Duramycin induced calcium release in cancer cells

Short title: Effect of duramycin on tumour cells

Laura J Broughton[1], Chris Crow[2], Anthony Maraveyas[3] and Leigh A Madden[4]*

[1]Hull York Medical School, University of Hull, Hull, UK. hylb3@hyms.ac.uk.

[2]Daisy Building Laboratory, University of Hull, Hull, UK. C.Crow@hull.ac.uk.

[3]Hull York Medical School, University of Hull, Hull, UK.

anthony.maraveyas@hey.nhs.uk.

[4]School of Biological, Biomedical and Environmental Sciences, University of Hull, Hull, UK. l.a.madden@hull.ac.uk.

*Corresponding author

Address for correspondence:

Dr Leigh A Madden

Allam Building

University of Hull

Cottingham Road

Hull

UK

HU6 7RX

Conflicts of Interest and Source of Funding

None Declared.

Abstract

Introduction: Duramycin through binding with phosphatidylethanolamine (PE) has shown potential to be an effective anti-tumour agent. However its mode of action in relation to tumour cells is not fully understood.

Methods: PE expression on the surface of a panel of cancer cell lines was analysed using duramycin and subsequent antibody labelling then analysed by flow cytometry. Cell viability was also assessed via flow cytometry using annexin V and propidium iodide (PI). Calcium ion (Ca^{2+}) release by tumour cells in response to duramycin was determined by spectrofluorometry following incubation with Fluo-3, AM. Confocal microscopy was performed on the cancer cell line AsPC-1 to assess real time cell response to duramycin treatment.

Results: Duramycin was able to detect cell surface PE expression on all 15 cancer cell lines screened, which was shown to be duramycin concentration dependent. However higher concentrations induced necrotic cell death. Duramycin induced calcium ion (Ca^{2+}) release from the cancer cell lines also in a concentration and time dependent manner. Confocal microscopy showed an influx of PI into the cells over time and induced morphological changes.

Conclusion: Duramycin induces Ca^{2+} release from cancer cell lines in a time and concentration dependent relationship.

Keywords

Duramycin, phosphatidylethanolamine, calcium release.

Introduction

Duramycin is a 19-amino acid tetracyclic peptide produced from *Streptoverticillium cinnamoneus* [1, 2]. It is one of very few known, relatively, small peptides to have a defined 3-dimensional structure. This structure is stable [3] due to the presence of 4 covalent intra-molecular bridges formed from the amino acids lanthionine, methyllanthionine, lysinoalanine and beta-hydroxyaspartic acid [4]. Due to the presence of these amino acids duramycin is designated as a Type B lantibiotic. Its stability also means that duramycin is resistant to both thermal and proteolytic degradation [3]. Duramycin's binding site confers specific physiochemical interactions that selectively recognise the ethanolamine headgroup of the aminophospholipid phosphatidylethanolamine (PE) [5]. The binding of duramycin to PE is on a 1:1 molar ratio and at high affinity with dissociation constants in the low nanomolar range (4-6nM) [6, 7]. PE is a phospholipid ubiquitously found in eukaryotic cell membranes. In quiescent cells PE is primarily located on the inner cell membrane along with the aminophospholipid phosphatidylserine (PS) [8-10]. This membrane asymmetry is enzymatically maintained [8, 11, 12] by ATP-dependent aminophospholipid translocases that are responsible for the inward and outward movement of PE and PS. Upon activation PE can become exposed on the outer cell membrane [13] which is important in a number of physiological processes for example in apoptosis and necrosis [14], membrane re-organisation in cytokinesis [15] and the initiation of coagulation [16, 17].

It has been reported in the literature that duramycin has an effect on cell membranes. Duramycin, through binding with PE, has been seen to change vesicle membranes into highly curved tubular structures [6], change the shape of PE containing monolayers and reduce lipid order [18]. It has been shown to have the ability to form channels in both artificial and biological membranes [19],

permeabilise cells at concentrations of >50μM [20, 21] and destroy cell membranes [22]. Adding to its ability to disturb cell membranes duramycin has been reported to effect ion movement in a range of cell types. Duramycin displayed inhibitory effects on chloride (Cl^-) transport in bovine clathrin-coated vesicles [23], the sodium-potassium ATPase pump through inhibition of phosphoenzyme formation [24] and a magnesium dependent ATPase in plasma membranes [20]. Duramycin also disrupted ATP dependent calcium ion (Ca^{2+}) uptake in vesicles from rabbit sarcoplasmic reticulum [21]. Duramycin has been shown to have stimulatory effects on ion movement leading to the release of intracellular ions or the elevation of intracellular ion concentration. This has been observed in human colonic epithelia [19, 25], cardiomyocytes [26] and airway epithelia [22, 27, 28]. A number of studies have investigated Ca^{2+} release in response to duramycin [22, 28] due to duramycin's ability to release this ion from the airway epithelia from intracellular stores at low concentrations and from both intracellular stores and extracellular influx at higher concentrations [28]. Due to its presumed action on calcium activated chloride channels (CACC) duramycin reached clinical trials for the treatment of cystic fibrosis (CF) [29, 30].

Exposure of cell surface PE has been reported to be up-regulated in cancer cells [31] and found to be expressed on the endothelium of tumour vasculature of many different forms of tumour *in vitro* and *in vivo* [1] and on the surface of cancer cell derived microparticles [32]. Through the targeting of PE duramycin has been used to successfully image prostate tumour endothelium in rats [1]. PE therefore has the potential to be a wide-ranging tumour marker for a variety of malignancies. Duramycin's specificity, stability and fast renal clearance time [33] make it an ideal probe for PE. Further duramycin has been shown to disrupt cancer cell derived microparticle mediated coagulation [34] and have anti-proliferative and anti-tumour effects on pancreatic cancer cell lines [31].

In this study the effect of duramycin on Ca^{2+} release in ovarian and pancreatic cancer cell lines was investigated. As duramycin is known to interact with cancer cell lines it may be important to discern the effects on ion movement, membrane integrity and channel formation in cancer cell lines as has been observed in other cell types. Duramycin has the potential to be an effective anti-tumour therapy agent however it's mode of action in relation to tumour cells is not fully understood.

Materials and Methods

Cell Culture

The cell lines AsPC-1, Caco-2, Colo320, HCT116, JJN3, Lovo, MCF7, MDA-MB-231, MIA PaCa-2, MM.1S and U266B1 were purchased from the American Type Culture Collection (UK). The cell lines A2780, SK-OV-3, T47D and U937 were purchased from European Collection of Cell Cultures (UK). All cell lines were cultured in accordance with distributor's recommendations. Each cell line was cultured using either DMEM, RPMI, IMDM or McCoy's 5a Medium Modified (Lonza, UK) with FBS (Bio-Sera, UK) and (v/v); 100 units/ml penicillin, 100 µg/ml streptomycin (P/S) (Lonza, UK). All serum, P/S and buffers added to media were filtered through a 0.2µm filter before addition. Between use all media was stored at 2-8°C. All cell lines were incubated at 37°C in a 5% CO_2 atmosphere except for MDA-MB-231 which was incubated at 37°C in a 0% CO_2 atmosphere. All cell lines were cultured in tissue culture flasks (Sarstedt, UK) and removed when cells were either adherent and in the logarithmic growth phase or removed when at a high enough growth density for suspension cell lines.

Flow Cytometric Phosphatidylethanolamine Detection

Cells (2×10^5) were re-suspended in 50µl PBS and added to a 5ml polypropylene tube (Sarstedt, UK). Then either 10µl of duramycin (duramycin from *Streptoverticillium cinnamoneus*, Sigma-Aldrich, UK) at appropriate concentration or 10µl normal rabbit serum (1:5000 dilution) as negative control was added to the tube and incubated at room temperature for 30 minutes. Cells were washed with PBS, centrifuged at 320g for 3 minutes and re-suspended in 300µl PBS and 10µl of anti-duramycin antibody (Abcam, UK) (final dilution 1:600) was added and incubated in the dark at room temperature for 30 minutes. The cells were then washed and re-suspended as before and 50µl of sheep anti-rabbit IgG:FITC

antibody (AbD Serotec®) (final dilution 1:600) added and incubated in the dark at room temperature for 30 minutes. Cells were washed, centrifuged and re-suspended as before and then analysed using a BD FACScalibur (BD Biosciences, UK), which was the flow cytometer, used for all flow cytometric analysis in this study. Data is expressed as median fluorescence intensity (MFI) ratio which is calculated by division of the MFI value for the positive sample by the MFI value of the negative control sample giving the level of shift in fluorescence intensity of a population of cells expressed as a ratio.

Flow Cytometric Cell Viability Analysis

Cell viability was analysed using the Annexin V: FITC Apoptosis Detection Kit I from BD Biosciences, UK and protocol followed manufacturer's instructions. Briefly, cells (2×10^5) were re-suspended in 50µl PBS and added to a 5ml polypropylene tube. The cells were treated with an appropriate concentration of duramycin and incubated for 30 minutes at room temperature. After washing cells were re-suspended in 100µl binding buffer (1:10 dilution) and incubated with 5µl Annexin V: FITC and 5µl propidium iodide (PI) for 15 minutes in the dark at room temperature. After this 300µl binding buffer was added and cells analysed by flow cytometry.

Forward Scatter and Side Scatter Data Collection

The data used for the forward and side scatter analysis was gathered from an investigation into the effect of duramycin treatment on cell viability at time points over a 2 hour time course (not included in this publication). These cells were processed using the flow cytometric cell viability analysis method described above.

Ca^{2+} Signalling Detection Assay

Cells were removed from tissue culture flask after treatment with 2ml trypsin incubated for 5 minutes at 37°C and 5% CO_2. After the cells were washed with PBS and centrifuged at 320g for 5 minutes they were re-suspended in 0.5ml fresh media, 1.3 µl of 100mM sulphinpyrazone and 5µl of Fluo-3, AM (stock solution 2.19mM) (Life Technologies, UK) and incubated for 30 minutes. Cells were then washed and centrifuged at 320g for 5 minutes and re-suspended in calcium buffer (150mM NaCl, 3mM KCl, 10mM glucose, 20mM HEPES and 2.5µM sulphinpyrazone, adjusted to pH 7.5). When required the buffer included 1.5mM calcium chloride. Cells (1×10^5) were added to a cuvette containing 1.9ml calcium buffer to make up to 2ml and placed in a spectrofluorometer. Duramycin (20µl) at appropriate concentration or calcium ionophore A23187 (Sigma-Aldrich, UK) (12µl) was added to the cuvette. All data was analysed using FeliX GX software (Photon Technology International, UK). The percentage calcium ion release from duramycin treated cells was calculated as a percentage of the total calcium ion release from the control cuvette treated with the calcium ionophore A23187 [28].

Confocal Microscopy

For study of cell viability and cell membrane appearance, cell membranes were dyed using the CellVue® Jade Cell Labelling Kit (eBioscience, UK) using manufacturer's instructions. Briefly, cells (2×10^7) were re-suspended in 1ml of diluent C and added to 1ml of diluent C containing 4µM dye solution and incubated for 3 minutes with periodic mixing. After being washed with PBS 250µl cells (2×10^5) were incubated with 12.5µl PI (BD Biosciences, UK) for cell nucleus staining for 15 minutes at room temperature in the dark. The cells were then transferred to lumox® dishes and analysed using a Zeiss LSM 710 confocal microscope and Zen software version X (Zeiss, UK).

For the study of Ca^{2+} present in cells, 2.6µl of 100mM sulphinpyrazone was added to cells ($1x10^6$) in 1ml PBS. The dye Fluo-3, AM (10µl of 2.19mM) (Life Technologies, UK) was added to the cell suspension and incubated at room temperature in the dark for 30 minutes while gently rocked. After being washed with PBS, cells (8×10^5) were re-suspended in 1ml warm PBS and incubated with 1x CellMask™ Orange Plasma membrane Stain (Life Technologies, UK) for 10 minutes in the dark at 37°C. After cells were washed and re-suspended in warm PBS, 250µl cells (2×10^5) were transferred to lumox® dishes and analysed using the Zeiss LSM 710 confocal microscope and Zen software version X (Zeiss, UK).

Results

Duramycin Detection of PE

A total of 15 different cancer cell lines, accounting for 6 different cancer types, were screened using flow cytometry for cell surface expression of PE. All the cell lines screened had detectable levels of PE on the cell surface (Figure 1). Median fluorescence intensity (MFI) ratios were used to express relative PE levels on the cancer cell lines. The majority of the cancer cell lines had relatively low MFI ratios i.e. between 1.00 (minimum detectable expression) and 1.80. An exception to this was the 3 multiple myeloma (MM) cell lines which had MFI ratios ranging from 2.15 to 3.75. The ovarian cancer cell lines A2780 and SK-OV-3 and the pancreatic cancer cell lines AsPC-1 and MIA PaCa-2 were investigated further. Due to the MM cell lines being a circulating form of tumour they were not investigated. All of the 4 cancer cell lines showed an increase in PE MFI ratio with increased duramycin concentration (Figure 2). The relationship between PE detection and duramycin concentration showed a strong correlation for all 4 cell lines with Pearson Correlation Coefficient's of r = 0.80, 0.84, 0.76 and 0.87 for A2780, AsPC-1, MIA PaCa-2 and SK-OV-3 respectively. AsPC-1 had the highest increased MFI ratio from 1.1 at the screening concentration of duramycin (2.5µM) to 4.5 at the highest duramycin concentration (1mM). The concentration of duramycin used in flow cytometric PE detection (2.5µM) was a compromise on what would be optimal detection concentration (200µM-1mM) due to the cytotoxic effects of duramycin at these higher concentrations.

Duramycin Induced Necrotic Cell Death

The 2 ovarian cancer cell lines and the 2 pancreatic cancer cell lines described above were assessed for cell viability after treatment with a series of duramycin

concentrations via flow cytometry with a dual Annexin V: FITC and PI stain. All 4 cell lines showed an increase in percentage necrotic cells in a duramycin concentration dependent manner (Figure 3). Duramycin had its highest cytotoxic effect (87-96% necrosis) at concentrations above 500μM. The threshold concentration for inducement of cell death in the cancer cell lines was 5μM; concentrations below this did not induce necrosis.

Ca^{2+} Release in Response to Duramycin

Intracellular calcium ion (Ca^{2+}) release upon treatment with duramycin was investigated using a spectrofluorometric method. The Ca^{2+} release was expressed as a percentage of the total Ca^{2+} released from cells that were treated with the control calcium ionophore. All 4 cell lines tested were found to release intracellular Ca^{2+} upon treatment with duramycin in a concentration dependent manner (Figure 4). Percentage Ca^{2+} release was investigated both in the presence and absence of extracellular Ca^{2+} but no significant difference was seen between the conditions. The amount of Ca^{2+} release and percentage necrosis of cells was not found to correlate when cells were treated with duramycin concentrations below 10μM. There was only a significant increase in Ca^{2+} release and necrotic cell death observed in the cancer cell lines when treated with 50μM duramycin.

The time taken to reach peak Ca^{2+} release also followed a duramycin concentration dependent trend (Figure 5). For all 4 cell lines investigated this time was extended when the cells were in the presence of extracellular Ca^{2+}. This extension in time taken to reach peak Ca^{2+} release varied between cell lines, for example when cells were treated with 50μM duramycin the time difference between cell lines ranged from 14 seconds (AsPC-1) to 91 seconds (A2780). Due to the release of Ca^{2+} in response to duramycin treatment it was plausible that Ca^{2+} release was a method of duramycin induced cell death. However, a sustained influx and efflux of Ca^{2+} was

seen up to 1hr after duramycin treatment suggesting maintained membrane integrity.

Duramycin Induced Cell Morphology Change

Analysis of forward scatter (FSC) and side scatter (SSC) data obtained from flow cytometry showed little change in cell size but some change in cell complexity when cancer cell lines were treated with a series of duramycin concentrations. The same 4 cancer cell lines were subjected to treatment with duramycin between 2.5μM and 1mM and analysed for change in scatter properties along a 2 hour time course. A2780, MIA PaCa-2 and SK-OV-3 showed no significant change in FSC over the time course for all duramycin concentrations. AsPC-1, however, showed a slight increase in FSC when treated with 50μM, 200μM and 1mM duramycin. For example, FSC at 30 minutes for untreated AsPC-1 cells was 351.1 compared to 528.2 when treated with 1mM duramycin. All 4 cancer cell lines increased in SSC when treated with 200μM and 1mM duramycin with peak increase occurring at 1 hour after treatment.

Confocal Microscopy

To further investigate the effect of duramycin on cancer cell membranes and any possible pore forming the pancreatic cancer cell line AsPC-1 was studied using confocal imaging. Over 20 minutes of 50μM duramycin treatment AsPC-1 cells showed a marked loss of cell membrane stain (Figure 6 and 7). There was an approximate 51% reduction of cells over the 20 minutes retaining the staining. This would suggest a loss in cell membrane integrity or cell death. Some cell membrane staining loss could also be accounted for by the destabilisation of the dye compound as the untreated control cells saw a 28% reduction in cell numbers with cell membrane staining. Uptake of PI from the buffer solution into the AsPC-1 cells over the 20 minutes can be seen by an increase in cell numbers with cell nucleus

staining (Figure 6 and 7). Over the time course there was a 62% increase in the number of cells with cell nucleus staining when treated with duramycin compared to the untreated control cells in which there was a 1.6% increase.

AsPC-1 cells were exposed to 50µM duramycin treatment over 30 minutes and imaged, using confocal microscopy, for Ca^{2+} release. Over the 30 minutes, from 2 minutes onwards, there was a significant loss in Fluo-3, AM dye (Figure 8) suggesting a leakage of Ca^{2+} out of the cell. There was a 13% increase in cell numbers dyed with cell membrane stain over the 30 minutes. Untreated control cells at 30 minutes and 60 minutes saw maintained membrane integrity with the Ca^{2+} dye remaining inside the cells (Figure 9).

Discussion

Duramycin was used to detect the cell surface presence of PE on a number of different cancer cell types. In this way it was used as a screening method for confirmation of the expression of PE by a diverse range of cancer cell types. All of the cancer cell lines showed detectable levels of PE to varying amounts. Levels of expression did not appear to be cancer type specific with the exception of the MM cell lines which had a relatively higher PE expression than the other cancer cell lines.

Further investigation into 4 of the cell lines: 2 ovarian and 2 pancreatic showed that PE detection was strongly correlated with duramycin concentration. This finding is confounded when it is taken in to account that duramycin has the ability to induce cell death and therefore potentially increase the cell surface expression of PE. The compromise between optimal PE detection and duramycin concentration was, however, necessary due to the cytotoxic effect of duramycin at higher concentrations. Investigations using the same 4 cancer cell lines, as above, showed that duramycin is able to induce necrosis in a concentration dependent manner. Concentrations above 500μM were able to induce 87-96% necrosis in the cancer cell lines. The pancreatic cancer cell line AsPC-1 showed a higher percentage of necrosis than the other cell lines at lower duramycin concentrations suggesting a higher sensitivity. However this may be due to naturally lower cell viability observed with this cell line. The level of cell death at the higher concentrations was cancer cell line dependent however all 4 cancer cell lines showed a uniform threshold concentration for inducement of cell death of 5μM. Concentrations of duramycin ≥3.3μM have been shown to cause 'leak' currents in cardiomyocytes [26] and concentrations of 100μM and 250μM duramycin inhibited Cl^- secretion in cystic fibrosis bronchial epithelial cells (CFBE) [22]. It was suggested that this was

due to a destruction of the membrane which would support our finding that higher duramycin concentrations induce cancer cell necrosis. It has been reported previously that pancreatic cancer cell lines undergo apoptosis and then ultimately necrosis when treated with relatively low concentrations of duramycin for 48 hours [31].

It is known that duramycin interacts with cell membranes. It was shown that duramycin binding to PE modified the membranes of multilamellar vesicles into highly curved tubular structures [6] and can form channels in artificial black lipid membranes and biological membranes [19]. Duramycin binding to PE has also been seen to change the shape of PE monolayers from circular to horseshoe-like and reduce lipid order [18]. It is also reported that the peptide elevates intracellular calcium levels [28] and in clinical trials, in the treatment of CF, was thought to work on CACC [27, 29, 30]. Further investigation by Oliynyk [22] found that duramycin did induce chloride (Cl^-) secretion from CFBE within a narrow concentration range of around 1μM and did not cause secretion in the non-CF epithelial counterparts. Intracellular Ca^{2+} concentration, however, was not found to increase in the CFBE cells and yet did in the non-CF human bronchial epithelial cells (16HBE). It was suggested that this inferred an efflux of ions from the non-specific changes of the cell membrane rather than a specific effect on CACC.

In this study it was investigated whether duramycin had any specific effect on Ca^{2+} release in cancer cell lines and whether the build-up of intracellular Ca^{2+} and its potential release from the cell could offer an explanation for the duramycin mediated cell death seen in the cancer cells. All 4 cell lines investigated were seen to release intracellular Ca^{2+} immediately following the addition of duramycin at concentrations of 5μM and above (Figure 4). The percentage Ca^{2+} release from the cells was duramycin concentration dependent. Time taken to reach the peak

amount of Ca^{2+} release increased as duramycin concentration increased and was prolonged when cells were in the presence of extracellular calcium. This may suggest that duramycin-induced pore formation may increase with an increase in duramycin concentration which may explain the higher loss of Ca^{2+} over a longer period of time. The data implies the pores would allow the movement of Ca^{2+} back into the cell due to the prolonged time to reach peak Ca^{2+} release in the presence of extracellular calcium, inferring an influx and efflux of Ca^{2+}. Also when the Ca^{2+} assay was allowed to run for 1 hour after duramycin addition the cancer cells did not return to their ground Ca^{2+} release state, seen with the control ionophore A23187, but instead showed a sustained influx and efflux of Ca^{2+}. This finding has been observed in airway epithelium in which the effect of duramycin on calcium release was sustained compared to the rapid increase seen with the control ionophore A23187 [28]. There was a lack of correlation between Ca^{2+} release and necrotic cell death. This would imply that, rather than a loss of Ca^{2+} triggering an internal cell death mechanism, duramycin creates pores that simply allows movement of calcium in and out of the cell. Oliynyk suggests a non-specific loss of Cl^- ions from CF epithelial cells due to duramycin induced membrane changes [22]. Duramycin concentrations of ≥0.3μM caused an increase in release of sodium ions (Na^+) from cardiomyocytes and ≥3.3μM caused an increase in leak current of hERG potassium channels and Na^+ from the cardiac cells [26]. It has also been seen that duramycin causes channel formation and ion release in colonic epithelial cells [19]. Due to the similar response of the release and leakage of ions from channels that are structurally different [26], from a range of cell types and from the immediacy of Ca^{2+} ion release seen here it is likely that duramycin causes a non-specific pore formation in cell membranes.

Alternatively, it is possible that duramycin's presence causes a destabilisation of the cell membrane that then leads to cell death. This is supported by flow cytometric

analysis of FSC/SSC data in which SSC immediately increased upon treatment with duramycin. This may have been due to internal changes in the cells or a destabilisation of the cell membrane. To investigate further the effect of duramycin on cancer cell membranes the pancreatic cancer cell line, AsPC-1, which increased in FSC was imaged along a time course of duramycin treatment using confocal microscopy. When AsPC-1 cells were stained with CellVue® Jade and imaged over a 30 minute time course of 50µM duramycin treatment there was a significant loss in cell numbers dyed with cell membrane stain. This may suggest a loss of membrane integrity or cell death. Some of the cells appeared to have increased in size supporting the finding of an increase in FSC and possible swelling of the cell. Interestingly, staining with CellMask™ Orange showed an increase in cell numbers with cell membrane staining over time compared to the decrease seen with CellVue® Jade.

Imaging of AsPC-1 cells with confocal microscopy also allowed for observation of an increase in PI staining of the cell nuclei and therefore movement of PI from the buffer solution into the cell and a loss of Ca^{2+} stain. Untreated control cells saw an increase of 1.6% in cell numbers with cell nucleus staining compared to the duramycin treated cells which showed an increase of 62%. The first 2 minutes of duramycin treatment increased Ca^{2+} staining. The most likely explanation for this is that as duramycin binds to cell surface PE in these first couple of minutes and the non-specific pores are formed there is a sudden increase in release of Ca^{2+} from intracellular stores. This is supported by results seen in the confocal imaging of 16HBE cells that saw an increase in intracellular Ca^{2+} concentration when treated with 1 and 3µM duramycin [22]. As duramycin, once bound to PE, may change the physical properties of the cell membrane and therefore disturb ion channel function [26] it is possible that duramycin induced cell death may be a mixture of both loss

of intracellular components through membrane pores and the destabilisation of the cell membrane.

In addition to its direct anti-tumour properties duramycin has potential as an imaging agent for tumours through the targeting of PE. The structure of duramycin includes 2 primary amines, cysteine and lysine, at the N-terminal which make conjugation of duramycin possible without disrupting the PE binding site [4]. This method has been employed to successfully radiolabel duramycin to Technitium-99m and image acute cell death *in vivo* in rat models of acute myocardial infarction [33]. This agent was found to have high specificity and stability and a fast renal clearance time [33]. Duramycin has also been radiolabelled with Gadolinium [4]. Conjugation of duramycin to fluorescent molecules has also been employed in the imaging of tumour cells *in vitro* [5] and the targeting of PE expressed on the endothelium of aortic flow dividers [35]. Here duramycin was firstly derivatised through biotinylation and then conjugated to an avidin molecule in a 2 step labelling technique [5, 35, 36]. This indicates that duramycin, at least in theory, has broad practical uses in the targeting of PE on tumour cells.

Conclusion

The current study provides evidence to suggest that upon binding to PE duramycin induces calcium release from cancer cell lines. It is known that duramycin causes ion release from many different cell types however the exact mechanism for this remains to be elucidated. The evidence from this study, and others, suggests the most likely explanation for this ion release is through duramycin mediated non-specific pore formation. The release of calcium from the cancer cells was duramycin concentration dependent. At higher concentrations duramycin caused cancer cells to undergo necrosis. This effect was almost instantaneous, occurring within minutes of duramycin treatment, suggesting a rapid loss of membrane stability. Within this timeframe it is more likely that duramycin's effect is that of forming a pore in the cell membrane rather than a physiological effect. However further study is required to be able to ascertain duramycin's mode of action on calcium release and inducement of cell death in cancer cells. Though yet to be established duramycin has the potential to be an effective anti-tumour agent or PE probe in the targeting of tumours.

Use of Duramycin

Duramycin is not approved for treatment of cancer and so the data presented in this work is purely investigational.

References

1. Stafford JH, Thorpe PE. Increased Exposure of Phosphatidylethanolamine on the Surface of Tumor Vascular Endothelium. Neoplasia. 2011;13(4):299-IN2.

2. Phoenix DA, Harris F, Mura M, Dennison SR. The increasing role of phosphatidylethanolamine as a lipid receptor in the action of host defence peptides. Prog Lipid Res. 2015;59:26-37.

3. Zimmermann N, Freund S, Fredenhagen A, Jung G. Solution structures of the lantibiotics duramycin B and C. Eur J Biochem. 1993;216(2):419-28.

4. Zhao M. Lantibiotics as probes for phosphatidylethanolamine. Amino Acids. 2011;41(5):1071-9.

5. Aoki Y, Uenaka T, Aoki J, Umeda M, Inoue K. A novel peptide probe for studying the transbilayer movement of phosphatidylethanolamine. J biochem. 1994;116(2):291-7.

6. Iwamoto K, Hayakawa T, Murate M, Makino A, Ito K, Fujisawa T, et al. Curvature-Dependent Recognition of Ethanolamine Phospholipids by Duramycin and Cinnamycin. Biophy J. 2007;93(5):1608-19.

7. Hayashi F, Nagashima K, Terui Y, Kawamura Y, Matsumoto K, Itazaki H. The Structure Of PA48009: The Revised Structure. J Antibiot. 1990;43(11):1421-30.

8. Daleke DL. Regulation of transbilayer plasma membrane phospholipid asymmetry. J Lipid Res. 2003;44(2):233-42.

9. Fadeel B, Xue D. The ins and outs of phospholipid asymmetry in the plasma membrane: roles in health and disease. Crit Rev Biochem Mol Biol. 2009;44(5):264-77.

10. van Meer G, Voelker DR, Feigenson GW. Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol. 2008;9(2):112-24.

11. Daleke DL. Phospholipid flippases. J Biol Chem. 2007;282(2):821-5.

12. Sahu SK, Gummadi SN, Manoj N, Aradhyam GK. Phospholipid scramblases: an overview. Arch Biochem Biophys. 2007;462(1):103-14.

13. Zwaal RFA, Schroit AJ. Pathophysiologic Implications of Membrane Phospholipid Asymmetry in Blood Cells. Blood. 1997;89(4):1121-32.

14. Emoto K, Toyama-Sorimachi N, Karasuyama H, Inoue K, Umeda M. Exposure of Phosphatidylethanolamine on the Surface of Apoptotic Cells. Exp Cell Res. 1997;232(2):430-4.

15. Emoto K, Kobayashi T, Yamaji A, Aizawa H, Yahara I, Inoue K, et al. Redistribution of phosphatidylethanolamine at the cleavage furrow of dividing cells during cytokinesis. P Natl Acad Sci USA. 1996;93(23):12867-72.

16. Falls LA, Furie B, Furie BC. Role of Phosphatidylethanolamine in Assembly and Function of the Factor IXa–Factor VIIIa Complex on Membrane Surfaces†. Biochemistry. 2000;39(43):13216-22.

17. Zwaal RFA, Comfurius P, Bevers EM. Lipid–protein interactions in blood coagulation. BBA - Biomembranes. 1998;1376(3):433-53.

18. Rzeźnicka II, Sovago M, Backus EHG, Bonn M, Yamada T, Kobayashi T, et al. Duramycin-Induced Destabilization of a Phosphatidylethanolamine Monolayer at the Air–Water Interface Observed by Vibrational Sum-Frequency Generation Spectroscopy. Langmuir. 2010;26(20):16055-62.

19. Sheth TR, Henderson RM, Hladky SB, Cuthbert AW. Ion channel formation by duramycin. BBA - Biomembranes. 1992;1107(1):179-85.

20. Racker E, Riegler C, Abdel-Ghany M. Stimulation of glycolysis by placental polypeptides and inhibition by duramycin. Cancer res. 1984;44(4):1364-7.

21. Navarro J, Chabot J, Sherrill K, Aneja R, Zahler SA, Racker E. Interaction of duramycin with artificial and natural membranes. Biochemistry. 1985;24(17):4645-50.

22. Oliynyk I, Varelogianni G, Roomans GM, Johannesson M. Effect of duramycin on chloride transport and intracellular calcium concentration in cystic fibrosis and non-cystic fibrosis epithelia. APMIS. 2010;118(12):982-90.

23. Stone D, Xie X, Racker E. Inhibition of clathrin-coated vesicle acidification by duramycin. J Biol Chem. 1984;259(5):2701-3.

24. Nakamura S, Racker E. Inhibitory effect of duramycin on partial reactions catalyzed by sodium-potassium adenosinetriphosphatase from dog kidney. Biochemistry. 1984;23(2):385-9.

25. Roberts M, Hladky S, Pickles R, Cuthbert A. Stimulation of sodium transport by duramycin in cultured human colonic epithelia. J Pharmacol Exp Ther. 1991;259(3):1050-8.

26. Zebedin E, Koenig X, Radenkovic M, Pankevych H, Todt H, Freissmuth M, et al. Effects of duramycin on cardiac voltage-gated ion channels. N-S Arch Pharmacol. 2008;377(1):87-100.

27. Cloutier MM, Guernsey L, Mattes P, Koeppen B. Duramycin enhances chloride secretion in airway epithelium. Am J Physiol-Cell Ph. 1990;259(3):C450-C4.

28. Cloutier MM, Guernsey L, Sha'afi RI. Duramycin increases intracellular calcium in airway epithelium. Mol Membr Biol. 1993;10(2):107-18.

29. Grasemann H, Stehling F, Brunar H, Widmann R, Laliberte TW, Molina L, et al. INhalation of moli1901 in patients with cystic fibrosis*. Chest. 2007;131(5):1461-6.

30. Zeitlin PL, Boyle MP, Guggino WB, Molina L. A phase I trial of intranasal Moli1901 for cystic fibrosis. Chest. 2004;125(1):143-9.

31. Yates KR, Welsh J, Udegbunam NO, Greenman J, Maraveyas A, Madden LA. Duramycin exhibits antiproliferative properties and induces apoptosis in tumour cells. Blood Coagul Fibrinolysis. 2012;23(5):396-401.

32. Larson MC, Woodliff JE, Hillery CA, Kearl TJ, Zhao M. Phosphatidylethanolamine is externalized at the surface of microparticles. BBA - Mol Cell Biol L. 2012;1821(12):1501-7.

33. Zhao M, Li Z, Bugenhagen S. 99mTc-labeled duramycin as a novel phosphatidylethanolamine-binding molecular probe. J Nucl Med. 2008;49(8):1345-52.

34. Yates KR, Welsh J, Echrish HH, Greenman J, Maraveyas A, Madden LA. Pancreatic cancer cell and microparticle procoagulant surface characterization: involvement of membrane-expressed tissue factor, phosphatidylserine and phosphatidylethanolamine. Blood Coagul Fibrinolysis. 2011;22(8):680-7.

35. Li Z, Wells CW, North PE, Kumar S, Duris CB, McIntyre JA, et al. Phosphatidylethanolamine at the luminal endothelial surface--implications for hemostasis and thrombotic autoimmunity. Clin Appl Thromb Hemost. 2011;17(2):158-63.

36. Zhao M, Li Z. A single-step kit formulation for the (99m)Tc-labeling of HYNIC-Duramycin. Nucl Med Biol. 2012;39(7):1006-11.

Legends to Figures

Figure 1. PE expression on 15 different cancer cell lines (6 different cancer types). PE expression is given as median fluorescence intensity (MFI) ratio. All of the cancer cell lines showed detectable levels of PE at relatively low levels with the exception of the multiple myeloma (MM) cell lines (JJN3, MM.1S and U266B1) which had a relatively higher PE expression than the other cancer cell lines. Readings are average, n=2.

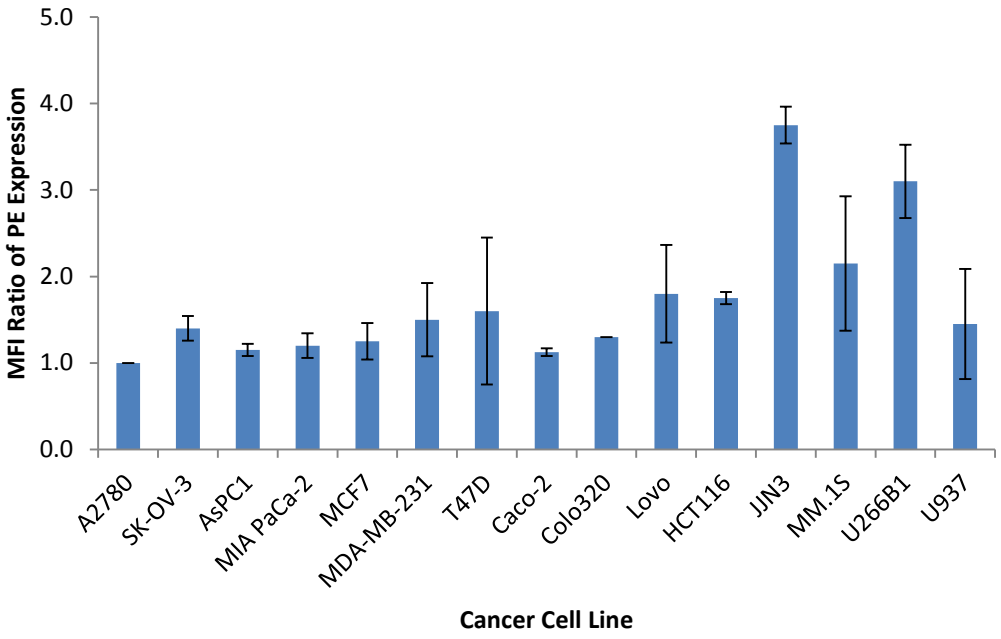

Figure 2. PE detection is duramycin concentration dependent. Optimal PE detection is seen at concentrations of 200µM duramycin and above. However the concentration used in this study to detect PE was 2.5µM due to duramycin's induced cell death at higher concentrations. Readings were performed in triplicate for each data point.

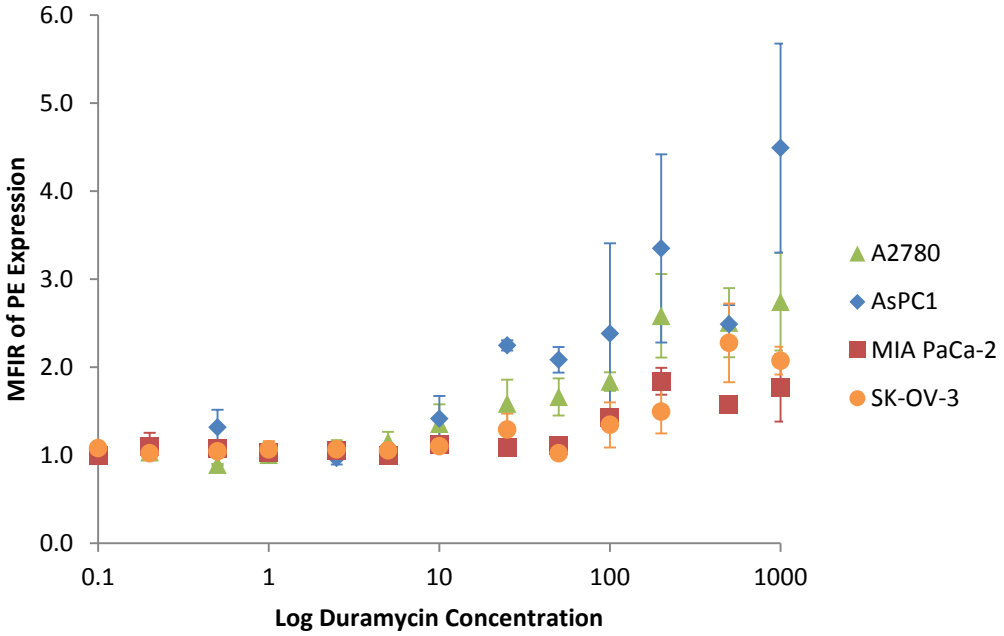

Figure 3. Duramycin mediated cancer cell death is duramycin concentration dependent. Concentrations above 500µM were able to induce 87-96% necrosis in the cancer cell lines. All 4 cancer cell lines showed a uniform threshold concentration for inducement of cell death of 5µM. AsPC-1 appear to have a higher sensitivity to duramycin however this may be due to a naturally lower viability observed in these cells. Readings were performed in triplicate for each data point.

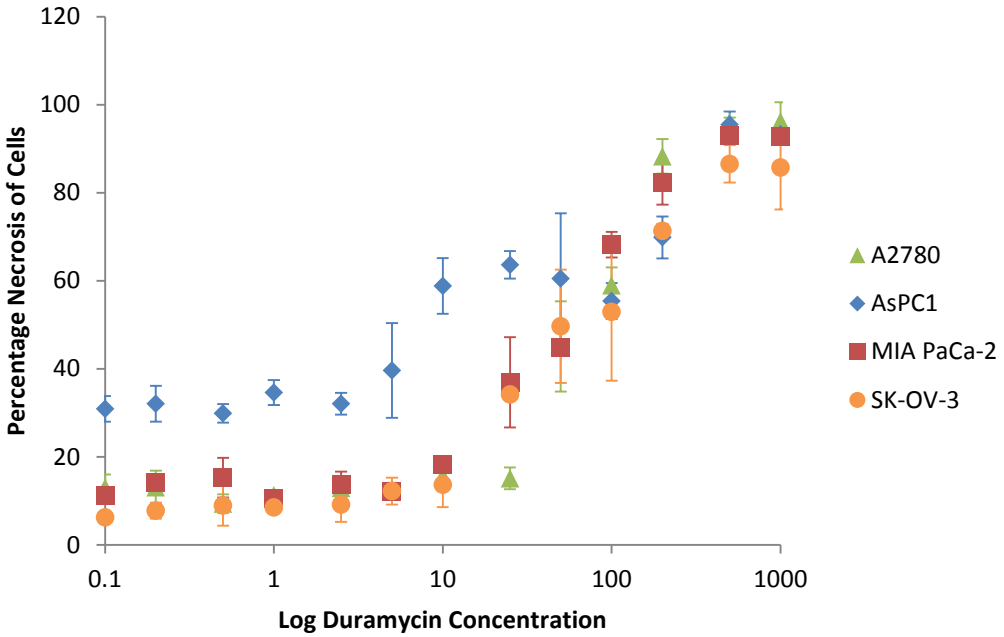

Figure 4. Intracellular calcium ion (Ca^{2+}) release upon treatment with duramycin. The Ca^{2+} release was expressed as a percentage of the total Ca^{2+} released from cells that were treated with the control calcium ionophore A23187. All 4 cell lines released Ca^{2+} in a duramycin concentration dependent manner. The closed data points represent the presence of extracellular calcium and the empty data points the absence. Each data point is the average of n=4.

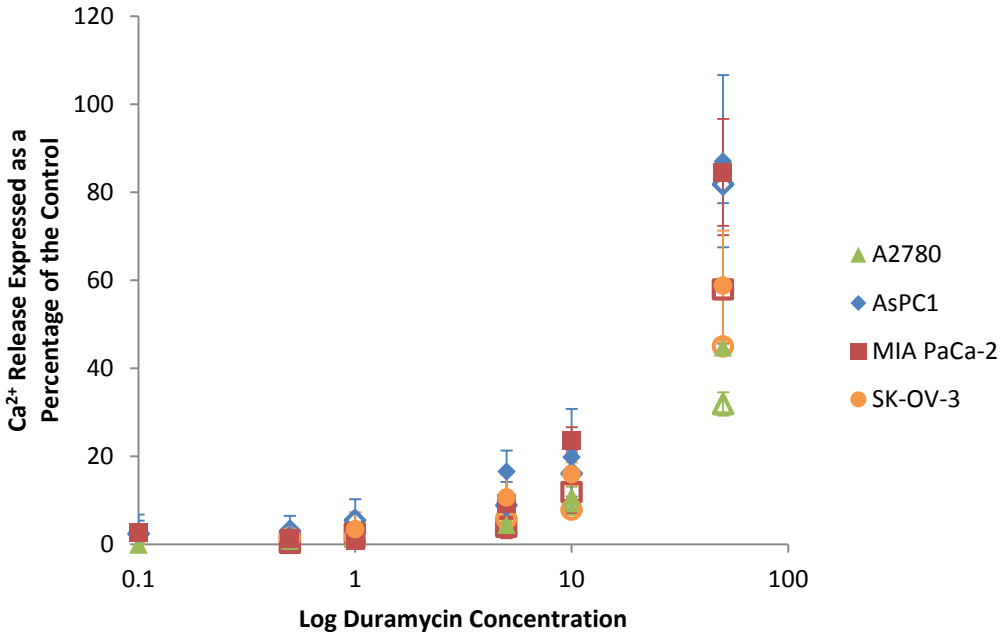

Figure 5. Time taken to reach peak Ca^{2+} release. Time taken to reach peak Ca^{2+} release was duramycin concentration dependent. For all 4 cell lines investigated the time to reach peak calcium release was extended when the cells were in the presence of extracellular Ca^{2+}. The closed data points represent the presence of extracellular calcium and the empty data points the absence. Each data point is an average of n=4.

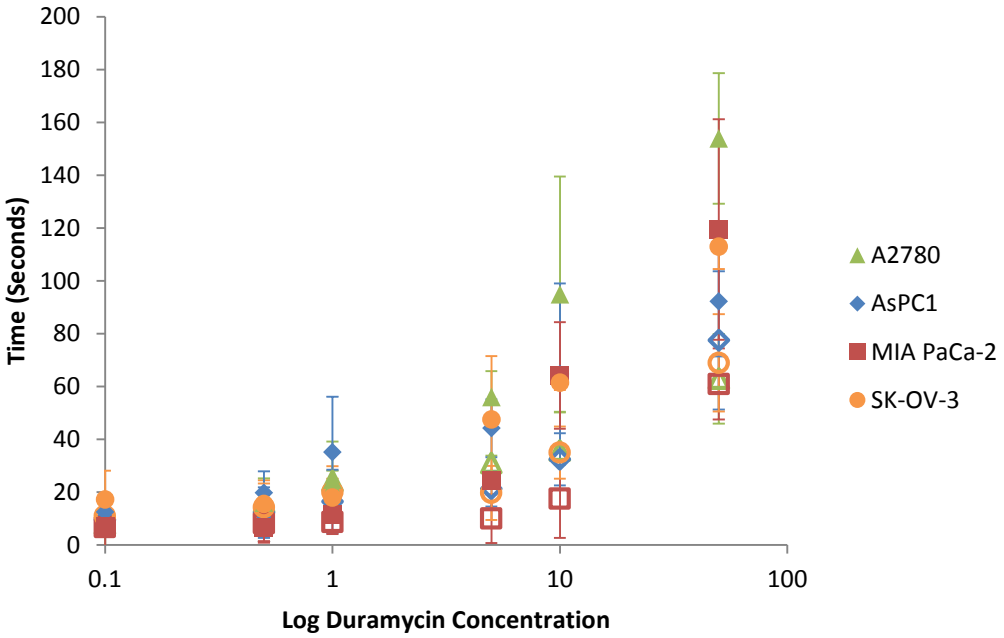

Figure 6. One minute-interval images of AsPC-1 cells over a 20 minute time-course of 50µM duramycin treatment. Cells were stained with cell membrane dye CellVue® Jade and cell nucleus stain propidium iodide (PI). There was an approximate 51% reduction over the 20 minutes of cells retaining membrane staining. Uptake of PI from the buffer solution into the AsPC-1 cells saw an increase of 62% in the number of cells with cell nucleus staining over the 20 minutes.

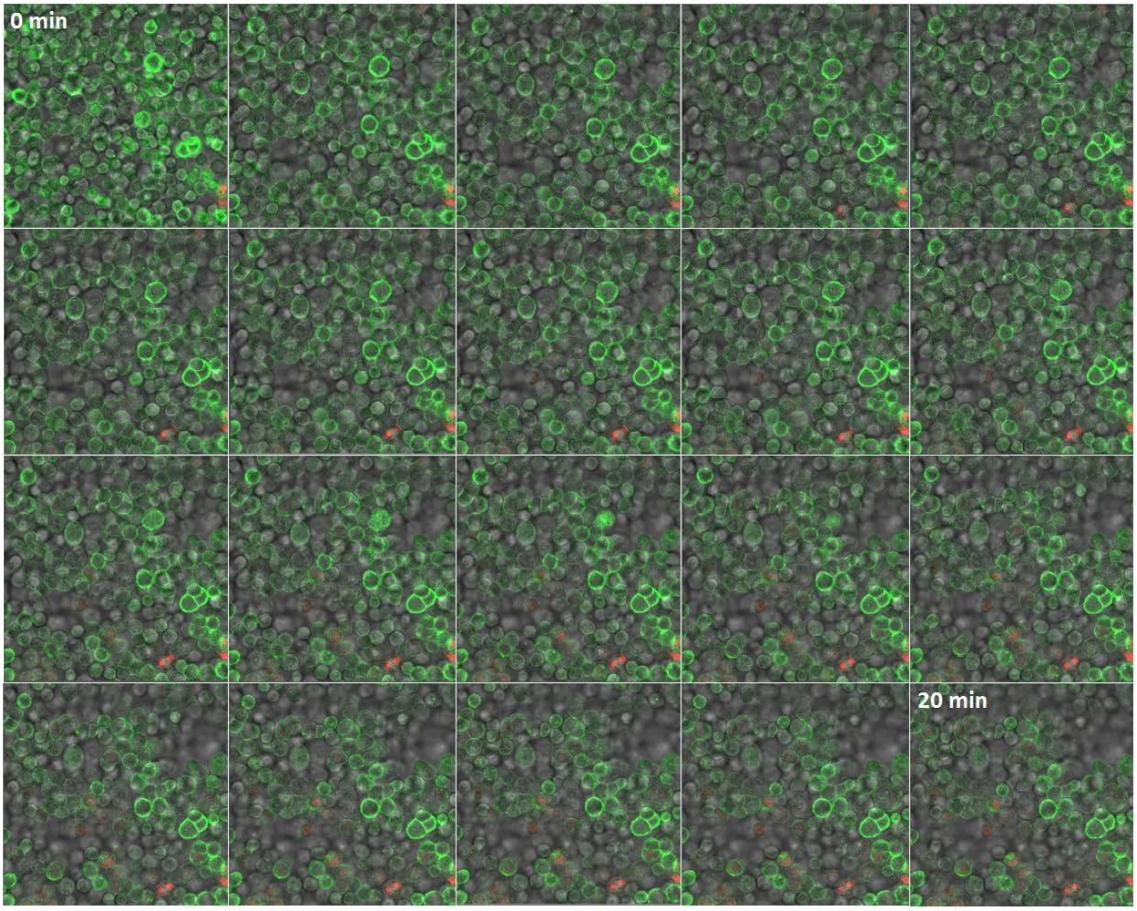

Figure 7. Confocal images of AsPC-1 cells. Cells were stained with cell membrane dye CellVue® Jade and cell nucleus stain PI. There was an approximate 51% reduction over the 20 minutes of cells retaining membrane staining. Uptake of PI from the buffer solution into the AsPC-1 cells saw an increase of 62% in the number of cells with cell nucleus staining over the 20 minutes.

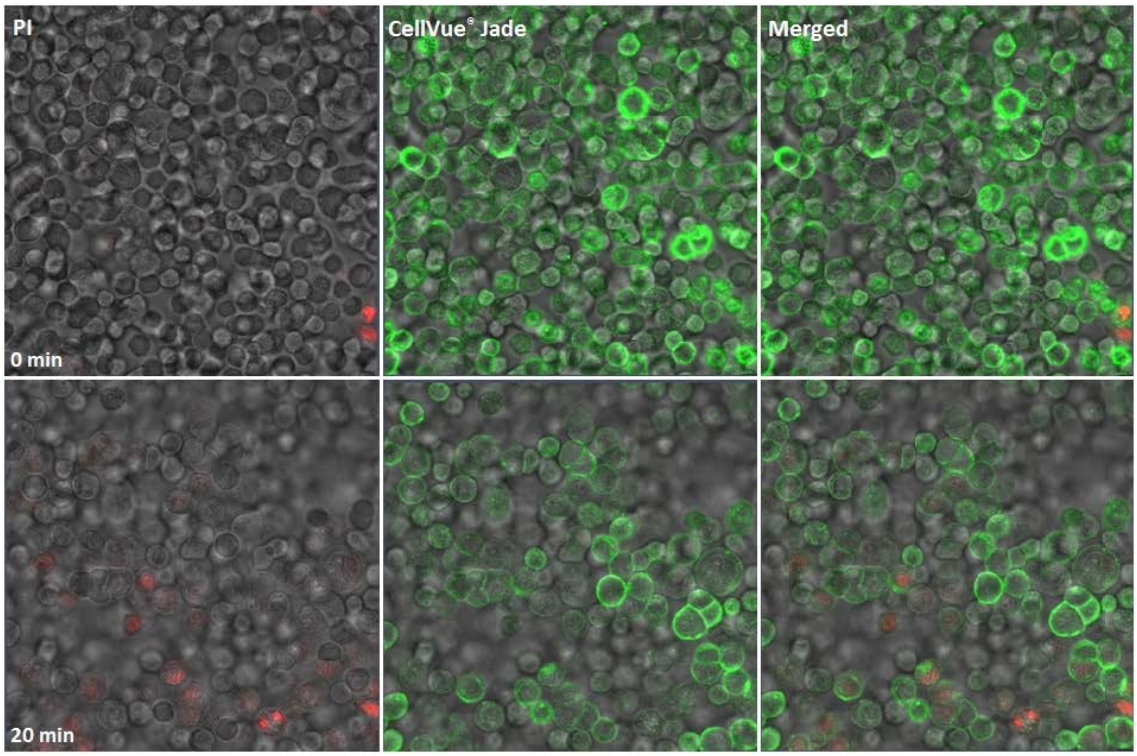

Figure 8. Confocal images of AsPC-1 cells exposed to 50µM duramycin treatment over 30 minutes. Cells were stained for cell membrane (CellMaskTM Orange) and Ca^{2+} (Fluo-3, AM). Over the 30 minutes there was a significant loss in Fluo-3, AM dye suggesting a leakage of Ca^{2+} out of the cell. There was a 13% increase in cell numbers dyed with cell membrane stain over the 30 minutes.

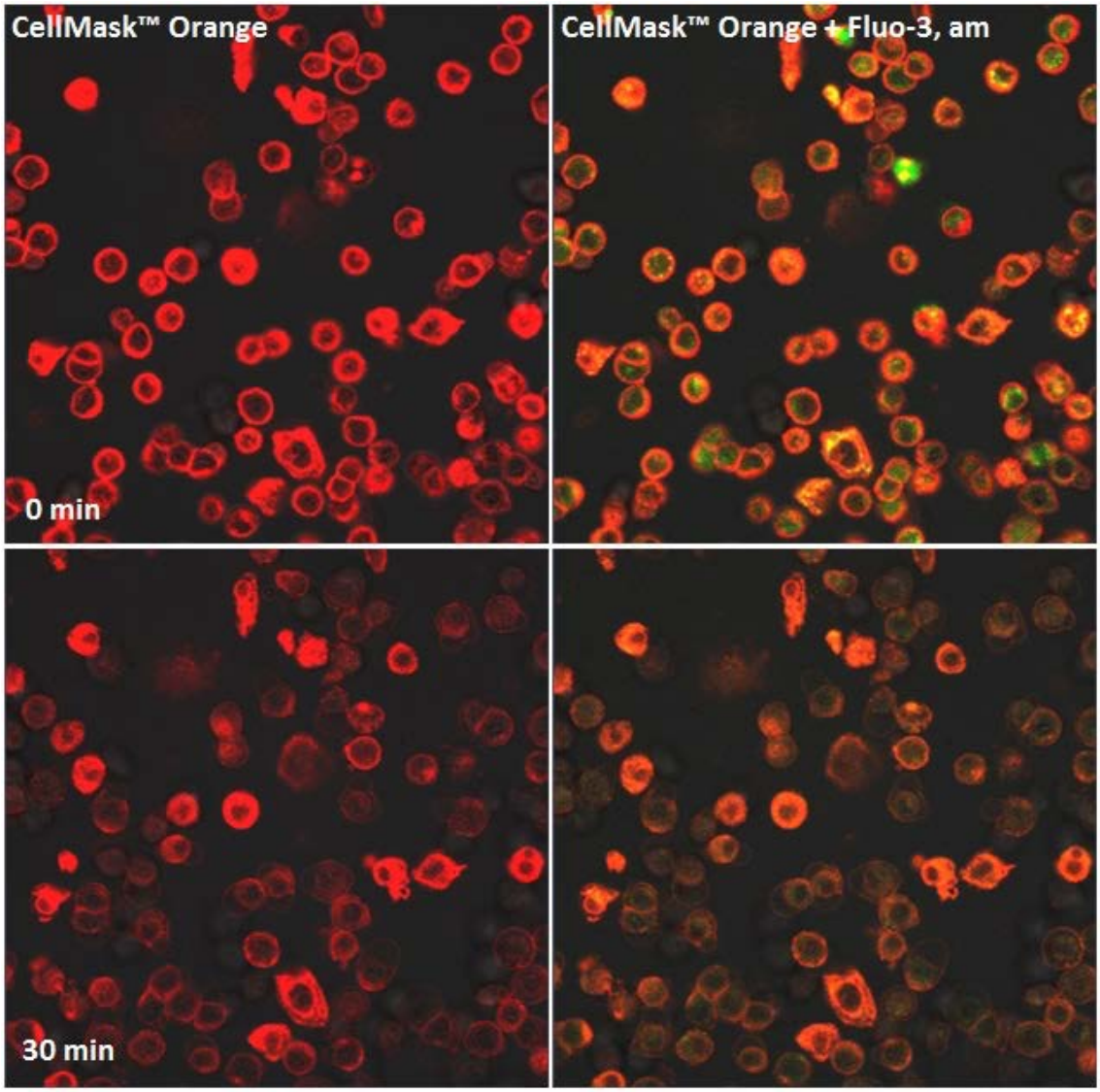

Figure 9. Confocal images of untreated control AsPC-1 cells. Images of cells were taken at 30 and 60 minutes under the same conditions as the duramycin treated AsPC-1 cells when undergoing confocal microscopy. Maintained cell membrane integrity and the Ca^{2+} dye remaining inside the cells can be seen.