBSCM, and cultured on ice for 45 min in blocking buffer. A working solution containing blocking buffer (1:3 in PBS) was used to dilute the primary antibodies (at 1:100). After an overnight incubation with primary antibody, cells were washed three times for 10 min each in PBSCM. Donkey anti-mouse FITC or donkey anti-rabbit rhodamine-conjugated antibodies (Santa Cruz Biotechnology) were applied as appropriate for 60 min at 1:500 or 1:1,000 depending on the primary antibody, and washed three times in PBSCM. Coverslips were mounted on slides using ProLong anti-fade/mounting media (Invitrogen), and then were visualized on a Nikon E600 fluorescent microscope.

Results

Mitogen-activated proliferation of mouse spleen cells is potently inhibited by AVP-13358

The ability of 2-PB compounds to suppress cell proliferation is illustrated in Fig. 1 wherein AVP-13358 substantially inhibits the proliferation of murine splenocytes in vitro at concentrations as low as 3 ng/ml. Growth suppression occurs regardless of the activation regimen employed: IL-4/anti-CD40 antibody, PMA/ionomycin, LPS, ConA, and anti-CD3 Ab. The diversity of these activation paradigms is evident in that anti-CD3 Ab and the combination of IL-4/anti-CD40 Ab activate cells via cell surface receptors, while PMA/ionomycin bypasses the cell surface receptor mediated process to directly activate protein kinase C and release intracellular Ca^{2+}. LPS is a bacteria-elicited endotoxin that binds the CD14/TLR4/MD2 receptor complex, and ConA activates cells via binding

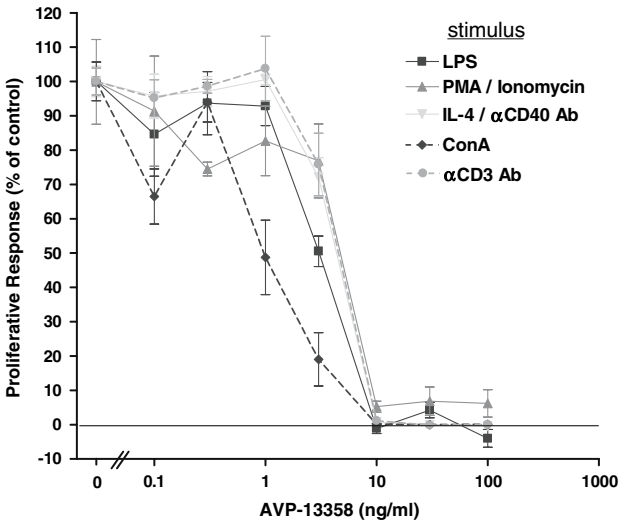


Fig. 1 Suppression of spleen cell proliferation following mitogen activation. Fresh BALB/c spleen cells were cultured for 4 days in the presence of the following stimuli: LPS (1 μg/ml), PMA (10 ng/ml) + ionomycin (100 nM), IL-4 (10 ng/ml) + anti-CD40 Ab (100 ng/ml), ConA (5 μg/ml), or anti-CD3 Ab (100 ng/ml). Cells were then harvested following a 6-h pulse with 3H-thymidine, and the cell-associated radioactivity was quantified. The IC_{50}s of AVP-13358 versus the proliferation of mitogen-activated spleen cells range between 2 and 10 nM. Data reflects means of quadruplicate samples ±SEM

carbohydrate residues on the plasma membrane. Moreover, LPS and the combination of IL-4/anti-CD40 antibody are more selective for B-cells while T-cells proliferate in response to either anti-CD3 Ab or ConA. Similar results were obtained when substituting human peripheral blood lymphocytes for mouse spleen cells (data not shown). Thus, the inhibition of these diverse proliferative responses with comparable potency suggesting that 2-PB compounds are acting on a target downstream of the activation event essential to the growth of both T and B lymphocytes.

The profile of tumor cell growth inhibition is consistent with a unique mode of action

The anti-proliferative effect of AVP-13358 against splenic lymphocytes prompted further inquiry into the therapeutic potential of 2-PB compounds against rapidly dividing tumor cells. An initial effort to evaluate this potential included testing of selected compounds at the NCI in their 60-cell line screening protocol, a program that was established to assess the activity and uniqueness of emerging cancer drugs. Data for the three compounds illustrated in Fig. 2 show a broad range of IC_{50}s against the 60 tumor cell lines ranging from <10 nM to over 10 μM. Although tissue specificity was weak, ovarian cancers as a group showed resistance while CNS and blood cancers were generally more susceptible to the inhibitory effects of the compounds. The sensitivity profile of the 60 cell lines to the action of AVP-893**,** AVP-13358**,** or AVP-25752 was significantly different from the therapeutic agents that have been previously tested by NCI and were available for comparison. Of the 38,000+ samples that had been tested against a minimum of 25 cell lines common to those tested with the 2-PB compounds, the highest correlation was a steroid compound (Pearson correlation coefficient = 0.793) while all others compared at $r = 0.727$ or less. In contrast, comparisons between each tested 2-PB compound resulted in correlation coefficients of >0.9, indicating that a common mechanism is shared by these agents to inhibit cell growth. This important evaluation supports the assertion that 2-PB compounds are acting on a heretofore unexplored target.

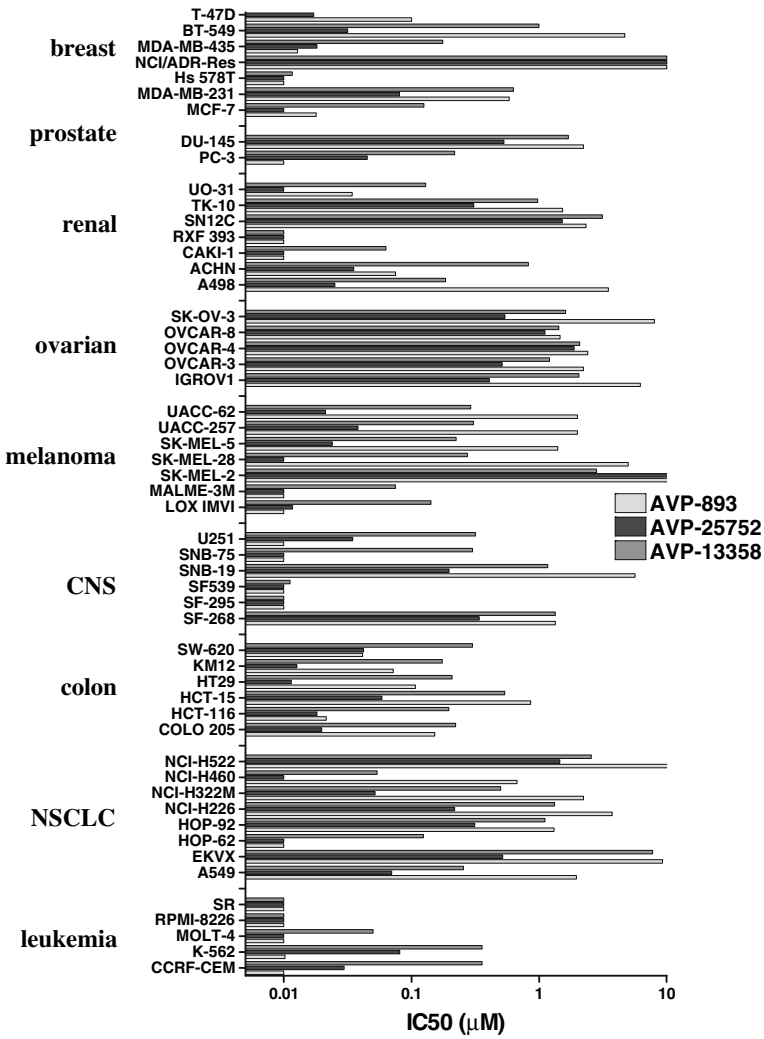


Fig. 2 NCI 60-cell screening assay. Three compounds, AVP-893, AVP-25752, and AVP-13358, were submitted to the National Cancer Institute (NCI) for testing in the 60-cell in vitro screening protocol. Each cell line was cultured for 2 days with compound in concentrations ranging from10 nM to 10 μM, and IC_{50}s were derived from the difference between the starting and ending protein concentrations measured in their sulforhodamine B assay. IC_{50} values outside the range of 10 nM and 10 μM are indicated as 10 nM and 10 μM, respectively

A group of compounds were selected from a library of agents containing the 2-phenyl benzimidazole backbone to canvas a broad spectrum of organ systems and cancer phenotypes, and test key functional group variants for their inhibitory potency against a panel of tumor cell lines (Table 1). Their anti-proliferative potency was compared to previously established structure–activity relationships (SAR) to assess whether a similar mechanism was used for suppression of IgE [16, 17]. Compounds are arranged based on the orientation of the amide linkages flanking the 2-phenyl benzimidazole core with the four possible combinations represented as groups 1–4 and the compounds as **1a–d**, **2a–c**, **3a–f**, and **4a–c**. Tumor cell lines tested in this paradigm included the human kidney carcinoma CAKI-1, human large cell carcinoma H460, murine melanoma B16-F10, human breast carcinoma Hs578t, human colorectal adenocarcinoma SW480, human breast carcinoma HCC1806, human T-cell leukemia MOLT-4, and human ovarian carcinoma OVCAR-3. Potencies for suppressing the proliferation of murine spleen cells activated by IL-4/anti-CD40 antibody are included for comparison. Consistent with the data provided by the NCI, the anti-proliferative potency of these compounds varied considerably depending on the cell line; i.e., H460, SW480, and OVCAR-3 were highly resistant to the anti-proliferative effects, while others like MOLT-4 and B16-F10 were potently inhibited. Moreover, as observed in the NCI 60-cell screen, proliferation was maximally inhibited >95% in all sensitive tumor cell lines. Although our results largely agreed with those provided by the NCI, quantitative differences were observed as for example the H460 cell line that we obtained from ATCC showed considerable more resistance to 2-PB anti-proliferative activity than the NCI-H460 line.

A significant feature of the 2-PB activity is that the relative potency of a compound across cell lines remained consistent, even as absolute effects differed. This parallel suggests that a common structure-activity relationship underlies the anti-proliferative activity of the 2-phenyl benzimidazole compounds. Moreover, their potency against the spleen cell IgE response determined previously [17] parallels their IC_{50} for cell proliferation (Electronic Supplementary Material); i.e., spleen cells (Spearman $r = 0.9020$), B16-F10 ($r = 0.9071$) CAKI-1 ($r = 0.9309$), SW480 ($r = 0.8698$), HCC1806 ($r = 0.9071$), H460 ($r = 0.8576$), OVCAR-3 ($r = 0.8574$), Hs578t ($r = 0.9145$), and MOLT-4 ($r = 0.8641$). Indeed, the positive slope and linearity of the data indicate that inhibition of IgE and cell proliferation is mediated by a common mechanism. This important point allows us to extend the extensive SAR developed for IgE inhibition [16, 17] to their anti-proliferative effects.

2-PB compounds slow tumor growth in vivo

Tumor models were selected for testing based on their consistency in producing robust tumors in host female Nu/Nu mice and their susceptibility to the anti-proliferative effects of 2-PB drugs in vitro (IC_{50} = 50 nM or less). Illustrated are the results of testing selected compounds in xenograph models of human melanoma Hs294t, human breast carcinoma HCC1569, and the human renal carcinoma CAKI-1 (Fig. 3a–c, respectively). Experimental outcomes were assessed via measurements of tumor size (Hs294t) or postponement of achieving end-point (HCC1569 and CAKI-1) defined by a tumor mass of 3,000 mm^3. Tumor growth was significantly reduced regardless of the 2-PB compound tested or the route of administration (AVP-893 i.p. or AVP-25752 p.o.). In the CAKI-1 renal tumor model, the anti-tumor effect of AVP-893 was additive with doxorubicin (Fig. 3c) indicating that the inhibition of proliferation by 2-PB compounds does not negatively impact the anti-tumor efficacy of this cytotoxic agent. Moreover, changes in body weight were not significantly different in mice receiving drug or vehicle suggesting that drug-related toxicity was not excessive (data not shown). Finally, 2-PB compounds do not inhibit tumor growth in tumor models of cells resistant to their anti-proliferative effects such as H460, HT-29, or SW480 (data not shown). These results illustrate the potential utility of these compounds as anti-cancer agents, and suggest that their effect on in vivo tumor growth translates from cell proliferation data in vitro.

Inhibition of cellular proliferation is neither cell cycle-specific nor coincident with cytotoxicity

We have previously shown that high concentrations (1 μg/ml) of AVP-13358 do not affect the viability of lymphocytes in vitro [16]. Here, the reversibility of the anti-proliferative effect was tested by culturing B16-F10 tumor cells for 4–72 h, removing media and washing the cells extensively. Cells were then counted and re-cultured at 50,000 per well for 72 h in the absence of drug. At the end of the drug-free culture period, cells were pulsed with ^{3}H-TdR for 6 h, harvested, and the cell-associated radioactivity quantified. Proliferation was absent in all cell cultures that were exposed to drug but not washed (data not shown). While doxorubicin (100 ng/ml) caused expected cytotoxicity and the consequent inability to grow following removal of drug, the proliferation of B16-F10 cells completely recovered following a 4- or 24-h exposure to 100 ng/ml AVP-893 (Fig. 4a). Longer exposures (48 h or more) or a very high concentration of AVP-893 (500 ng/ml), however, resulted in a partial or complete loss of reversibility. Similar experiments were performed with Hs578t and Du145 tumor cells resulting in a comparable pattern of reversibility (data not

Table 1 Structure and anti-proliferative potency of 2-PB compounds

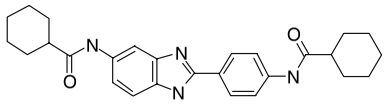
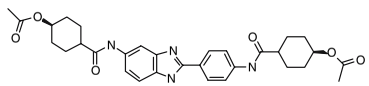
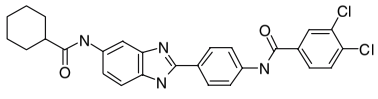
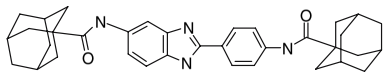
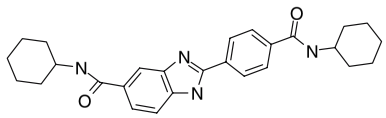
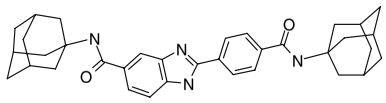
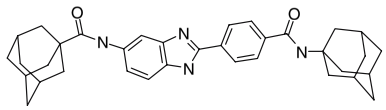
Numbers	IC_{50} (nM)[a]									
	Structure	Spleen	CAKI-1	H460	B16-F10	Hs578t	SW480	HCC1806	MOLT4	OVCAR-3
1a (AVP-893)		4	50	1,400	15	13	800	45	13	400
1b		1,000	7,000	6,200	700	700	6,500	800	600	5,000
1c		3	30	110	6	6	300	3	3	90
1d		0.6	13	300	2	5	200	4	6	200
2a		60	1,000	6,000	400	1,000	8,000	140	250	7,000
2b		6	100	250	5	50	150	15	60	750
2c		60	500	800	50	150	1,100	130	250	10,000

Table 1 continued

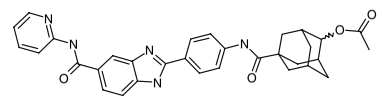
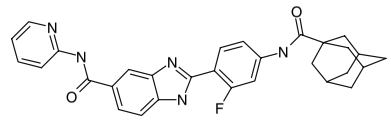
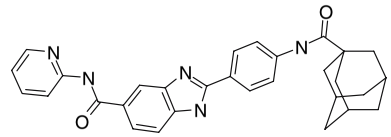
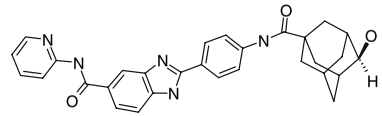
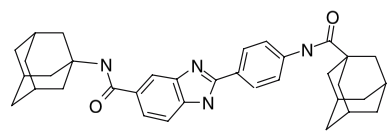
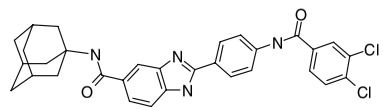
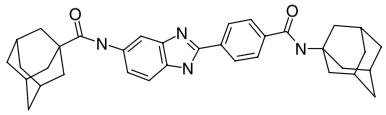
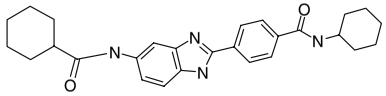
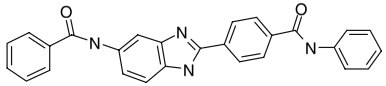
Numbers	IC_{50} (nM)[a]									
	Structure	Spleen	CAKI-1	H460	B16-F10	Hs578t	SW480	HCC1806	MOLT4	OVCAR-3
3a		9	80	600	50	55	500	50	60	450
3b (AVP-25752)		4	350	600	9	120	650	130	70	800
3c (AVP-13358)		9	75	350	12	40	400	45	60	1,300
3d		6	110	600	45	45	420	35	50	375
3e		1	15	85	3.5	12	110	6	35	245
3f		1	3	25	0.7	0.2	20	3	1	24

Table 1 continued

Numbers	IC_{50} (nM)[a]									
	Structure	Spleen	CAKI-1	H460	B16-F10	Hs578t	SW480	HCC1806	MOLT4	OVCAR-3
4a		3	7	55	1.3	6	150	8	6	180
4b		60	500	1,400	65	140	1,400	115	50	6,000
4c		600	3,000	7,500	620	1,500	5,500	1,200	1,300	19,000

[a] IC_{50} values represent 50% suppression of cell growth following a 48–96 h culture measured by the SRB assay; spleen cells were stimulated with IL-4 and αCD40 Ab

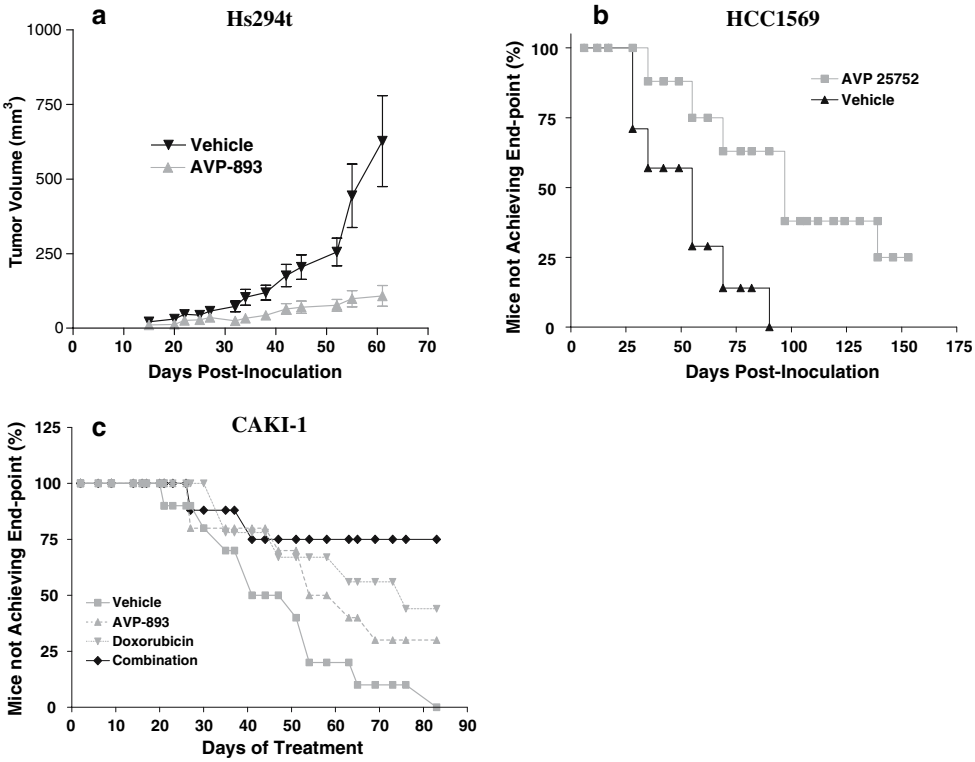


Fig. 3 Inhibition of tumor growth in vivo. Efficacy of AVP-893 and AVP-25752 in human tumor models was tested following subcutaneous inoculation of the following cell lines in the flank of female Nu/Nu mice: **a** 10 million Hs294t melanoma cells, **b** 7 million HCC1569 breast tumor cells, and **c** 2 million CAKI-1 renal tumor cells. Daily treatment with vehicle or 8 mg/kg AVP-893 i.p. or 6 mg/kg AVP-25752 p.o. was initiated 3–7 days later when tumors became visible. Both drug and vehicle were administered once daily, 6 days a week. The endpoint for parts **b** and **c** is defined as a tumor volume of 3,000 mm^3. Two-way ANOVA analyses of data curves show significant differences for the treatment groups: **a** $P < 0.0001$; **b** $P = 0.0114$; **c** $P = 0.0846$ (Vehicle versus AVP-893), $P = 0.0263$ (Vehicle versus doxorubicin), and $P = 0.0017$ (Vehicle versus combination). Less than 10% variation in mean body weight resulted in each experiment. Data reflects mean observations of 7–10 mice per treatment group ±SEM

shown). Finally, analysis of the pro-apoptotic effects of AVP-893 and AVP-25752 in several tumor cell lines using propidium iodide and annexin also yielded a negative result (data not shown). These results thus support previous findings demonstrating the reversibility of the pharmacological effects and the lack of cellular toxicity attributable to 2-PB compounds.

The influence of 2-PB drugs on the cell cycle of B16-F10 cells was also tested. Following overnight exposure to AVP-25752 (1–1,000 ng/ml), B16-F10 cells were harvested and the nuclear material stained with propidium iodide. A parallel culture was incubated with 50 ng/ml paclitaxel, which halts cell proliferation in G2/M through prevention of mitotic spindle formation [20]. AVP-25752 did not modify cell cycle in comparison to untreated cells, whereas paclitaxel caused an accumulation of cells in G2/M-phase (data not shown). To test the possibility that 2-PB compounds arrest cells in G_0/G_1, B16-F10 cells were treated with AVP-25752 for 1 h prior to addition of paclitaxel and then cultured overnight (Fig. 4b). Cells exposed to both drugs behaved as though treated with paclitaxel alone. These findings were repeated when testing AVP-893, and in other sensitive tumor cell lines (data not shown). Thus the effect of 2-PB compounds on cell proliferation is not cell cycle specific.

The influence of 2-PB compounds on tumor cell proliferation is determined by their quantitative effects on resident Golgi proteins

Previous proteomic work has shown that 2-PB compounds selectively deplete resident Golgi proteins while leaving proteins of other organelles involved in synthesis, processing, and transport apparently unperturbed (see footnote 1). Subsequent work has shown that these compounds displace resident Golgi proteins from the juxtanuclear region followed by their degradation (either through the proteosome or lysosome dependent on the individual cell), and that this action occurs in the same concentrations that give rise to their suppression of cytokines and IgE. This action results in a glycosylation defect that gives rise to EndoH sensitivity thus further implicating enzymes localized to the Golgi. Specific to their anti-proliferative properties, 2-PB compounds do not bind nuclear material or affect microtubules

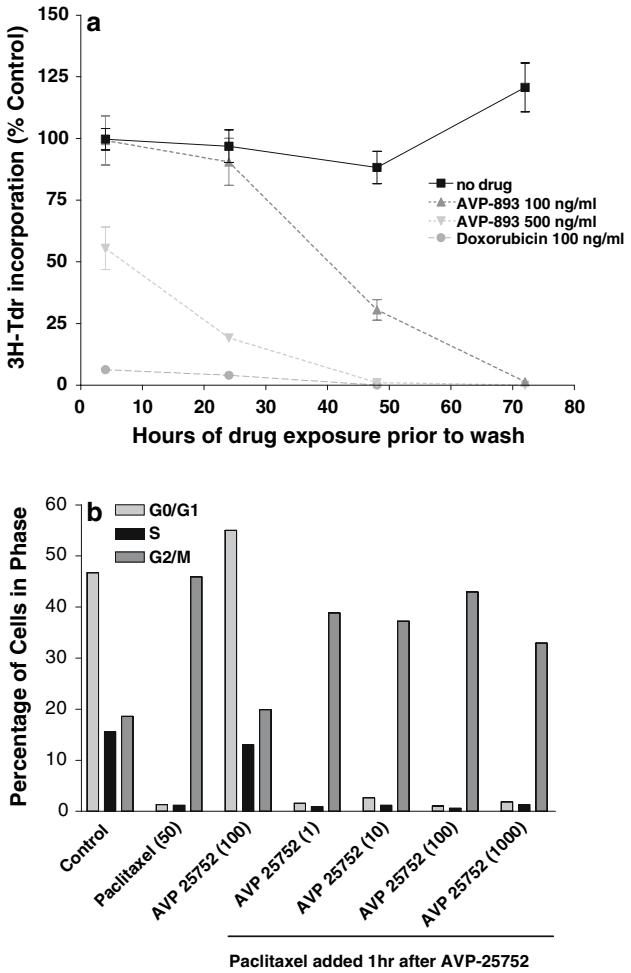


Fig. 4 **a** Reversibility of AVP-893 effect on B16-F10 proliferation. B16-F10 cells were cultured for 4–72 h in the presence and absence of AVP-893. Cells were washed extensively, counted, and re-cultured at 50,000 cells/well for an additional 72 h after wash. This was followed by a 6-h pulse with 3H-thymidine and harvesting. Mean proliferation data is shown with SEM. The IC_{50} of AVP-893 for B16-F10 cell proliferation is 15 nM (~7 ng/ml). **b** AVP-25752 inhibits cell growth without modifying cell cycle. B16-F10 melanoma cells were cultured with AVP-25752 (1–1,000 ng/ml) for 1 h followed by paclitaxel (50 ng/ml) for 24 h prior to processing for propidium iodide staining and FACS analysis. Five thousand cells were analyzed for each sample and the experiment has been repeated with similar results. The IC_{50} of AVP-25752 for B16-F10 cell proliferation is 9 nM (~5 ng/ml). The experiment has been repeated with equivalent results

(data not shown). We have shown that inhibition of proliferation of many cells occurs in the same concentrations that result in inhibition of cytokine and IgE responses. However, many cell lines show considerable resistance to the anti-proliferative effects of 2-PB compounds suggesting that multiple mechanisms may be involved or that resistant cells have developed a means for growth without Golgi proteins.

To better understand the role of the Golgi in the action of 2-PB compounds and perhaps reveal the source of tumor cell resistance to these agents, the localization and total cellular expression of Golgi proteins was tested in a group of tumor cell lines that are distinguished by their variable growth sensitivity to AVP-893. For these experiments we have examined the expression of the Golgi SNARE proteins GS15 and GS28, and the Golgi phosphoprotein GPP130. Although these are but a handful of the proteins residing within the Golgi they have been followed because of their high level of expression and the availability of reagents that can detect these proteins by Western blot and immunofluorescence. We have previously shown that the initial cellular change noted in cells treated with 2-PB compounds is the displacement of resident Golgi proteins from the juxtanuclear region (see footnote 1). Indeed resident Golgi proteins leave the perinuclear zone of all primary and transformed cells treated with 2-PB compounds. Even in the highly resistant cell lines, H460 and HT-29, the expression of GPP130 and GS28 are no longer concentrated in the juxtanuclear region following an overnight exposure to AVP-893 (Fig. 5a, b). In contrast, the expression pattern of the Golgi matrix (non-resident) protein GM130 is unaffected. Thus although AVP-893 causes the displacement of resident proteins from the Golgi region, they continue to proliferate.

The predictable effect of these compounds on the localization of resident Golgi proteins, however, contrasts with their fate following displacement. To illustrate this, the expression of three resident Golgi proteins were measured by Western blot in cell lines differentiated by their proliferation sensitivity to AVP-893, including: Hs578t (IC_{50} = 13 nM), MOLT-4 (13 nM), HCC1806 (45 nM), OVCAR3 (400 nM), SW480 (800 nM), H460 (1,400 nM), and HT-29 (2,000 nM). Because of their high expression level in these cell lines, GS15, GS28, and GPP130 were measured to illustrate the effect on resident Golgi proteins. The endoplasmic reticulum protein calnexin is included as a negative control. Sensitive cell lines show the typical depletion of GS15, GS28, and GPP130 (Fig. 5c). The loss of Golgi proteins in the highly sensitive Hs578t and MOLT-4 cells occurs in concentrations that roughly parallel their inhibition of proliferation. Cells such as HCC1806 and OVCAR3 are moderately sensitive to the anti-proliferative effect of AVP-893 and show a mixed effect on Golgi proteins. For example, GS15 is reduced in OVCAR3 cells but not eliminated; in HCC1806 cells GS28 is suppressed but GS15 is poorly expressed and apparently unaffected by drug. Interestingly, in OVCAR3 cells the IC_{50} for inhibition of resident Golgi proteins is considerably lower than the IC_{50} for proliferation. However, residual levels of GS15 and GS28 remain in OVCAR3 cells following overnight culture with high concentrations of AVP-893, which may indicate that lower levels of Golgi protein continue to support proliferation albeit at a lower rate. Finally, the three resistant cell

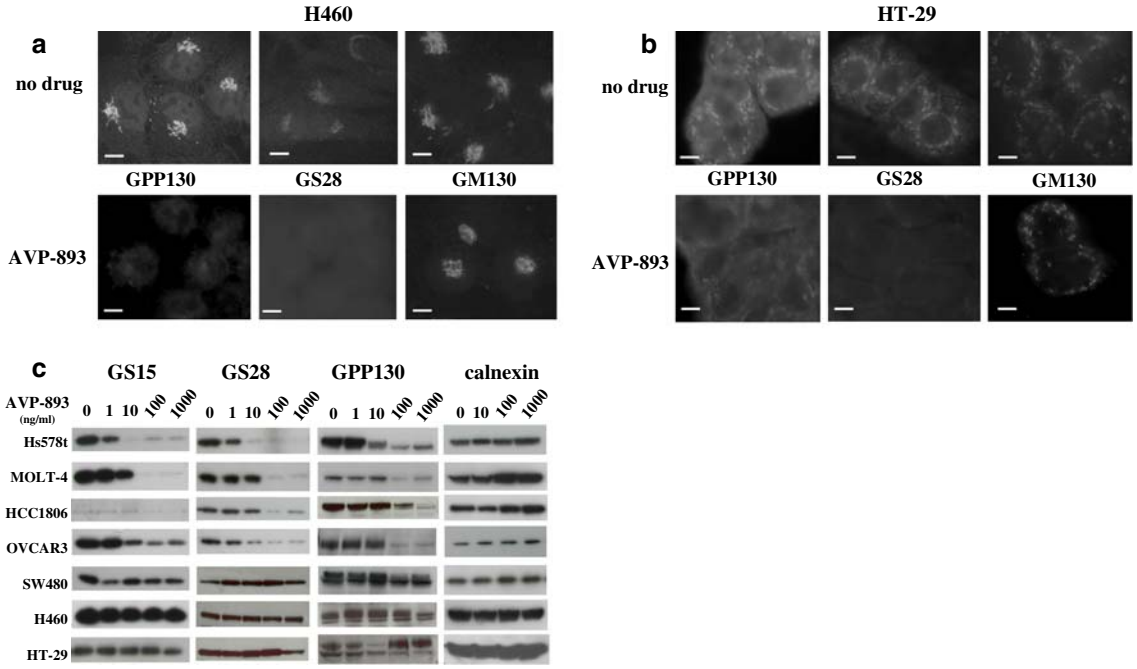


Fig. 5 Resident Golgi proteins are displaced from the juxtanuclear region of tumor cells that are resistant to the anti-proliferative effects of 2-PB compounds. AVP-893 (100 ng/ml) was added to cultures of H460 (**a**) and HT-29 (**b**) and incubated for 24 h on cover-slips in 24-well culture dishes. Samples were then fixed, washed extensively, and tested for expression of the resident Golgi proteins, GS28 and GPP130, as well as the control cellular protein GM130. *White bars* represent 5 μM. **c** Cells sensitive to anti-proliferative effect of AVP-893 also lose total cellular expression of resident Golgi proteins. Cell lines were seeded at subconfluency in 100 mm culture plates and allowed to attach. AVP-893 was added in the indicated concentrations and cultured overnight before harvesting and preparing lysates for Western blot as described in the Methods. For each cell line, equivalent total protein (20–30 mg) was loaded in each lane. Calnexin is included as a loading control. Full-length blots with markers are presented in Supplemental Figure S10

lines (SW480, H460, and HT-29) experienced minimal suppression in their expression of GS15, GS28, and GPP130 following overnight culture with AVP-893. These results indicate that the anti-proliferative effect of 2-PB compounds is a consequence of a secondary effect on Golgi protein depletion rather than their displacement. Cellular sensitivity to the anti-proliferative effects of 2-PB compounds, thus, is dependent on a cells ability to resist the depletion of resident Golgi proteins.

Discussion

This report describes the use of a novel group of 2-phenyl benzimidazole compounds for the treatment of experimental cancer. These agents potently inhibit tumor cell growth in vitro and suppress tumor growth in vivo. The novelty of these 2-PB compounds is reflected in the unique profile of the anti-tumor cell activity in the NCI 60-cell screen, and their selective effects on the Golgi. Finally, it is shown that inhibition of tumor cell proliferation by 2-PB compounds is coincident with the loss of resident Golgi proteins, while resistant cells share a unique ability to protect these proteins from degradation.

Using a proteomic approach, the molecular activity of the 2-PB compounds has previously been localized to a group of Golgi proteins (see footnote 1). These analyses included the measurement of responses of over 3,500 proteins in a time-frame of drug exposure extending from 2.5 to 16 h. Follow-up experiments verified the effect on Golgi proteins and further focused the attention on those proteins that function within the Golgi and are recycled back to early Golgi membranes. These resident Golgi proteins normally move in an anterograde fashion coincident with the natural process of Golgi maturation [21–23]. To conserve these resident proteins, they are recycled back to proximal Golgi membranes by coatomer-coated vesicles known as COPI [23, 24]. In 2-PB compound-treated cells, depletion of resident Golgi proteins appears to be initiated by the cell's inability to recycle these proteins from the distal membranes (trans-Golgi or trans-Golgi network) to early membranes of the Golgi apparatus (see footnote 1). The morphology of Golgi cisternae also changes in a time-frame that is consistent with the loss of Golgi proteins from the juxtanuclear region. The sequence of the 2-PB-initiated events suggests that the loss of these proteins is secondary to their displacement and the normal cellular clean-up process, ultimately leading to their degradation within either the proteosome or lysosome.

The connection between the anti-proliferative activity of 2-PB compounds and their site of action provides insight into potentially important new approaches to cancer chemotherapy. The profile of anti-proliferative activity in NCI's 60-cell panel and the breadth of other pharmacological effects exhibited by these agents further support their action via a unique mechanism. In primary cells and sensitive tumor cell lines treated with 2-PB compounds, resident Golgi proteins are degraded. Thus, their anti-proliferative activity appears to be determined by the quantitative cellular expression of the resident Golgi proteins rather than changes in their location. Indeed, resistance to the anti-proliferative action of 2-PB compounds is observed in cells that protect resident Golgi proteins from degradation following their displacement from the juxtanuclear region (Fig. 5). Since 2-PB compounds cause a disruption of Golgi cisternae and displacement of Golgi proteins in all cells, it is likely that resistant cells have developed a mechanism to protect these proteins possibly through sequestration in the endoplasmic reticulum, endosomes, or other organelles in order to support continued proliferation. Thus Golgi cisternae can be dissociated and Golgi proteins redistributed without drastically affecting cell proliferation. It can also be concluded that at least some resident Golgi proteins are essential for proliferation, and that they remain viable targets for unrestricted cellular proliferation.

How perturbation of the Golgi influences cell proliferation is not completely understood. Golgi enzymes carry out numerous roles in the processing of proteins and lipids as well as sorting cargo proteins during their transit to peripheral structures such as the plasma membrane or secretory granules. Indeed, since the Golgi participates in the building and trafficking of lipids required for plasma membrane formation [25, 26] this action would be expected to have pronounced effects on the proliferation of cancer cells, which rely heavily on the supply and processing of lipids through the Golgi to sustain their growth. Alternatively, the anti-proliferative action of 2-PB compounds may be related to an effect on the transport and processing of glycoproteins. Indeed, post-translational modification and trafficking of proteins through the Golgi has been suggested as a comprehensive approach to cancer therapy since these processes are likely to play a fundamental role in modulating the movement of aberrant or over-expressed proteins that contribute to tumor growth and metastasis [27]. A clearer understanding of how the disruption of these processes might impact cell growth awaits further study.

Like many chemotherapy drugs already in use, the molecular target of the 2-PB compounds is not known. Candidates include any of a plethora of Golgi proteins with poorly understood functions. However, recent studies of cells lacking one or more of a cassette of proteins that comprise the hetero-octamer Conserved Oligomeric Golgi (COG) complex reveal numerous parallels with the phenotype of 2-PB-treated cells, ranging from the loss of resident Golgi proteins to specific deficits in glycoprotein processing and disrupted Golgi morphology [28–30]. A similar phenotype is also observed in HeLa cells wherein a COPI vesicle coat protein (γ-COP) is depleted using siRNA knock-down [30]. COPI proteins putatively interact with the COG complex to return resident Golgi proteins to early Golgi membranes thus conserving their expression [24, 31, 32]. Indeed, depletion of selected COG homologs in yeast causes growth inhibition [33]. These striking phenotypic similarities strongly support a role for Golgi protein recycling in the action of these drugs, perhaps through COPI vesicles and/or COG proteins.

Finally, a comment about the potential toxicity of these compounds is warranted. As shown, 2-PB compounds suppress tumor growth when administered in doses that are not associated with overt toxicity. However, as expected from their short serum half-life ($\sim$1 h) and the reversible non-cytotoxic nature of their anti-proliferative effect, once-daily administration of these agents does not completely inhibit tumor growth (Fig. 3). Higher doses of AVP-25752 sufficient to abort growth and reduce tumor volume also cause body weight loss (data not shown), suggesting that while tumor reduction is possible with these compounds in mice, it is not without toxic consequences for the host. Extensive pre-clinical toxicological evaluation of these agents has identified the gut as the target organ for toxicity. The effect is usually mild presenting as diarrhea or mucosal hyperplasia, and is both dose-dependent and reversible. In mice this effect appears only after chronic use of high doses of compound. No changes in lymphocyte populations, organ weights, mutagenesis, liver enzymes or renal function were associated with the chronic use of this compound. Moreover, Phase-I multiple rising dose clinical trials have not yet identified drug-related toxicity.

Thus these compounds have potential utility in treating cancer and other diseases associated with uncontrolled cellular proliferation. Moreover, improvements in our understanding of the target of these compounds can lead to the development of new anti-cancer treatments, and well as improving our knowledge of the role of Golgi proteins in cell growth.

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