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Inhibition of protein synthesis and JNK activation are not required for cell death induced by anisomycin and anisomycin analogues

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ABSTRACT
Anisomycin was identified in a screen of clinical compounds as a drug that kills breast cancer cells (MDA16 cells, derived from the triple negative breast cancer cell line, MDA-MB-468) that express high levels of an efflux pump, ABCB1. We show the MDA16 cells died by a caspase-independent mechanism, while MDA-MB-468 cells died by apoptosis. There was no correlation between cell death and either protein synthesis or JNK activation, which had previously been implicated in anisomycin-induced cell death. In addition, anisomycin analogues that did not inhibit protein synthesis or activate JNK retained the ability to induce cell death. These data suggest that either a ribosome-ANS complex is a death signal in the absence of JNK activation or ANS kills cells by binding to an as yet unidentified target.

ABBREVIATIONS
ANS, anisomycin; Compound 26, (2R,3S,4S)-1-benzyl-4-benzyloxy-3-hydroxy-2-(4-methoxybenzyl)pyrrolidine; Compound 27, (2R,3S,4S)-3-acetoxy-1-benzyl-4-benzyloxy-2-(4-methoxybenzyl)pyrrolidine; 25, (2R,3R)-3-acetoxy-1-benzyl-2-(4-methoxybenzyl)pyrrolidine; 17, (2R,3R,4S)-3-acetoxy-1-benzyl-2-(4-methoxybenzyl)-4-methylpyrrolidine; CHX, Cycloheximide; JNK, Jun kinase; DMSO, dimethyl sulfoxide; TNBC, Triple Negative Breast Cancer; PBS, phosphate buffered saline; TBS, Tris buffered saline; HBSS, Hanks’ balanced salt solution; DTT, dithiolthreitol; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; PVDF, polyvinylidene difluoride.

INTRODUCTION
Triple negative breast cancer (TNBC) accounts for 10-15% of all breast cancer, and it is usually more aggressive than other types of breast cancer. While TNBCs typically respond well to chemotherapy, many patients relapse and treatment of relapsed metastatic disease is currently very difficult due to drug-resistance [1]. Drug-resistance in breast cancers is likely to be caused by several different types of mechanism [2], but it has been shown to correlate with increased expression of the efflux pumps such as ABCB1 [3], which is also a characteristic of cancer stem cell populations [4], and with the expression of YHWAZ and LAPTM4B which confer resistance to doxorubicin in TNBC [5]. TNBCs also often lose p53 function. These types of molecular change are part of the reason that the prognosis for TNBC is the poorest of all types of breast cancer [6].

The Johns Hopkins Clinical Compound Library (JHCL) of more than 1,200 approved compounds was screened using a multi-drug-resistant TNBC cell line (MDA16) which expresses high levels of ABCB1 and is derived from MDA-MB468 cells [7]. The aim was to discover an existing drug with activity against multi-drug resistant cancer cells and/or cancer stem cells that could be repurposed [8-9] as an anti-cancer agent or could be used as a lead molecule for the development of a new drug.

The most potent drug identified by the screen was an anti-protozoal drug, anisomycin (ANS). ANS binds the ribosome, inhibits peptidyl transferase activity and so blocks protein synthesis [10]. ANS bound to the
ribosome also activates stress kinases such as JNK, a response that has been termed ribotoxic stress [11]. Agents beside ANS can induce ribotoxic shock, including trichothecenes like deoxynivalenol [12], and aminohexose pyrimidine nucleoside antibiotics but other protein synthesis inhibitors such as cycloheximide and puromycin do not trigger this response [11]. ANS is also known to induce cell death and JNK activation is required for ANS-induced apoptosis [13] and anoikis in cancer cells [14], linking this death to ribotoxic stress. In addition ANS can sensitize cells to apoptotic stimuli that act via the death receptor pathway by inhibiting protein synthesis and causing a decrease in short-half life anti-apoptotic proteins [15-16].

Here we show that ANS induced caspase-independent cell death in MDA16 cells. In addition, the cell-death inducing activity of two ANS analogues is shown. These data significantly strengthen the case for the continued investigation into the potential use of ANS and its analogues as anti-cancer agents. However, our data are not consistent with a model in which ANS-induced cell death is dependent on protein synthesis inhibition or JNK or p38 activation. This suggests that either ANS-induced ribotoxic shock can be uncoupled from JNK activation or that ANS kills cells through interaction with an unidentified target.

MATERIALS AND METHODS

Cell lines
MDA-MB-468 and its derivative, MDA16 breast cancer cell lines were supplied by Dr. T. Gant (Medical Research Council Toxicology Unit, University of Leicester). MDA-MB-231 cells were supplied by the National Breast Cancer Research Institute, NUI Galway. The identity of the MDA-MB-468 and MDA-MB-231 cells was confirmed by analysis of its short tandem repeat profile (LGC). All cell lines were maintained in DMEM medium supplemented with 10% FBS. All culture media contained 100 U/ml penicillin and 100 µg/ml streptomycin. Cell lines were incubated in a humidified incubator (5% CO₂, 37 °C) and passaged every 2-3 days.

Synthesis of ANS and ANS analogues
ANS and ANS analogues were prepared as previously described [17].

Colony formation assay
Cells were seeded (3 x 10⁴/T25 flask), incubated overnight. Cells were treated with drug or DMSO (1% v/v final concentration) for 24 hours, the drug washed away and fresh medium added. After one week colonies were washed in ice cold PBS, fixed in methanol for 5 minutes and stained with crystal violet dye for 5 minutes before de-staining in water. The number of colonies per flask was determined by scoring colonies containing >10 cells in a total of 20-30 fields (200-300 colonies counted per flask). MDA-MB-231 cell survival was assessed by extracting crystal violet from stained cells using 30% acetic acid, 10% methanol and measuring the absorbance at 590 nm.

Caspase-3 like activity assay
Caspase activity was assessed as previously described [18] and caspase-3-like activity was expressed as arbitrary fluorescent units (AFU) per minute per mg protein.
**Annexin V staining**

Cells (1 x 10^6 cells per group) were treated with ANS for 24 hours. Floating and adherent cells were harvested and centrifuged (300 x g, 5 mins). The cells were washed twice in Annexin V buffer and then resuspended in 0.3 ml of Annexin V buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂). One microlitre of Annexin V stock solution was added the cells were incubated for 15 minutes at room temperature before being placed on ice. Propidium iodide (1:100 dilution of a 5 mg/ml stock) was added immediately before analysis using a BD Accuri™ C6 flowcytometer.

**Immunoblotting**

Samples were then immunoblotted as previously described [19]. Antigens of interest were visualised either by chemiluminescence and a CCD camera (Alpha Technology) or using an infrared scanner (Licor).

**SUnSET assay and assay for JNK phosphorylation**

5 x 10⁵ cells/well were seeded in 6-well plates and incubated overnight. Cells were then incubated for 1 hour with test compounds or DMSO as vehicle control (final concentration 1% v/v). After 1 hour puromycin was added (final concentration of 18 mM) and cells incubated for a further 10 minutes to label nascent polypeptide chains. Background labelling was determined by incubating cells without puromycin. Cells were then washed in HBSS, harvested by scraping and centrifuged (300xg, 5 minutes). Cells were resuspended in 0.5 ml 50 mM DTT containing phosphatase inhibitors and incubated at 95 °C for 10 minutes. Samples were then snap frozen in liquid nitrogen and stored at -20 °C until blotted. Samples (20-30 mg protein/sample) were blotted onto a PVDF membrane using a vacuum manifold. The membrane was blocked and incubated either with anti-puromycin antibody (KeraFAST) (SUnSET assay[20]) or anti-phospho-Thr183/Tyr185-JNK anti-body (Cell Signaling Technoloiges) overnight at 4 °C. Secondary antibodies conjugated to a fluorophore were used to label the primary antibody and detected using an infrared scanner (Licor). Equal loading was confirmed by stripping the membrane and probing for beta-actin (SUnSET assay) or total JNK. The intensity of the fluorescence signal for anti-puromycin and anti-phospho-JNK antibody was background corrected and normalized for loading.

**Statistical analysis**

Statistically significant differences (P<0.05) were tested using Kruskal-Wallis test, followed by *post-hoc* test using a modified Mann-Whitney test [21] where experiments involved more than two groups.

**RESULTS**

**Anisomycin kills multi-drug resistant MDA16 cells**

We used MDA16, a cell line derived by fluorescence activated cell sorting using an antibody against ABCB1 from the triple negative breast cancer cell line MDA-MB-468 [7], to screen the JHCCL (at 10 mM).

Anisomycin (ANS) was identified as the most potent drug with an LC₅₀ estimated to be ~10⁻⁷ M (see supplementary data for the other hits identified and hit validation).
ANS inhibits of protein synthesis [16] and death may occur by inhibiting the synthesis of a short half-life pro-survival protein. A SUNSET assay [20], which detects the incorporation of puromycin into nascent polypeptide chains, confirmed that ANS blocked protein synthesis at concentrations that killed cells (Figure 1A). In contrast, another protein synthesis inhibitor, cycloheximide (CHX) caused comparatively little reduction in cell death assessed by colony formation (Figure 1B).

Next, the relationship between colony formation and the degree of protein synthesis inhibition was examined (Figure 1C). A range of different ANS (0.3-10 mM) and CHX (1-30 mM) were tested and the level of protein synthesis inhibition assessed by SUNSET assay plotted against cell death assessed by colony formation. This showed that concentrations of CHX that blocked protein synthesis as effectively as ANS had a far smaller effect on colony formation than ANS. Thus, ANS induced cell death while another protein synthesis inhibitor, CHX did not. These data suggest that the cell-death observed was not due to a decrease in the levels of a pro-survival protein.

JNK activation by ANS is indicative of ribotoxic stress[11]. ANS also activated the stress kinase JNK in both cell lines (Figure 1D) and p38 (supplementary data). However, a colony formation assay showed that neither the JNK inhibitor, SP600125 (Figure 1E) nor the p38 inhibitor, SB203580 (data not shown) protected cells from ANS-induced cell death, even at high concentrations. This is not consistent with reports using other cell lines and suggests that ribotoxic shock cannot explain the cell-death induced by ANS.

Modes of ANS-induced cell death
ANS is reported to induce apoptosis [13] and so the Annexin V assay was used to test for ANS-induced phosphatidylserine exposure on MDA16 and MDA-MB-468 cells by flow cytometry (Figure 2A-C). This showed that ANS caused an increase the number of apoptotic cells in treated MDA-MB-468 cultures, but not in MDA16 cultures. These data were corroborated using a biochemical assay for caspase-3 activity which showed that ANS activated caspase-3 in MDA-MB-468 cells, but not in MDA16 cells (Figure 2D). These data show that ANS induces caspase-dependent apoptosis in one cell type but a caspase-independent cell-death in the other.

Structure activity relationships of ANS and analogues
JNK activation by ANS analogues has been reported [17, 22]. To build on this work a panel of ANS analogues was tested against MDA16, MDA-MB-468 and MDA-MB-231 cells using the colony formation assay. This showed that compound 27 (numbering based on Rosser et al. [17]) killed all three lines, while compound 26 was less effective against MDA16 cells than ANS (Figure 3A). The cell death induced by compound 27 and 26 in MDA-MB-468 cells was concentration dependent with LC_{50} values being ~3 mM and ~7 mM respectively (Figure 3B). The levels of caspase-3 like activity in MDA-MB-468 cells treated with...
ANS, compound 27 and 26 for 24 hours were then measured. Both compound 27 and 26 induced a statistically significant increase in caspase-3 like activity in MDA-MB-468 cells, although the level was less than that induced by ANS (Figure 2C).

Next, the effect of ANS, compound 27 and 26 on cell death, protein synthesis and JNK phosphorylation in MDA-MB-468 cells was investigated. Two ANS analogues with the closest structural similarity to compound 27 and 26 (compounds 25 and 17) were also included as control molecules. ANS, compound 27 and 26 killed MDA-MB-468 cells as expected (Figure 4A). Protein synthesis in treated cells was measured in parallel using the SUnSET assay. Both ANS and compound 27 inhibited protein synthesis at concentrations which killed MDA-MB-468 cells (Figure 4B). In contrast, none of the other ANS-analogues affected protein synthesis even though compound 26 induced cell death.

To test whether JNK activation correlated with analogue-induced cell death we examined the phosphorylation state of JNK using a phospho-specific antibody. To accomplish this a dot-blot assay was established to more easily quantify any effect on JNK activation caused by the ANS-analogues. JNK-activation induced by ANS was readily detectable using this assay, however the ANS-analogues did not activate JNK (Figure 4C), irrespective of their effect on cell death (Figure 4A).

DISCUSSION

Repurposing ANS for drug-resistant TNBC

ANS emerged from the screen as an agent that killed a multidrug-resistant TNBC cell line, MDA16 that expresses high levels of ABCB1 [7]. ABCB1 expression decreases the efficacy of a range of chemotherapeutics and is detected in >50% of drug resistant tumours [4]. In addition, cancer stem cells are inherently drug-resistant and in some cases express high levels of ABC proteins [23]. Moreover, ANS killed the TNBC cell line MDA-MB-231. This line shows amplification of chromosome 8q22 an event common in drug-resistant TNBC [5] that leads to increased expression of the doxorubicin-resistance genes, YHWAZ and LAPTM4B. ANS is known to inhibit tumour growth in a mouse model at concentrations that reported to be non-toxic [13, 24] and activation of oncogenes myc [25], ras [26] or src [12] confers increased sensitivity to ANS. In addition ANS crosses the blood brain barrier [27] and therefore is likely to be effective against brain metastases. Overall, these data and ours strengthen the case for repurposing ANS as an anti-cancer agent.

The data do not fit the current models for ANS-induced cell death

ANS binds ribosomes and inhibits peptidyl-transferase activity, so blocking protein synthesis [10]. Inhibition of protein synthesis sensitizes cells to apoptosis induced by Death Receptor signalling and to anoikis [14-15] and this sensitization has been linked to decreases in the levels of short-half life pro-survival molecules such as cFLIP [14]. ANS can also activate stress-activated kinases p38 and JNK and so induce apoptosis by increasing expression of FOXP3 [13]. ANS-induced JNK activation has been considered indicative of
ribotoxic stress [11, 28] and to lead to JNK-dependent apoptosis [15], although the role of JNK in cell death is complicated with both pro- and anti-death roles being described [29-30].

The ANS-induced cell death shown here did not correlate with protein synthesis inhibition or with JNK activation. Thus the data shown here are not consistent with the current understanding of ANS’s mechanism of action. One possible explanation is that the binding of ANS to the ribosome is sufficient to generate a death-inducing signal (which does not require protein synthesis inhibition or kinase activation) or an alternative mechanism is in play. In the former case, the link between protein synthesis, JNK activation and ribotoxic stress is called into question. In the latter case there must be an as yet unidentified ANS-target, in addition to the ribosome, that is required for ANS-induced cell death.

**ANS induced caspase-dependent and independent cell death**

The data presented here show that while ANS induced apoptosis in MDA-MB-468 cells (consistent with previous reports), MDA16 cells died without caspase activation or exposure of phosphatidylserine and indicating a non-apoptotic process. Although the mechanism of cell death in MDA16 has not been identified, over-expression of ABCB1 in HeLa cells switches cell death induced by irradiation from apoptosis to mitotic catastrophe [31] and the high levels of ABCB1 in MDA16 may be influencing the mode of death chosen by these cells in a similar way.

**Identification of two ANS analogues with cell-death inducing activity**

The reported crystal structure of ANS bound to the peptidyl transferase site of the ribosome shows the importance of both the pyrrolidine ring and its hydroxyl substituents in binding [32]; it has also been shown that deacetyl-ANS does not block protein synthesis [33]. Compound 27 was demonstrated to block protein synthesis yet the pyrrolidine ring is different from that of ANS in three ways; it is modified with an N-benzyl group, the C(4) hydroxyl is benzylated and the C(3) acetyl group of ANS is missing. The N-benzyl group of 27 removes the hydrogen bond from the pyrrolidine nitrogen to C2487 in the A-site crevice of the ribosome which is observed for ANS [32]. Similarly, the presence of the C(4) O-benzyl group prevents the hydrogen bond from this hydroxyl to U2539 which is observed for ANS [32]. Thus the observed activity of 27 can be reconciled with the published ANS-ribosome interaction if we conclude that compound 27 binds to the ribosome through an alternative binding mode, perhaps more closely aligned with that of the related protein synthesis inhibitor puromycin, or that it blocks protein synthesis via a mechanism different from that of ANS.

The pyrrolidine ring of 26, like that of compound 27 is modified with both an N-benzyl group and a C(4) O-benzyl group. Unlike compound 27 however, 26 retains the C(3) acetyl group of ANS. Despite the presence of the acetyl group, compound 26 does not inhibit protein synthesis, possibly because the two benzyl groups prevent binding to the peptidyl-transferase site, although from the current data different intracellular concentrations (due to differences in log P, H-bonding, etc) cannot be excluded as an alternative explanation.
for the different outcomes caused by compounds 26 and 27 [34]. The difference between the structures of compound 27 and 26 suggest that the hydroxyl group of compound 27 is an important determinant for binding to the target involved in protein synthesis.

The presence of an N-benzyl group combined with the lack of the C(4) hydroxyl in compounds 25 and 17 is sufficient to explain the inability of these molecules to block protein synthesis. The differences between compound 26, which killed cells and contained an C(4) O-benzyl group, and both 25 and 17, which did not kill cells and lacked this group, suggests that the O-benzyl group conferred cytotoxicity. At the same time, compound 27 was more cytotoxic than 26 suggesting that removal of the acetyl group at C(3) to give an unsubstituted hydroxyl group increases cell killing activity. The current data do not allow us to determine whether the free hydroxyl group at C(3) affects cytotoxicity due to enhanced intracellular availability, by altering affinity for ABCB1, or for the target relevant for cell death or through a combination of these factors.

Compound 27 has been previously described as a weak JNK activator (at 22 mM) [17]. In MDA-MB-468 cells, only ANS increased JNK phosphorylation and compound 27 (10 mM) and 26 (10 mM) did not do not produce a detectable increase in JNK phosphorylation. Thus, JNK phosphorylation did not correlate with either protein synthesis inhibition by compound 27 or cell death induced by compound 27 and 26. Neither compound 27 nor 26 are predicted to bind to the ribosome in the way that ANS does (see above). However, puromycin, which binds to the same site as ANS and inhibits protein synthesis did not increase JNK phosphorylation (data not shown), suggesting that increased JNK phosphorylation is not simply triggered by drug-binding to the site of peptidyl-transferase activity.

In conclusion, these data significantly strengthen the case for repurposing ANS as an anti-cancer agent for advanced multi-drug resistant TNBC and identify an anisomycin analogue, compound 27, as a potential lead molecule for development as a new therapy for drug resistant TNBC. The data presented are not consistent with current explanations of ANS’s mechanism of action and suggest that either ribosome-ANS complex is itself a death signal or that ANS acts by binding to an unidentified target.
Figure 1. (A) ANS (3 µM) decreased protein synthesis in MDA16 and MDA-MB-468 cells as assessed by the SUnSET assay. (B) ANS but not CHX reduce colony formation by MDA-MB-468 cells. (C) Both ANS and CHX reduced protein synthesis in MDA-MB-468 cells to a similar extent, but ANS also reduced colony formation. ANS (●); small to large symbol are concentrations of 0.3, 1.0, 3.0 and 10 µM. CHX (○); small to large symbol are concentrations of 1, 3, 10 and 30 µM. (D) ANS (3 µM) increased levels of phospho-JNK in MDA16 and MDA-MB-468 cells. (E) JNK inhibitor (30 µM) did not rescue cell viability in MDA-MB-468 cells. All data shown are mean ± SEM of ≥3 independent experiments. * P<0.05.
Figure 2. Treatment with ANS (10 μM) increased phosphatidyl serine exposure by MDA-MB-468 cells but not MDA16 cells. A representative dot plot is shown (A). The quantitation of >3 independent experiments are shown in B and C. (D) ANS increased caspase-3 like activity. All data shown are mean ± SEM of ≥3 independent experiments. * P<0.05.
Figure 3. (A) Compounds 27 (10 µM) and 26 (10 µM) affected colony formation by MDA16 and MDA-MB-468 cells and the survival of MDA-MB-231 cells. (B) The concentration-dependent cell death induced by compound 27 (○) and 26 (●) in MDA-MB-468 cells. (C) ANS and ANS analogues (10 µM) increased caspase-3 like activity in MDA-MB-468 cells. All data shown are mean ± SEM of 3 independent experiments, except (B), where n =2. * P<0.05.
Figure 4. (A) ANS and ANS analogues (all at 10 µM) affected colony formation by MDA-MB-468 cells. (B) The effect of ANS and ANS analogues on protein synthesis in MDA-MB-468 cells as assessed by SUnSET assay. (C) The effect of ANS and ANS analogues on JNK phosphorylation in MDA-MB-468 cells. For (B) and (C) the inset graph shows the relationship between fluorescence intensity and protein amount for each assay. All experimental samples shown in A-C fall within the range shown. All data shown are mean ± SEM of ≥3 independent experiments. * P<0.05.
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