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Characterization of a membrane-active anti-tumor agent, UA8967

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Summary

Deletions or mutations in the tumor suppressor gene DPC4 (*deleted in pancreatic carcinoma locus 4*) are common in colon and pancreatic cancers. Using the Target-related Affinity Profiling (TRAP) chemical library screening method, a novel agent, UA8967, was selected for further

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studies because it showed greater potency in DPC4-deleted HCT-116 colon cancer cells. Cytotoxicity studies in six pancreatic cancer cell lines (MiaPaca-2, Panc-1, BxPC3, CF-PAC1, AsPC1, and T3M4), one normal human pancreatic ductal epithelial line (HPDE-6) and the HCT-116 DPC4 (+/+) and HCT-116 DPC4(−/−) colon cancer cells showed IC₅₀s ranging from 12–61 μM for exposure times of 72 h. Analysis of schedule dependence showed no advantage for long drug exposure times. There was also no selective inhibition of DNA, RNA or protein synthesis after exposure to UA8967. At 24–48 h, there was an accumulation of cells in G₀/G₁-phase and a proportionate reduction in S-phase cells. Within 1–6 h of exposure, cells were found to undergo an autophagic response, followed at 24 h by a low level of caspase-independent apoptosis with some necrosis. Because of the relatively non-specific mechanistic effects of UA8967, plasma membrane viability was evaluated using uptake of trypan blue and Sytox® Green dyes, and leakage of LDH. There was a dose dependent increase in Sytox® Green staining, trypan blue uptake and LDH leakage with increasing concentrations of UA8967, suggesting that UA8967 is affecting the plasma membrane. The DPC4(−/−) cells were more sensitive to UA8967 but not to DMSO, suggesting a drug-specific effect on cell membrane integrity.

Keywords

DPC4; Membrane integrity; SMAD4; TRAP; UA8967; Autophagy

Introduction

There are only two widely used drugs for the treatment of advanced pancreatic adenocarcinomas in the United States, gemcitabine (Gemzar®, Eli Lilly) and erlotinib (Tarceva®, OSI Pharma). Neither agent increases survival substantially, and despite advances in the molecular understanding of the disease, pancreatic cancer remains the fourth leading cause of cancer death in the United States [1, 2]. Because most patients are diagnosed with advanced disease that is not amenable to surgical intervention, new therapies for systemic pancreatic cancer are vitally needed. Recently, a better understanding of the genetic basis of this disease has suggested new molecular targets for therapeutic development [3]. These include specifically targeting several unique genetic lesions found in pancreatic cancer, such as the ubiquitous gain of function *Kras* mutations, or loss of function mutations in tumor suppressor genes. One such tumor suppressor gene is the *deleted in pancreas cancer locus 4 (DPC4)* gene which functions in the TGF-beta/DPC4 (Mothers against decapentaplegic homolog 4) or SMAD4 signaling pathway [4]. Inactivation of this tumor suppressor gene by either homozygous deletion or loss of heterozygosity is seen in up to 55 % of pancreatic tumors and 31–70 % of metastatic colon cancers [4]. Furthermore, those pancreatic cancer patients with normal DPC4 expression have a significantly longer median survival of 19.2 months compared to 14.7 months in patients with DPC4 (SMAD4) deficient tumors [5].

DPC4 is therefore an attractive target for therapeutic development since it is inactivated in many pancreatic and colon cancers and is associated with a negative impact on survival. An agent with increased activity in DPC4(−/−) cancers could potentially target more aggressive cancers while sparing normal tissues that tend to be DPC4(+/+). Wang et al., have developed a matched-pair of BxPC3 pancreatic cancer cells lines that are isogenic except for DPC4 expression [6]. This was accomplished by genetically restoring wild-type DPC4 protein expression in the parental, homozygously deleted DPC4(−/−) BxPC3 cell line. The resulting isogenic cell line pair, BxPC3/DPC4(+/+) and BxPC3/DPC4(−/−) was previously used to screen a commercial Nanosyn library of 20,000 compounds. This yielded 2 agents with enhanced cytotoxicity for the DPC4(−/−) cell line over the DPC4(+/+) cell line:

UA62001 [6] and UA62784 [7]. These two agents demonstrated proof of principle, but had weak specificity for the DPC4(−/−) cell line.

In the current report, we have expanded our screening capacity using Target-related Affinity Profiling (TRAP), a computationally-driven iterative chemical library screening technology to identify a new lead, UA8967 [8]. This agent demonstrates cytotoxic potency in the low μM range, with a cytotoxic action that involves a drug-specific compromise of plasma membrane integrity in DPC4(−/−) HCT-116 colon cancer cells.

Materials and methods

Chemicals

Chemical libraries selected by the TRAP algorithms were evaluated for cell growth inhibition in the HCT-116 DPC4 isogenic cell lines using the sulforhodamine B (SRB) assay as described previously [9]. After each set of compounds is assayed, the growth inhibitory activities for the compounds are incorporated into a computational model for subsequent compound selection. Details of the TRAP compound libraries (Telik, Inc., Palo Alto, CA) and the adaptive selection algorithms are described elsewhere [8]. In this screening campaign, three iterations of compound selection and screening were performed, involving 275 compounds in total.

Compound UA8967, 3(4-benzylpiperazino)methyl]-1H-indole (Fig. 1) was identified from the screening campaign, and additional supplies were purchased from Maybridge Fine Chemicals Division (Cornwall, UK). The pan-caspase inhibitor (Q-VD-OPh), the caspase 3 inhibitor (Q-DEVD-OPh), and the caspase inhibitor negative control for Q-VD-OPh were purchased from BioVision (Milpitas, CA). Caspase inhibitors were added to cells 30 min prior to the addition of UA8967 at a final concentration of 5 μM .

Cell lines and culture conditions

The pancreatic MiaPaCa-2 (CRL-1420), BxPC3 (CRL-1687), Panc-1 (CRL-1469), CF-PAC1 (CRL-1918) cells were purchased from the American Type Culture Collection (Manassas, VA). The AsPC1 pancreatic adenocarcinoma and the HPDE-6 normal pancreatic ductal epithelial lines [10] were obtained from the Experimental Mouse Shared Service (EMSS) at the University of Arizona Cancer Center. The T3M4 pancreatic cancer cell line [11] was a generous gift from Dr. Surinder Batra, University of Nebraska Medical Center, Lincoln, NE, USA.

The HCT-116 DPC4(+/+) and HCT-116 DPC4(−/−) colon cancer cell lines were generously provided by Dr. Bert Vogelstein (Johns Hopkins University) [12]. The identity of all cell lines was validated by short tandem repeat (STR) analysis by the University of Arizona Genetics Core. All cells were negative for 44 species of mollicutes, including the six most common mycoplasma species found in cell culture, using MycoAlert™ (Lonza, Walkersville, MD).

Growth inhibition assays

Growth inhibition assays were performed according to Mosmann, wherein the activity of mitochondrial reductases are measured using 3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyl-tetrazolium bromide (MTT) dye [13]. Cell growth inhibition data are expressed as percent survival, compared to untreated cells. Results are mean $\pm$ SEM ($n=3$). Enhanced cytotoxic potency for the HCT-116 DPC4(−/−) cells was defined as the ratio of IC₅₀ values for DPC4(+/+) cells/DPC4(−/−) cells.

Measurement of cell cycle

Cell cycle analysis was done by flow cytometry using propidium iodide (PI) staining (Sigma Chemical Company, St Louis, MO). Briefly, cells were treated with UA8967 for 24-72 h, fixed with 70 % ethanol over-night, then stained with 40 $\mu\text{g/ml}$ of PI and 0.5 mg/ml RNase A for 30 min at 37 °C. DNA intercalation of PI was measured on a Becton Dickinson FACScan® (San Jose, CA) and analyzed using Mod-Fit (Verify Software, Topsham, ME).

Measurement of apoptosis and necrosis

Apoptosis and necrosis were measured by flow cytometry using dual staining with AnnexinV-Alexa488 (Invitrogen) and propidium iodide (PI), (BioVision, Milpitas, CA) after 24-72 h treatment with UA8967. Fluorescence was measured on a Becton Dickinson FACScan using CellQuest Pro® software (BD Biosciences, San Jose, CA). Unstained cells are deemed alive, Alexa488 positive cells are deemed apoptotic, and dual Alexa488/PI stained cells are deemed necrotic.

Measurement of macromolecular synthesis

We measured RNA, DNA and protein synthesis after 24-72 h of exposure to UA8967. Inhibition of RNA, DNA and protein synthesis was measured by standard macro-molecular precursor incorporation assays using ^3H uridine (MP Bio, Solon, OH), ^3H thymidine (GE Healthcare, Piscataway, NJ), and ^{14}C valine (GE Healthcare, Piscataway, NJ), respectively. Cells were plated in 96-well plates and exposed to drug for 24-72 h, at which time 0.5 $\mu\text{Ci/ml}$ (for RNA and DNA) or 0.025 $\mu\text{Ci/ml}$ (for protein) of radioactivity was added. Cells were harvested on a Packard FilterMate Harvester (Perkin Elmer, Waltham, MA).

Measurement of autophagy protein, LC3

Autophagy was evaluated by western blot analysis of the autophagic vesicle protein, LC3 [15] and by examining the cellular distribution pattern of LC3 by microscopy using immunofluorescent staining.

For western blot analysis, MiaPaCa-2 cells were exposed to UA8967 for 0 to 24 h, lysed, and separated by SDS-PAGE. Proteins were visualized using standard immunoblot techniques using anti-LC3 (Abgent, San Diego, CA) and quantitated using ImageQuant® software (Molecular Dynamics, Sunnyvale, CA) after normalizing for β -actin loading (Sigma Chemical Company, St. Louis, MO).

Measurement of plasma membrane integrity

Plasma membrane integrity was evaluated by three methods: (a) trypan blue dye exclusion (Sigma Chemical Company, St Louis, MO), (b) fluorescence of Sytox® Green nucleic acid stain (Invitrogen, Carlsbad, CA), and (c) lactate dehydrogenase (LDH) release into the cell culture medium (BioVision, Milpitas, CA).

Sytox® Green is membrane impermeant to live cells, but stains nucleic acids of cells with compromised plasma membranes [14]. For Sytox® Green staining, cells are treated with UA8967 for various times, and stained with 5 nM Sytox® Green for 10 min at 37 °C, and analyzed by flow cytometry on a Becton Dickinson FACScan (San Jose, CA) using CellQuest Pro® software (BD BioSciences, San Jose, CA). Lactate dehydrogenase (LDH) is a cytosolic enzyme that is released from the cell into the culture medium when plasma membrane integrity is compromised. To measure LDH release, cells were treated for 24-72 h with UA8967, centrifuged, and 100 μl of the cell culture supernatant transferred to a clean 96-well plate. The LDH detection reagent was added and incubated for 15 min in the dark, and absorbance read at 490 nm (reference filter 600 nm). LDH leakage (% cytotoxicity) was

calculated as follows: $((\text{OD sample} - \text{OD background}) / (\text{OD cells treated with 1 \% Triton}^{\text{TM}} \text{ X-100} - \text{OD background})) \times 100$.

Measurement of Mitochondrial Membrane Potential (MMP)

MMP was measured in cells treated with 0–100 μM of UA8967 for 24 h. The cells were stained with 10 $\mu\text{g/ml}$ of JC-1 dye (Invitrogen) for 10 min at 37 $^{\circ}\text{C}$, and washed thoroughly with PBS before analyzing by Flow cytometry on a Becton Dickinson FACScan (San Jose, CA) using Cell-Quest Pro[®] software (BD BioSciences, San Jose, CA).

Immunofluorescent analysis

Cells were immobilized and fixed in 4 % paraformaldehyde, washed in DPBS, and permeabilized in DPBS containing 0.1 % gelatin and 0.1 % TritonTM X-100 for 30 min. Samples were stained with anti-LC3 and Alexa Fluor 488 goat antirabbit (Molecular Probes, Carlsbad, CA). Confocal images were collected using a Nikon PCM 2000[®] inverted confocal microscope. Cells incubated for 4 h in Earle's Balanced Salts Solution (EBSS, Invitrogen) served as a positive control.

Subcellular fractionation and western blot

Cells treated with 0, 25, 50 or 100 μM of UA8967 for 24 h were fractionated to enrich the cytosolic, mitochondrial and insoluble portions of the cell. At the indicated time point, cells were centrifuged at 1,000 rpm and washed in ice cold DPBS. Cells were resuspended in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 0.01 % (w/v) digitonin, 2 mM sodium orthovanadate, 10 $\mu\text{g/ml}$ cytochalasin D and protease inhibitors) and incubated on ice for 30 min with vortexing every 15 min. Cell lysates were then centrifuged at 1,000 $\times$ g for 5 min and the supernatant collected as the cytosolic fraction. The pellet was resuspended in mitochondrial extraction buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl_2 , 2 mM EGTA, 2 mM EDTA, 1 % (v/v) NP-40, 10 % (v/v) glycerol) and centrifuged at 10,000 $\times$ g for 10 min. The supernatant was collected as the mitochondrial fraction and the resulting insoluble pellet was resuspended in RIPA Buffer (30 mM Hepes, pH 7.5, 10 mM NaCl, 5 mM MgCl_2 , 25 mM sodium fluoride, 1 mM EGTA, 1 % TritonTM X-100, 10 % glycerol, and protease inhibitors) and was considered the nuclear/insoluble fraction. Equivalent lane loading and confirmation of each fraction was validated by immunoblotting for cytoplasmic fractions (β -actin, Sigma Chemical Co.) or mitochondrial fractions (Mn-SOD, Stress-Gen, San Diego, CA). Extracts were separated by SDS-PAGE for immunoblotting and analysis.

Results

The chemical screening libraries were selected through the application of TRAP, an approach for lead identification that enables an efficient alternative to high-throughput screening [8]. A total of 275 discrete compounds in the Telik library were evaluated for cytotoxicity in 3 sequential tranches using the SRB assays in the paired isogenic (DPC4(+/-) and DPC4(-/-)) HCT-116 cell lines. The three sequential sets of compounds were provided by Telik using the TRAP chemical-space mapping technique, which informs compound selection for the next set based on results from the prior set. The DPC (-/-) selectivity factors for the library of compounds examined ranged from <2-fold to approximately 5-fold for UA8967, and overall growth inhibitory potencies (IC_{50}) ranged from 2.2 μM . to >100 μM . On the basis of the approximately 5-fold greater cytotoxicity for DPC4(-/-) HCT-116 cells, UA8967 was selected from the third tranche of Telik-supplied compounds for further studies of potential mechanisms of action in pancreatic and colon cancer cell lines. The structure of UA8967 is comprised of an indole nucleus to which an N-benzyl-piperazinylmethyl substituent is attached at the 3-position (Fig. 1).

Cytotoxicity profile of UA8967

We examined the cytotoxicity profile of UA8967 in six pancreatic carcinoma cell lines, one normal pancreatic ductal epithelial line, and two colon carcinoma lines, isogenic except for DPC4 status. Wild type DPC4 (+/+) cell lines included the MiaPaca-2, Panc-1, AsPC1 and T3M4. The cell lines with spontaneous DPC4 loss of function mutations were BxPC3 and CF-PAC1 (by homozygous gene deletion). We also utilized a colon carcinoma cell line, HCT-116, with wild type DPC4 expression (HCT-116 (+/+)), and its' isogenic clone in which the DPC4 gene was deleted by homologous recombination (HCT-116 (-/-)) [12].

The IC₅₀s ranged from 12 to 61 μ M in the pancreatic and colon cancer cell lines for exposure times of 72 h (Table 1). Analysis of the schedule dependence (exposure time $\times$ dose integrated AUC's to achieve 50 % cell kill) showed that there was no advantage for prolonged exposures (Supplemental Table 1). This analysis assumes that the drug remains stable in culture conditions for the entire duration of the exposure, however drug stability in culture medium was not measured. The lack of schedule dependence suggests that cytotoxicity by UA8967 is not dependent upon a particular phase of cell division, to be effected by the drug. A comparison between IC₅₀ and DPC4 status shows that the DPC4 deleted HCT-116 (-/-) colon carcinoma line was 4.75-fold more sensitive to UA8967. However, there was no significant difference in the IC₅₀ for the two pancreatic cell lines with spontaneous loss of DPC4 function (CF-PAC1 and BxPC3) compared to the other four DPC4(+/+) pancreatic cell lines (Table 1). The mean (SEM) IC₅₀ for UA8967 in the HPDE-6 normal immortalized pancreas epithelial cell line, (41.66 (10.5) μ M), was similar to that of the mean IC₅₀ in the 6 pancreatic cancer cell lines (44.9 μ M, Table 1). This shows that UA8967 has equal potency for cancer cells and normal immortalized pancreatic epithelial cells. Therefore, cytotoxicity from UA8967 may be dependent on proliferation rates, rather than the malignant transformation process, since both cell types have similar doubling times.

UA8967 does not inhibit macromolecule synthesis

To determine if UA8967 has selective inhibitory effects on RNA, DNA or protein synthesis, we used standard macromolecular precursor incorporation assays. These studies showed that there was no selective inhibition of DNA, RNA or protein synthesis after 24, 48 or 72 h exposure to the drug in two pancreatic cancer cell lines. For example, the IC₅₀ values for inhibiting RNA, DNA and protein synthesis for a 72 h drug exposure in the MiaPaca-2 cell line were 40 μ M, 40 μ M and 28 μ M, respectively. For comparison, the IC₅₀ for growth inhibition in the MTT assay was 37 μ M for the same drug exposure time (Table 1). This shows that the syntheses of all three macromolecules were similarly affected, and therefore, no single macromolecular synthesis pathway is identified as being in the critical pathway for mediating cell growth inhibition by UA8967.

Cell cycle effects of UA8967

Cell cycle analyses of HCT-116 (+/+) and HCT-116 (-/-) cells treated with 0 to 50 μ M of UA8967 for 24–48 h showed a small accumulation of cells in G₀/G₁ and a slight reduction in cells in S-phase (Fig. 2a, b, d, e). This pattern continued at 72 h in the HCT-116 (-/-) cells (Fig. 2f), but was absent in the HCT-116 (+/+) cells (Fig. 2c). The reduction in the percent of cells in S-phase was more pronounced and dose-dependent in the HCT-116 (-/-) cell line which lacks DPC4, compared to the HCT-116 (+/+) cell line.

UA8967 induces non-apoptotic cell death

When exposed to up to 100 μ M of UA8967 for 24 h, we saw less than 20 % cell death by either necrosis or apoptosis in both HCT-116 DPC4(+/+) and HCT-116 DPC4(-/-) cells

(Fig. 3). We also added caspase inhibitors 30 min prior to exposing these cells to UA8967. As shown in Fig. 3, the addition of the pan-caspase inhibitor (Q-VD-OPh), or the caspase 3 inhibitor (Q-DEVD-OPh), had no effect on apoptotic cell death in either cell line. We also explored whether the addition of these caspase inhibitors would alter the cytotoxicity of UA8967, as measured by MTT at 72 h, but again found no significant inhibition (Supplemental Fig 1). Western blot analyses confirmed no activation of caspases 3 or 6 in the MiaPaCa-2 pancreatic cell line treated with UA8967 for 24 h (data not shown).

Because we did not see caspase activation in UA8967 treated cells, we investigated an alternative, caspase-independent mode of death or survival response known as autophagy. Cells were incubated with 50 μ M UA8967 for 0 to 24 h, and analyzed for the autophagic vesicle marker, LC3. When autophagy is induced, LC3 is cleaved and conjugated to phosphatidyl ethanolamine for insertion into the autophagosomal membrane [15]. Cleaved LC3 increased in a dose- and time dependent fashion in all four cell lines examined, when exposed to 50 μ M of UA8967. Even as early as 1 h after exposure, there is a notable increase in cleaved LC3 (LC3II—14 kDa), which continues to increase up to 24 h (Fig. 4a). Of note, the increase in cleaved LC3 was more prominent in the HCT-116 (–/–) cell line (Fig. 4a).

Immunohistochemistry studies of autophagy markers demonstrates diffuse expression of LC3 in untreated MiaPaca-2 cells while the MiaPaCa-2 cells treated with 100 μ M of UA8967 accumulated large, punctuate structures characteristic of autophagosomes (Fig. 4b, top right panel). Control cells incubated in EBSS, which induces autophagy by amino acid deprivation, showed similar effects of autophagy (Fig. 4b, bottom left panel).

Effects of UA8967 on membrane integrity

Because of the relatively non-specific mechanistic effects of UA8967 on macromolecular synthesis and the lack of any schedule-dependency, we investigated whether UA8967 might be damaging the plasma membrane, as we had observed for a bisphosphonate-based cytotoxic compound Apomine™, which similarly did not activate caspase 3 in melanoma cells [16].

First, LDH leakage was compared in the HCT-116 DPC4 (+/+) and HCT-116 DPC4 (–/–) cells after 24 h exposure to UA8967. There was a dose-dependent increase in LDH leakage in the DPC4 (–/–) cell line, whereas the parental DPC4 (+/+) cell line showed minimal LDH leakage (Fig. 5a). To ensure that the LDH release observed was not due to the DMSO vehicle, an agent known to affect membrane permeability [17], we also measured LDH release with equivalent DMSO concentrations and no drug (Fig. 5b). These results confirm that DMSO is not contributing to the LDH release observed with UA8967. When we used sub IC₅₀ doses of UA8967 for an extended period of time (72 h), we did not see any dose dependent increase in LDH release, however, we did note that the DPC4 (–/–) cells showed slightly higher basal LDH leakage compared to their DPC4 (+/+) counterparts (Fig. 5c).

We also used trypan blue dye exclusion and Sytox® Green nucleic acid stain to assess plasma membrane integrity. UA8967 caused a concentration-dependent increase in trypan blue dye uptake, an increase in nucleic acid staining by Sytox® Green, and elevated LDH leakage into the culture media (Fig. 6). The two DPC4 (–/–) cell lines, BxPC3 and HCT-116 DPC4 (–/–), were clearly more sensitive to the membrane effects of UA8967 (Fig. 6b and d, respectively).

To determine if the mitochondrial membrane of DPC4 (–/–) cells is affected by UA8967, we measured fluorescence of the cationic dye JC-1 [18, 19]. On initial uptake, JC-1 accumulates in intact mitochondria forming J-aggregates that fluoresce at 590 nm (red). When the

mitochondrial membrane undergoes a permeability transition, JC-1 leaks into the cytoplasm wherein the monomeric form is detected at 529 nm (green). Thus, a decrease in the ratio of red:green fluorescence indicates a loss of mitochondrial membrane potential (MMP). This red:green ratio is dependent solely on membrane potential, and is not affected by other factors such as mitochondrial size or overall dye uptake [18]. The data shows that 20-30 % of the cells had a loss of MMP at the highest drug concentration for the 2 pancreas cancer cell lines (Fig. 7a, b). In contrast, the HCT-116(−/−) cell line exhibits a marked and dose-dependent loss of MMP following exposure to UA8967. At the highest drug concentration, 80 % of these cells exhibit a loss of MMP (Fig. 7d).

Because of the increased mitochondrial membrane permeability noted in the DPC4(−/−) cells, we used subcellular fractionation and western blot analysis to investigate the release of apoptogenic factors. Cytochrome C release from the inner mitochondrial membrane space is a well established feature of caspase dependent apoptosis, while the release of apoptosis inducing factor (AIF) has been shown to contribute to one form of caspase-independent apoptosis [20]. Nuclear, mitochondrial and cytoplasmic fractions were isolated using selective lysis and differential centrifugation. Western blot analysis demonstrated no substantial release of either cytochrome C or AIF in any of the cell lines following incubation with cytotoxic concentrations of UA8967 (Fig. 8). β -actin and Mn-SOD were used to validate the purity of the fractions. Of note, there was some mitochondrial leakage (increased Mn-SOD) observed in the cytoplasmic samples of the HCT-116 DPC4 (−/−) cells following exposure to 50 and 100 μ M UA8967. That may explain the faint cytochrome C bands observed in the cytoplasmic fractions (Fig. 8d).

Discussion

Current strategies for drug development in cancer often rely on discovering compounds selectively targeted to growth signaling pathways up-regulated by gain of function mutations in an oncogenic pathway, such as imatinib for the *bcr/Abi* mutation in CML [21, 22]. Conversely, loss of function mutations in a tumor suppressor pathway such as *p53* or an anti-apoptotic pathway such as with the MDM2 oncoprotein [23] also represent opportunities for therapeutic intervention, as in the current case with UA8967 in DPC4(−/−) cells. However, the recently reported set of 12 core cellular signaling pathways and 541 differentially expressed genes in pancreatic cancer [1], suggests that no singly-targeted agent is likely to provide comprehensive inhibition of these different oncogenic pathways and genes. Therefore, drugs with broad inhibitory activities are needed.

The compound identified in the current study, UA8967, was very efficiently identified from a chemical diversity library as being selectively active in pancreatic and colon cancer cell lines with the DPC4 (*SMAD4*) deletion. Only 3 sequential iterations involving <300 total compounds were required to identify UA8967 using the TRAP technology. By comparison, a prior random library search for agents active in DPC4(−/−) Panc-1 cells required high throughput screening of 16,320 compounds to identify 11 compounds with 5–12 fold increased potency [24]. In contrast to random screening, the Telik TRAP-based analog selection system adjusts each sequential library based on physico-chemical properties of analog “hits” in the preceding iteration. Of note, the selection criteria in the current study was based on cytotoxicity and not a particular molecular mechanism. Thus, despite using selection criteria based on DPC4 deletion, the cytotoxic action of UA8967 may not involve a direct interaction with known elements of the *TGF-beta/SMAD4* signaling pathway. Rather, it appears to involve autophagy and a disruption of the integrity of the plasma membrane. This membrane effect, accompanied by an early autophagic response, eventually leads to cell lysis via a caspase-independent, mostly non-apoptotic form of cell death. It is not known whether the autophagy response comprises a cell death mechanism, or a survival response to

the drug. Importantly, the increased membrane instability with DPC4(–/–) cells appears to be specific to UA8967, since the cells were not more sensitive to cytotoxicity induced by high concentrations of DMSO (Fig. 5b). A similar pattern involving membrane lysis and indiscriminant effects on macromolecular synthesis was noted by us in a prior study of the mechanism of action of a purported purely-apoptotic bisphosphonate compound, Apomine™, in human melanoma cells [16].

The slow accumulation of cells in G₀/G₁-phase and the slow onset of both apoptosis and membrane instability suggest that UA8967 is not acting as a classical detergent, but rather that there is an active process going on which ultimately leads to compromised plasma membrane integrity. Overall, the results suggest a cytotoxic action of UA8967 involving cytoplasmic membrane disruption. Our observation that UA8967 induces mitochondrial membrane permeability but does not activate caspase 3 is unusual. Furthermore, AIF, which functions independent of caspases as both an NADH oxidase as well as an executioner of apoptosis [25] was also not involved in mediating UA8967 cytotoxicity. Thus, we cannot definitively determine if caspase activity is required for UA8967-induced cytotoxicity, but the negative caspase inhibitor studies suggest that caspase activation is not required.

We did not find schedule dependence with UA8967, which suggests cytotoxicity is not cell cycle dependent. However, this analysis assumes that the drug remains stable in culture conditions for the entire duration of the exposure. And, because dialkyl aminomethylindoles of this type may be prone to fragmentation resulting in an electrophilic indolinium ion, instability of UA8967 in cell culture conditions may be a consideration.

With the knowledge gained from our *in vitro* studies on the effect of UA8967 on membrane integrity and cell cycle, our next step is to examine whether UA8967 is active in mice bearing human cancer DPC4(–/–) xenografts.

In conclusion, we show that UA8967 induces early markers of autophagy and to a lesser extent, apoptosis, yet caspase activation was not seen. Furthermore, adding a caspase 3 inhibitor or a pan-caspase inhibitor did not affect cytotoxicity or LDH release. This suggests that cell death from UA8967 is occurring through a non-typical, largely non-apoptotic mechanism. The findings show that in a search for agents with specificity for a common genetic deletion in human gastrointestinal cancers, we have identified a compound which induces a loss of membrane integrity in gastrointestinal cancer cells that have lost the DPC4 tumor suppressor gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Structure of UA8967, 3-[(1-benzylpiperazin-4-yl)methyl]-1H-indole, MW=305.42, Molecular formula $C_{20}H_{23}N_3$, Maybridge MFCD 00396990. This compound has an indole nucleus with a benzylpiperazine side chain

Fig. 2.

UA8967 causes cell cycle accumulation in G₀/G₁ and consequent loss in S phase. HCT-116 DPC4(+/+) and HCT-116 DPC4(-/-) cells exposed to 0–50 μM of UA8967 for 24 h or 48 h **a-d** or to 0–10 μM of UA8967 for 72 h **e-f**, then stained for DNA content using PI and analyzed by flow cytometry. Results shown are the mean±SEM (*n*=3)

Fig. 3.

Caspase inhibitors do not block UA8967-induced cell death. **a** HCT-116 DPC4(+/+) and **b** HCT-116 DPC4(-/-) cells were exposed to 0–100 μ M of UA8967 for 24 h alone or after a 30 min pre-incubation with a caspase 3 inhibitor (Q-DEVD-Oph) or a pan-caspase inhibitor (Q-VD-Oph). Cells were stained for AnnexinV/PI, then analyzed by flow cytometry. Results shown are the mean $\pm$ SEM ($n=3$)

Fig. 4.

Markers of the autophagic stress response. **a** MiaPaCa-2, BxPC3, HCT-116 DPC4(+/+), or HCT-116 DPC4(-/-) cells were incubated with 50 μ M UA8967 for up to 24 h and LC3 processing examined by Western blot. Autophagic vesicle formation requires the cleavage and lipidation of the 16 kDa protein, LC3I, to produce the 14 kDa LC3II. **b** MiaPaCa-2 cells were incubated in complete media with either 0.01 % DMSO (vehicle control), 100 μ M UA8967, or in EBSS for 4 h, fixed in paraformaldehyde, and stained with anti-LC3 and goat anti-rabbit conjugated to Alexa-488. This LC3 antibody only recognizes the smaller 14KDa LC3II form. EBSS incubation induces amino acid starvation and serves as a positive control for autophagic vesicle formation

Fig. 5.

Plasma membrane integrity is compromised in DPC4(-/-) cells. HCT-116 DPC4(+/+) and HCT-116 DPC4(-/-) cells were exposed to increasing concentrations of UA8967 for **a** 24 h, 0–250 μ M or **c** 72 h, 0–20 μ M and LDH leakage into the supernatant was measured. **b** An equivalent amount of DMSO vehicle used to prepare UA8967 was also tested at 24 h. Results shown are the mean $\pm$ SD ($n=2$) for 24 h, mean $\pm$ SEM ($n=3$) for 72 h

Fig 6. Correlation between cell death and plasma membrane integrity. **a** MiaPaCa-2, **b** BxPC3, **c** HCT-116(+/+), and **d** HCT-116(-/-) cells were exposed to increasing concentrations of UA8967 for 24 h. Cell viability was measured using trypan blue exclusion, and membrane integrity was measured using Sytox® green and LDH leakage assays

Fig. 7. Analysis of mitochondrial membrane potential by JC-1. **a** MiaPaCa-2, **b** BxPC3, **c** HCT-116 DPC4(+/+), and **d** HCT-116 DPC4(-/-) cells were incubated with 0–100 μ M of UA8967 for 24 h followed by staining with the cationic dye JC-1, and analyzed by flow cytometry. Cells with loss of MMP are detected by fluorescence at 529 nm (green). Data shown is the mean percent of total cells with green fluorescence (mean $\pm$ SEM, $n=3$)

Fig. 8. Subcellular fractionation of **a** MiaPaCa-2, **b** BxPC3, **c** HCT-116 DPC4(+/+), and **d** HCT-116 DPC4(-/-) cells treated with increasing doses (0, 25, 50, and 100 μ M) of UA8967 for 24 h. Cells were harvested and lysates separated into either the cytosolic or mitochondrial fraction by selective lysis and differential centrifugation. Western blot was used to examine whether AIF (55, 67 kDa) or cytochrome C (14 kDa) would translocate from the mitochondria due to UA8967 exposure. β -actin (42 kDa) and Mn-SOD (25 kDa) were examined to assess the purity of each subcellular fraction

Table 1

Cytotoxicity of UA8967

Cell line	DPC4 status	72 h IC ₅₀ , pM (Mean±SEM)
MiaPaca-2	+	36.81 ±3.33
Panc-1	+	51.24±3.31
AsPC1	+	51.51 ±5.05
T3M4	+	24.13±0.98
CF-PAC1	–	61.20±3.74
BxPC3	–	50.72±3.28
HCT-116 (+/+)	+	57.50±10.00
HCT-116 (–/–)	–	12.10±2.80
HPDE-6	+	41.66±10.50