Massive induction of apoptosis of multicellular tumor spheroids by a novel compound with a calmodulin inhibitor-like mechanism

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Abstract

Background: A number of anticancer drug candidates have been identified by cell-based screens utilizing tumor cells grown in monolayer culture. Since such cultures are poor models of 3-D tumor micro-environments, we here aimed to identify novel compounds showing anti-proliferative activity on multicellular spheroids.

Methods: A chemical library was used to screen for compounds capable of reducing viability and inducing apoptosis of colon carcinoma cells grown as multicellular spheroids. Assessment of possible mechanism of action was performed using gene expression profiling.

Results: The screen identified NSC647889 as a potent apoptotic compound. NSC647889 induced dramatic increases in tumor cell apoptosis in multicellular spheroids compared to standard antineoplastic agents. Interestingly, quiescent cells in spheroid cores were resistant to NSC647889-induced apoptosis and appeared to die by other mechanisms. The cellular phenotypic response to NSC647889 was similar to that of known calmodulin inhibitors and the compound stimulated rapid increases of intracellular calcium levels. NSC647889 was, however, not a direct inhibitor of calmodulin-dependent calcineurin activity. Finally, NSC647889 induced tumor apoptosis in a xenograft tumor model.

Conclusions: Novel drugs that show considerably stronger cytotoxic activity in 3-D culture compared to standard agents can be identified. Further development of such drugs may be a useful strategy for improved treatment of solid tumors.

Keywords: Multicellular tumor spheroids, apoptosis, calcium signaling, cmap

Introduction

It is well known that a significant discrepancy exists between the efficacy of anticancer drugs *in vitro* and *in vivo* [1]. This discrepancy could, in part, be due to the three dimensional (3-D) nature of tumors as compared to the two dimensional (2-D) nature of standard cell culture systems. To be effective *in vivo*, anticancer drugs must penetrate into tumor cell masses to reach cancer cells at toxic concentrations. Experiments using multicellular *in vitro* models have shown that the tissue is a barrier to the distribution of many anticancer agents [2-4]. Furthermore, tumor cells exist in an organized 3-D matrix, where nutrients and oxygen will not be easily accessible to all cells. Differences in proliferation, gene expression pattern, cell surface receptor expression and extracellular matrix synthesis of cells are grown in 2-D or 3-D culture have indeed been reported [5-7]. An outstanding question is

which strategy to use to achieve optimal anti-proliferative effect on hypoxic and nutrition limited cells present in solid tumors. An increased understanding is expected to result in the development of drugs with an improved therapeutic efficacy.

Multicellular spheroids (MCS) and other 3-D culture systems mimic human solid tumors better than 2-D monolayer cultures [8-11]. MCS are known to be more resistant to drug effects compared to monolayer cultures [12-15] and many clinically used drugs show limited potency on spheroids [1,16,17]. Therefore, MCS are better suited than monolayer cultures for drugs screening projects aimed to identify compounds active on solid tumors. We have established a screening procedure with good assay performance for the identification of drugs that induce apoptosis of tumor cells grown in MCS [18]. We here describe a novel small molecule (NSC647889)

capable of inducing an unprecedented extent of apoptosis in MCS. Interestingly, however, the novel drug did not induce apoptosis in the core areas of MCS, suggesting that cells in hypoxic and nutrient limited areas are resistant to apoptosis. The gene expression profile induced by this drug suggested a molecular mechanism of action similar to that of calmodulin inhibitors. The result of our study show that novel drugs can be identified which show considerably stronger apoptotic activity on tumor cells grown in 3-D culture compared to standard agents.

Materials and methods

Compound library

The Mechanistic set was obtained from the Developmental Therapeutics Program of the US National Cancer Institute (<http://www.dtp.nci.nih.gov>). All compounds were dissolved in DMSO. A final concentration of 0.5% DMSO was reached in cell cultures; control wells received solvent only.

Cell culture, generation of spheroids and screening

HCT116 colon carcinoma cells were maintained in McCoy's 5A modified medium/10% fetal calf serum at 37°C in 5% CO₂. Spheroids were prepared as described previously [19]. Briefly, a cell suspension containing 10,000 cells was added to each well of poly-HEMA-coated 96-well plates. Wells were then filled by adding culture medium to acquire a convex surface. Plates were inverted to allow the cells to sediment to the liquid–air interface. After 24 h incubation on a rotatory shaker, plates were returned to normal and excess medium was removed. Plates were then incubated for a further 4 days. This procedure was both for screening and all other experiments. At the day of treatment, the medium was removed and 200 µL fresh medium was added per well. After 24 hours of drug treatment, NP40 was added to the culture medium to 0.1% in order to extract caspase-cleaved K18 (cck18) from spheroids and to include material released to the medium from dead cells. cck18 was determined using 25 µL medium/extract using the M30 Apoptosense® ELISA [20] (www.peviva.com, VLVbio AB, Sundbyberg, Sweden). Signals were factorised to percent of a staurosporine reference (quadruple wells used on each plate at 1 µM). Viability measurements were performed using the acid phosphatase (APH) method described by Friedrich et al., [21]. Background activity was subtracted.

Immunological assays

Spheroids produced by the hanging drop method in 96 well plates were fixed in paraformaldehyde, dehydrated, embedded in paraffin and sectioned. Each sample contained 32 spheroids (spheroids from each 96 well plate were pooled into 3 groups). The sections were deparaffinized with xylene, rehydrated and microwaved and then incubated over-night with the monoclonal primary antibodies diluted in 1% (wt/vol) bovine serum albumin and visualized by standard avidin–biotin–peroxidase complex technique (Vector

Laboratories, Burlingame, CA, USA). Counterstaining was performed with Mayer's haematoxylin. Antibody against active caspase-3 was from Pharmingen and used at 1:50, Antibody to Ki67 was from Dako (Glostrup, Denmark) and to p27/Kip1 from BD Pharmingen (San Jose, CA). Hypoxic regions were detected after incubation of MCS with 100 mM of pimonidazole hydrochloride. Sectioned spheroids were incubated with Hypoxyprobe-1 (HPI Inc., Burlington, MA, USA) to detect pimonidazole adducts. Serial sectioning was not required since the hanging drop procedure generates spheroids of homogenous size [19].

Connectivity map

The Connectivity Map (Cmap) (www.broad.mit.edu/cmap) build 02 contains genome-wide expression data for 1300 compounds (6100 instances, including replicates, different doses and cell lines). We followed the original protocol using MCF-7 breast cancer cells as described by Lamb et al., [22]. Briefly, cells were plated in 6-well plates at a density of 0.4 x 10⁶ cells per well. Cells were left to attach for 24 h, followed by exposure to NSC647889 at a final concentration of 10 µM, or to vehicle control (DMSO). After 6 h treatment, the cells were washed with PBS and total RNA was prepared using RNeasy miniprep kit (Qiagen, Chatsworth, CA). Starting from two micrograms of total RNA, gene expression analysis was performed using Genome U133 Plus 2.0 Arrays according to the GeneChip Expression Analysis Technical Manual (Rev. 5, Affymetrix Inc., Santa Clara, CA). Raw data was normalized with MAS5 (Affymetrix) and gene expression ratios for drug treated vs. vehicle control cells were calculated to generate lists of regulated genes. Only probes present on HG U133A were used, for Cmap compatibility. The 40 most up and down regulated genes (i.e., probes) for each compound were uploaded into the CMAP and compared to the 6,100 instances in the Cmap database, to retrieve a ranked compound list. Raw and normalized data will be made available at Gene Expression Omnibus.

Calcineurin activity assay

HCT116 cells were exposed to NSC647889 (10 µM) or DMSO vehicle control for 1 hour. W7-treated cells (10 µM) were used as a positive control. Phosphatase assay was performed according to the manufacturer's instructions of the calcineurin cellular activity assay kit (Calbiochem, Merck4Biosciences). Cells were lysed using cold lysis buffer (50 mM Tris, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 0.2% NP-40, pH 7.5) freshly supplemented with protease inhibitor from the kit. Lysates were cleared by centrifugation at 15,000 rpm for 30 mins at 4°C and the protein concentrations were measured by the Bradford assay (Bio-Rad, Richmond, CA, USA). The RII phosphopeptide substrate (Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-pSer-Val-Ala-Ala-Glu) was activated with calmodulin in the assay buffer (200 mM NaCl, 100 mM Tris, 12 mM MgCl₂, 1 mM CaCl₂, 1 mM DTT, 0.05% NP-40, pH 7.5) for 10 min at 30°C. Supernatants

were added to the reaction mixture and incubated for 30 mins at 30°C. EGTA in the assay buffer was used to distinguish between calcineurin and other phosphatases as recommended by the kit manufacturer. The enzyme activity was measured as phosphatase released per mg of protein from the substrate.

Calcium uptake measurement

A Fluo-4 Direct Calcium Assay Kit (Molecular Probes) was used to measure the uptake of extracellular calcium ions. HCT116 cells were treated with 10 μ M NSC647889, 10 μ M W7 or DMSO (vehicle control) for 1 hour after which the cells were loaded with the Fluo-4 direct calcium assay reagent supplemented with probenecid, following the manufacturer's instruction and incubated for 60 seconds. The fluorescence was measured at excitation and emission of 465 nm and 535 nm respectively in a Tecan multiplate reader.

Treatment of mouse xenografts and determination of tumor-derived K18 in mouse plasma

A FaDu head-neck carcinoma model (p53mut) was used for testing of *in vivo* antitumor activity. This model has previously been validated to be useful for determining human tumor cell apoptosis in SCID mice using blood samples [23]. FaDu cells express high levels of K18 which is released to the extracellular compartment and into the blood from dying cells [23]. When FaDu tumors had grown to a size of approximately 400 mm³, mice were subcutaneously injected with NSC647889 dissolved in DMSO (final dose 5 mg/kg). Mice were sacrificed 48 hours after injection of drug and EDTA-plasma was collected. Caspase-cleaved K18 (K18-Asp396) and total K18 were measured in 12.5 μ L plasma using the Peviva M30-Apoptosense and M65 ELISA assays (www.peviva.com, VLVbio AB, Sundbyberg, Sweden) as described [23]. Each sample was mixed with 0.4 mL of heterophilic blocking reagent (HBR-Plus purified, part#3KC579; Scantibodies laboratory Inc, Santee CA, USA). Animal experiments were conducted in full accordance with Swedish governmental statutory regulations on animal welfare under permission from ethical committees.

Results

Screening multicellular tumor spheroids for apoptosis-inducing compounds identified the compound NSC647889

Five days after seeding HCT116 colon carcinoma cells in 96 well plates for formation of multicellular spheroids (MCS), spheroids showed a compact morphology and a diameter of ~ 500 μ m (Figure 1A). MCS contained a hypoxic core consisting of non-proliferating cells (Figure 1A). The response of HCT116 MCS to three anticancer agents, cisplatin, melphalan and vinblastine is shown in (Figure 1B). These drugs elicited only limited activation of caspase-3 at the spheroid periphery, consistent with previous reports [24,25].

Note, however, that DNA damaging drugs such as cisplatin induce complex cellular responses (i.e., both apoptosis and senescence [26,27]). Therefore, the ability of cisplatin to reduce clonogenicity of MCS was larger than expected from the limited induction of apoptosis (see Figure 2C below; discussed in [25]).

HCT116 MCS were used to screen the NCI Mechanistic Set (827 compounds) for compounds that induce apoptosis. This library has been preselected for compounds with antiproliferative activity in the NCI60 cell line screen. Apoptosis was assessed as generation of an endogenous apoptosis product, caspase-cleaved keratin 18 (cck18) [18]. In a secondary screen, 29 compounds were tested both for their ability to induce apoptosis and to induce loss of cell viability (Figure 1C). The most active compound found in the secondary screen was NSC647889 (N-(17b-dimethylamino-3b-androstanyl)-N'-[2-phenyl-1-(2-thiazolyl)ethyl]urea). Compounds with scaffolds similar to NSC647889 have not previously been described to induce apoptosis of tumor cells. NSC647889 has a molecular weight of 548.8 Dalton (Figure 1D).

NSC647889 is strongly apoptotic to spheroids

Dose response determination of induction of apoptosis and loss of cell viability are shown in (Figures 2A and 2B). An ~ 2.5-fold difference was observed in the potency of inducing loss of cell viability between monolayer and spheroid cultures (Figure 2B). The clonogenicity of cells dispersed from MCS exposed to 10 μ M NSC647889 was < 5% of that of cells dispersed from solvent-treated MCS (Figure 2C).

The ability of NSC647889 to induce apoptosis in MCS was examined by sectioning spheroids exposed to a concentration of 10 μ M, followed by staining for active caspase-3 (Figure 3). At 6 h of exposure to NSC647889, increased apoptosis was observed of cell populations 0-100 μ m from the spheroid surface. Interestingly, apoptosis was not observed at deeper layers at later time points (8 and 10 h). At even later time-points, MCS disintegrated and could not be sectioned. We conclude that 10 μ M NSC647889 induced an apoptotic response in ~80% of the cells in HCT116 spheroids (calculated on volume and assuming the same cell volume in different layers) whereas clonogenicity was reduced to ~1% (Figure 2C).

NSC647889 induces a phenotypic response similar to that of calmodulin inhibitors

To generate hypotheses regarding the molecular mechanisms of action of NSC647889, we used the Connectivity Map (Cmap) [22], a large compendium of gene expression signatures from drug-treated tumor cell lines. The gene expression response to NSC647889 was most similar to that of the calmodulin antagonist calmidazolium (Figure 4A). Furthermore, the expression profile was also similar to those of a number of other compounds reported to be

Figure 1. Identification of NSC647889 by spheroid screening. (A). Sections of HCT116 multicellular spheroids (MCS) 5 days after seeding of cells in hanging drops. Sectioned MCS were stained by hematoxylin, for pimonidazole adducts and for Ki67 and p27^{Kip1} as indicated. (B). HCT116 MCS are resistant to standard chemotherapeutic drugs. MCS were exposed to 10 μ M cisplatin, 1 μ M vinblastine or 10 μ M melphalan for 24 or 48 h, sectioned and stained by an antibody to active caspase-3. (C). Identification of NSC647889 using spheroid screening. A selected subset of the NCI Mechanistic Set was used to treat HCT116 colon carcinoma cells at 5 μ M of each compound. Viability and apoptosis induction was assessed for each compound at 24 h. Viability was determined using the acid phosphatase [21] (plotted right to left) and M30 Apoptosense® methods [45]. (D). Structure of NSC647889 (N-(17 β -dimethylamino-3 β androstanyl)-N'-[2-phenyl-1-(2-thiazolyl)ethyl]urea). Lower structure derived using Marvin Sketch for Mac. The compound has a molecular weight of 548.8 Daltons, a XLogP of 7.3, 6 rotatable bonds and a topological surface area of 85.5 Å².

calmodulin antagonist such as phenoxybenzamine [28] and trifluoperazine [29,30], thioridazine [31] and prochlorperazine [32].

We determined Ca²⁺/calmodulin-dependent calcineurin (Ser/Thr protein phosphatase 2B (PP-2B)) activity in cells exposed to NSC647889. The assay was based on the phosphopeptide substrate Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-(phospho)Ser-Val-Ala-Ala-Glu. To distinguish calcineurin from other phosphatases recognizing the same substrates, assays were performed in the presence and absence of EGTA. In contrast to the calmodulin antagonist W7, and contrary to our working

hypothesis, NSC647889 increased calcineurin activity in HCT116 cells (Figure 4B).

NSC647889 increases intracellular calcium concentrations

The increased activity of calcineurin in NSC647889-exposed cells was hypothesized to be due to increases in cellular calcium. Cellular Ca²⁺ overload is known to trigger apoptotic cell death [33], potentially explaining the mechanism of cytotoxic activity. We determined intracellular calcium (Ca²⁺) using the probe Fluo-4. Increased concentration of Ca²⁺ were indeed observed after 1 h of drug exposure (Figure 5). The

dose-response relationships of the increases in $[Ca^{2+}]_i$ and induction of cell death/apoptosis were similar (Figures 2 and 5).

Assessment of *in vivo* apoptotic activity

We were interested in determining whether NSC647889 shows antitumor activity. The drug has a low solubility in aqueous solution ($XLogP = 7.3$) and could not be formulated for repeated injections. We therefore used NSC647889 dissolved in DMSO and injected the drug s.c. (5 mg/kg). This approach is not feasible for performing standard tumor growth inhibition experiments that require repeated drug injections. Since NSC647889 elicits generation of ccK18 *in vitro* (Figures 1 and 2), we examined whether the drug induces increased levels of circulating ccK18 in SCID mice with FaDu head-neck carcinoma xenografts. FaDu xenografts were chosen (instead of HCT116) due to previous findings of this model releasing large amounts of K18 after treatment with cytotoxic drugs [23]. The relative level of ccK18 in relation

to total K18 reflects cell death mode (apoptosis/necrosis) [34], and we also determined total plasma K18 [34,35]. The specificity of the antibodies to human K18 makes this a sensitive method for determination tumor apoptosis/cell death independently of host toxicity in mouse xenograft models [23]. Statistically significant increases in both ccK18 and total K18 were observed 48 hours after injection of NSC647889 (Figure 6), showing induction of tumor apoptosis and providing proof-of-principle for antitumor activity.

Discussion

Screening of the NCI Mechanistic Set identified the compound NSC647889 to be strongly apoptotic to HCT116 colon carcinoma multicellular spheroids. Apoptosis induction in spheroids was dramatic compared to that observed using standard compounds, and the strongest spheroid activity encountered by our laboratory. The scaffold of NSC647889 has to our knowledge not been previously reported to show

Figure 4. NSC647889 has a mechanism of action similar to that of calmodulin inhibitors. **A.** Evaluation of molecular mechanism of activity of NSC647889 using gene expression profiling. Shown are the top ten compounds (out of 1309) that induce the most similar gene expression profiles as NSC647889 after 6h of drug exposure using the Connectivity Map (Cmap) [22]. Score according to the Cmap database. **B.** NSC647889 increases Ca^{2+} /calmodulin-dependent calcineurin activity after 1 hour of drug exposure. HCT116 cells were exposed to 10 μM NSC647889 or 10 μM W7 and enzyme activity measured in cell extracts (units/mg protein).

antineoplastic activity. NSC647889 shows antiproliferative activity in the NCI60 cell panel (average GI₅₀: 1.1 μM ; strongest activity on acute lymphoblastic leukaemia, promyelocytic leukemia and chronic myelogenous leukemia cell lines (<http://dtp.nci.nih.gov/>)). We previously reported that compounds active on multicellular spheroids generally are hydrophobic [19] and the hydrophobicity (XLogP 7.3) of NSC647889 is consistent with this observation. The high degree of hydrophobicity complicated systematic *in vivo* assessments of antineoplastic effects. We were, however, able to document induction of tumor apoptosis at a dose of 5 mg/kg using circulating cell death biomarkers (Figure 6). This was close to the maximally tolerated dose (10 mg/kg) and it is unclear whether NSC647889 has a sufficient therapeutic window to make it a candidate for further development.

The Cmap analysis suggested a mechanism of action related to calmodulin inhibition. A number of CaM antagonists have been described (for reviews, see [36,37]) and the CaM antagonist calmidazolium has previously been

Figure 5. NSC647889 stimulates increases in intracellular calcium. HCT116 cells were exposed to different concentration of NSC647889 or W7 (10 μM) for 1 h and cellular calcium was determined using Fluo-4.

Figure 6. *In vivo* induction of apoptosis by NSC647889 in FaDu head-neck carcinoma xenografts. SCID mice were injected with 5 mg/kg NSC647889 (subcutaneous injection, compound dissolved in DMSO). Levels of human caspase-cleaved (ccK18, K18 cleaved at DALD-396 by caspases) and total K18 were determined in mouse plasma after 48 h by ELISA. The antibodies used to detect ccK18 and K18 in plasma do not recognize the mouse protein. Control mice were injected with DMSO.

found to induce apoptosis [38]. NSC647889 did not, however, inhibit CaM-dependent calcineurin (protein phosphatase 2B) activity in cells. Rather, calcineurin phosphatase activity was stimulated in exposed cells. These increases are likely to be due to elevations of intracellular calcium concentrations.

A number of ion channels are regulated by CaM and CaM can be considered as an integral ion-channel subunit [39]. Ion channels regulated by calmodulin include the voltage-gated calcium channel (VGCC, CaV) and ryanodine and IP3 receptors [40]. It is therefore possible that the similar gene expression pattern induced by NSC647889 and CaM inhibitors reflect a general cellular response to influx of Ca^{2+} into cells during the early phases of the apoptotic process. Sustained increases in $[\text{Ca}^{2+}]_i$ are well known to be associated with apoptosis [33,41,42] but generally occurring during late stages [43]. The consequences of these more universal changes of calcium fluxes during the apoptotic execution phases will not be detected by the Cmap approach which uses 6 h exposure times.

Activation of caspase-3 was not observed in tumor spheroid core regions. However, the same concentration (10 μM) of drug that was incapable of inducing apoptosis in core cells (a cell population constituting ~20% of total) was found to reduce clonogenicity of dispersed MCS cells to ~ 1%. These findings are likely to be explained by core cells dying by other mechanisms (i.e., presumably necrosis). Analysis of the pattern of apoptosis-induction after drug treatment (Figure 3) showed a sharp “boundary” at a depth of ~100 μm , below which the compound did not induce caspase-3 activation. This “boundary” was visible after 6 h and remained essentially at the same depth at 10 h. This pattern is not expected from a simple lack of drug penetration since the drug-gradient should have changed during this period. We believe it to be more likely that core cells are intrinsically resistant to apoptosis and die by other mechanisms. Core cells are hypoxic (Figure 1A) and express the ER stress marker Grp78 (our unpublished data). Since apoptosis requires an adequate cellular ATP supply [44], it is interesting to speculate whether cells in the MCS cores are compromised with regard to energy status and unable to undergo apoptosis. If this phenomenon is generally applicable to the response of hypoxic cell populations, apoptosis may not be an adequate read-out for the effects of drugs on solid tumors.

We conclude from this study that cell-based screening using multicellular spheroids identified a novel small molecule with an unprecedented ability to induce apoptosis of 3-D cultured cells. The results show that compounds can be identified that are significantly more powerful than drugs currently used in the clinic. Although the exact mechanism of action of apoptosis induction by NSC647889 is unclear, induction of calcium signaling is likely to be involved. We believe that the identification of drugs that show strong cytotoxic activity on hypoxic and nutrient limited tumor cell populations is a useful strategy for improved treatment of solid tumors.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	STL	CM	WF	MHO	RL	AD	MF
Research concept and design	✓	✓	✓	✓	✓	✓	✓
Collection and/or assembly of data	--	✓	✓	✓	--	--	✓
Data analysis and interpretation	✓	✓	✓	✓	✓	✓	✓
Writing the article	✓	✓	✓	✓	✓	✓	✓
Critical revision of the article	✓	✓	--	--	--	--	✓
Final approval of article	✓	✓	✓	✓	✓	✓	✓
Statistical analysis	✓	✓	--	--	--	--	✓

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