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Antimicrobials and In Vitro Systems: Antibiotics and Antimycotics Alter the Proteome of MCF-7 Cells in Culture

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ABSTRACT

Cell culture is widely used to study gene or protein changes in response to experimental conditions. The value of such experiments depends on stringent control and understanding of the in vitro environment. Despite well-documented evidence describing toxic effects in the clinical setting, antibiotics and antimycotics are routinely used in cell culture without regard for their potential toxicity. We cultured MCF-7 breast cancer cells in the presence/absence of antibiotics (penicillin/streptomycin) and/or the antimycotic amphotericin B. Differential protein expression was assessed using 2D-DIGE and MALDI-MS/MS. Antibiotics caused 8/488 spots (1.3% of the protein) to be generally down-regulated. The affected proteins were principally chaperones and cytoskeletal. In marked contrast, amphotericin B induced a more dramatic response, with 33/488 spots (9.5% of the total protein) generally up-regulated. The proteins were mostly involved in chaperoning and protein turnover. Combining antibiotics and amphotericin B had little overall effect, with only one (unidentified) protein being up-regulated. As this study identifies differential protein expression attributable to antibiotics/antimycotics, we urge caution when comparing and interpreting proteomic results from different laboratories where antibiotics/antimycotics have been used. We conclude that as antibiotics and antimycotics alter the proteome of cultured cells in markedly different ways their use should be avoided where possible. J. Cell. Biochem. 112: 2170–2178, 2011.

KEY WORDS: ANTIBIOTICS; AMPHOTERICIN B; CELL CULTURE; FUNGIZONE; PENICILLIN; PROTEOMICS; STREPTOMYCIN

Contamination of cell cultures with bacteria and fungi is a major concern for cell biologists. Consequently, the routine addition of an antimicrobial cocktail, typically consisting of the β-lactam antibiotic penicillin, the aminoglycoside antibiotic streptomycin and the polyene macrolide antimycotic amphotericin B (also known as Fungizone) is commonplace [Lincoln and Gabridge, 1998]. However, there are disadvantages in that over reliance on antimicrobials can lead to poor aseptic laboratory techniques, mask latent infections and lead to the development of resistant bacteria and fungi.

There is also a substantial body of evidence that demonstrates that antimicrobials are toxic, both in vivo and in vitro. In the clinical setting, neurotoxicity is caused when the normal binding of γ-aminobutyric acid to its receptors is compromised by β-lactam antibiotics [Chow et al., 2005]. Levels of aminoglycoside-induced nephrotoxicity have been assessed at 10–25% [Laurent et al., 1990; Leehey et al., 1993] and ototoxicity at 2–45% [Fausti et al., 1999; Matz, 1993]. In cell culture, penicillin, gentamicin, and streptomycin have variously been shown to impair the proliferation and differentiation of embryonic stem cells, decrease nuclear maturation in murine oocytes, alter calcium homeostasis and induce apoptosis in renal cell lines [Bird et al., 1994; Cohen et al., 2006; Lemeire et al., 2007]. Amphotericin B is also toxic, its propensity to induce nephrotoxicity, anaphylaxis, phlebitis and other complications driving the development of alternative formulations and delivery mechanisms in medicine [Hartsel and Bolard, 1996]. In the laboratory, a variety of toxic effects have been attributed to amphotericin B, from inducing apoptosis in proximal tubular and medullary interstitial cells to inhibiting the infectivity of larval parasites [Varlam et al., 2001; Mone et al., 2010].

Although it is clear that the toxic effects of antimicrobials are multifaceted, no study (to our knowledge) has previously been carried out to assess how typical concentrations of commonly used antimicrobials affect the proteome of cells in culture. This is an important question because cell culture is widely used to model in vivo systems, and consequently its value is dependent on the culture

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conditions accurately representing those found in vivo. We addressed this question by culturing the widely used MCF-7 breast cancer cell line with typical concentrations of (i) a penicillin/streptomycin mixture, (ii) amphotericin B, (iii) a combination of penicillin/streptomycin and amphotericin B, and (iv) no antimicrobials. We then used two-dimensional difference gel electrophoresis (2D-DIGE) followed by matrix assisted laser desorption and ionization tandem mass spectrometry (MALDI-MS/MS) to identify proteins whose expression levels were altered.

MATERIALS AND METHODS

REAGENTS AND APPARATUS

The MCF-7 breast cancer cells were a gift from Julia Gee, Welsh School of Pharmacy, Cardiff University, United Kingdom. Cell culture reagents were purchased from Gibco, Paisley, UK. FACS was carried out using a LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ). The 2-D Clean-Up kit, 2-D Quant kit, CyDye DIGE Fluor minimal dyes and reagents, the isoelectric focussing Immobiline Drystrips and IPG buffer were all from GE Healthcare, Little Chalfont, UK. The Criterion gels and running buffer were from Bio-Rad, Hemel Hempstead, UK. Isoelectric focusing was carried out in an Ettan IPGphor 3 (GE Healthcare), the gels were imaged using a Dyversity imaging system (Syngene, Cambridge, UK) and the mass spectrometry was carried out in an Ultraflex III MALDI ToF/ToF (Bruker Corporation, Coventry, UK).

CELL CULTURE

MCF-7 cells, certified mycoplasma-free by the European Collection of Cell Cultures, Salisbury, UK, were cultured for five passages in RPMI-1640 containing 10% FCS and 2 mM Glutamax® to expunge the effects of historical culture in medium containing antibiotics and antimycotics. Each passage consisted of seeding 650,000 cells in 75 cm^2 vented culture flasks with 15 ml culture medium then culturing them to 70% confluence. The cells were counted prior to seeding using a haemocytometer. To test for the effects of antimicrobials, cells from the 5th passage in antimicrobial-free medium were cultured for a further 8 passages in the same medium with the addition of (i) Penicillin 100 Units/ml and Streptomycin 100 μg/ml, source-format (Ab^{+ve}), (ii) Amphotericin B 2.5 μg/ml, source-format (My^{+ve}), (iii) Penicillin 100 Units/ml, Streptomycin 100 μg/ml and Amphotericin B 2.5 μg/ml (Ab/My^{+ve}), and (iv) no additives (Neg). Thus, all the antimicrobials were added at the manufacturer's recommended concentrations. Each culture condition (i–iv) was performed in quadruplicate without pooling between passages, so there were 16 culture flasks in total, representing four biological replicates for each culture condition. Each culture flask was checked daily under an inverted compound microscope for evidence of microbial contamination and at each passage the cell mortality rate was assessed by trypan blue exclusion.

CELL CYCLE ANALYSIS

In order to establish whether the antimicrobials induced changes to the cell cycle, a second series of four biological replicates of Neg, Ab^{+ve}, My^{+ve}, and Ab/My^{+ve} were cultured. Aliquots containing 1.5×10^6 cells were fixed for 1 h in a 75% ethanol/3.75% acetic acid solution at −20 °C. The cells were then washed, re-suspended in 1 ml of staining solution (PBS containing 50 μg/ml propidium iodide, 3.8 mM sodium citrate and 0.5 μg/ml RNase A) and left overnight in the dark at 4 °C. Cells at different stages of their cell cycle were detected by flow cytometry, with 50,000 cells being analyzed per preparation after cellular debris and doublets had been excluded by gating from the FSC/SSC and the 610/20 YG-W/ 610/20 YG-A plots respectively. The percentage of cells in G_1, S and G_2/M phase were calculated using FloJo analysis software (v 9).

PROTEIN EXTRACTION AND LABELING

For the final passage, cells were cultured in 10 cm diameter culture dishes. When 50-90% confluent, the cells were washed three times with PBS then lysed in the culture dish with 1.5 ml of cell lysis solution (7 M urea, 2 M thiourea, 30 mM Tris base and 4% CHAPS). Total cell lysis was verified using an inverted compound microscope. A cell scraper was used to ensure that all cellular debris was recovered. Insoluble material and contaminants likely to interfere with electrophoresis were removed with a 2-D Clean-Up kit and then protein concentrations assayed using a 2-D Quant kit. The pH of each sample was assessed using pH 7.5-9.5 indicator strips and adjusted to pH 8.5 using 50 mM NaOH. Aliquots containing 50 μg protein from each sample were then labeled with 400 pmol CyDye DIGE Fluor minimal dyes according to the manufacturer's protocol, such that two of the quadruplicates of each preparation (Ab^{+ve}, My^{+ve}, Ab/My^{+ve}, and Neg) were labeled with Cy3 and two labeled with Cy5. An internal standard, pooled from 25 μg of each sample (400 μg in total) was labeled with Cy2. The labeling reaction was carried out on ice for 30 min then terminated by adding 1 μl of 10 mM lysine per reaction. Eight sample mixtures were then prepared, such that each mixture contained one Cy3-labeled sample, a Cy5-labeled sample from a different culture condition and 50 μg of the Cy2-labeled internal standard (150 μg in total).

TWO-DIMENSIONAL ELECTROPHORESIS

Isoelectric focussing was carried out on 11 cm, pH 3-11, non-linear Immobiline Drystrips. Each strip was re-hydrated overnight in 200 μl of rehydration solution (8 M urea, 2% CHAPS, 280 mM DTT, 0.05% bromophenol blue and 0.5%, pH 3-11 IPG buffer), and then transferred to the manifold of the Ettan IPGphor 3. The sample mixtures were loaded onto the strips using the IPGphor cup-loading protocol and then separated by charge using the following protocol: (i) 500 V for 6 h, (ii) a gradient to 1000 V over 1 h, (iii) a gradient to 6000 V over 2 h, and (iv) 6000 V for 9.5 h (69,620 Vh in total). Immediately following isoelectric focussing, the strips were incubated for 20 min in equilibrium solution (6 M Urea, 75 mM Tris-HCl, 30% glycerol, 2% SDS and 0.01% bromophenol blue) containing 1% DTT and then for a further 30 min in equilibrium solution containing 4% iodoacetamide. The strips were then immediately transferred to 8.7 × 13.3 cm Criterion XT, 4–12% Bis-Tris precise gels, which were run in XT-MES running buffer at 150 V for 90 min, until the dye ran to the bottom of the gel.

IMAGE ACQUISITION AND ANALYSIS

The gels were imaged on a Dyversity imaging system, with the exposure time optimized to obtain the maximum dynamic range.

The gel images were exported to Progenesis SameSpots v. 3 (Nonlinear Dynamics, Newcastle, UK) and the spot patterns aligned using the program's automatic alignment vector calculations after seven landmark vectors were set manually. The aligned images were then grouped according to the culture conditions (Ab^{+ve}, My^{+ve}, Ab/My^{+ve}, and Neg) and the spots ranked according to the greatest fold change across the groups at 95% confidence level, using the pooled internal standard as a reference. Spots that were differentially expressed in either the Ab^{+ve}, the My^{+ve}, or the Ab/My^{+ve} group compared with the Neg group were identified by the software and ranked by p-value from one way ANOVA analysis.

GENERATING PROTEIN IDENTITIES USING MALDI-MS/MS

In order to visualize the spots that were differentially expressed, the gels were re-stained with Instant Blue (Expedeon, Harston, UK) and the differentially expressed spots excised manually using a spot cutter (The Gel Company, Tübingen, Germany). Gel pieces were washed two times with 50% aqueous acetonitrile containing 25 mM ammonium bicarbonate, then once with acetonitrile and dried in a vacuum concentrator (Thermo Scientific, Basingstoke, UK) for 20 min. Sequencing-grade, modified porcine trypsin (Promega, Southampton, UK) was dissolved in the 50 mM acetic acid, then diluted 5-fold by adding 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.02 μg/μL. Gel pieces were re-hydrated by adding 10 μL of trypsin solution, and after 30 min enough 25 mM ammonium bicarbonate solution was added to cover the gel pieces. Digests were incubated overnight at 37 °C. A 1 μL aliquot of each peptide mixture was then applied directly to the ground steel MALDI target plate, followed immediately by an equal volume of a freshly-prepared 5 mg/ml solution of 4-hydroxy-α-cyano-cinnamic acid in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid.

Positive-ion MALDI mass spectra were obtained using a Bruker Ultraflex III in reflectron mode, equipped with an Nd:YAG smart beam laser. MS spectra were acquired over a mass range of m/z 800-4000. Final mass spectra were externally calibrated against an adjacent spot containing 6 peptides (des-Arg^1-Bradykinin, 904.681; Angiotensin I, 1296.685; Glu^1-Fibrinopeptide B, 1750.677; ACTH (1-17 clip), 2093.086; ACTH (18-39 clip), 2465.198; ACTH (7-38 clip), 3657.929). Monoisotopic masses were obtained using a SNAP averaging algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and a signal-to-noise ratio threshold of 2.

For each spot, the ten strongest peaks of interest with a signal-to-noise ratio greater than 30 were selected for MS/MS fragmentation. Fragmentation was performed in LIFT mode without the introduction of a collision gas. The default calibration was used for MS/MS spectra, which were baseline-subtracted and smoothed (Savitsky-Golay, width 0.15 m/z, cycles 4); monoisotopic peak detection used a SNAP averaging algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) with a minimum signal-to-noise ratio of 6. Brukerflex Analysis software (version 3.0) was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra.

Tandem mass spectral data were submitted to database searching against using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.1), through the Bruker BioTools interface (version 3.2). Search criteria included: Enzyme, Trypsin; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M); Peptide tolerance, 250 ppm; MS/MS tolerance, 0.5 Da; Instrument, MALDI-TOF-TOF. Searches were made against the IPI Human v3.x and NCBInr databases. Spot identities were accepted if their total ion scores were such that a significance threshold of 99% was exceeded.

RESULTS

CELL CULTURE

We are satisfied that our results were not influenced by microbial infections. There was no visual evidence of any contamination in any of our cultures and nor was there a difference in the proportion of dead cells in any of the preparations ($< 2\%$ in all instances). There were no unique spots in any of the gels that would suggest a potential microbial infection in that culture and nor were there any microbial proteins identified when we carried out MS/MS searches against the NCBInr database.

CELL CYCLE ANALYSIS

In order to minimize preparation-induced variation, all sixteen cultures were processed simultaneously for DIGE. It was therefore impossible to avoid between-culture variations in confluence at the point of cell lysis. Consequently, a cell cycle analysis was carried out to establish whether there were any differences in cell cycle phase between the cultures that might account for differential protein expression. In Neg cultures, 41.8% of the cells were in G_1, 35.5% in S and 21.7% in G_2/M phase (Supplementary Figure 1). There were no statistically significant differences between these percentages and those in any of the antimicrobial-containing cultures ($P = 0.74$–0.14 by Student's t-test).

AMPHOTERICIN B ALTERS PROTEIN EXPRESSION LEVELS IN CULTURED CELLS MORE THAN PENICILLIN/STREPTOMYCIN

In total, 428 different spots were detected in the gels. Although the normalized volume of each spot varied from gel to gel, all of the spots were present in all of the gels. Therefore, there was inter-sample variation in protein expression levels but there were no instances where a protein was present in one culture but not in another. In order to compare the spot patterns between the groups, the normalized spot volume for each spot in the Ab^{+ve}, My^{+ve}, Ab/My^{+ve}, and Neg set of gels was averaged by one-way ANNOVA and any spots in either the Ab^{+ve}, My^{+ve}, or Ab/My^{+ve} group of gels with a statistically different volume compared with the Neg gels were highlighted. In the Ab^{+ve} gels, 8 spots, totaling 1.3% of the total spot-volume, were differentially expressed compared with the Neg gels. In My^{+ve} gels, 33 spots representing 9.5% of the total spot volume were differentially expressed. The Ab/My^{+ve} gels were the most similar to Neg gels in that only one spot (0.2% of the total volume) was up-regulated. This spot was also differentially expressed in both the Ab^{+ve} and the My^{+ve} gels. Four of the seven remaining Ab^{+ve} spots were also amongst those differentially expressed in the My^{+ve} gels. In addition to numbers of spots, the spot volume dynamics differed between the preparations. In the Ab^{+ve} gels, 7 out of the 8 spots were down-regulated compared with those

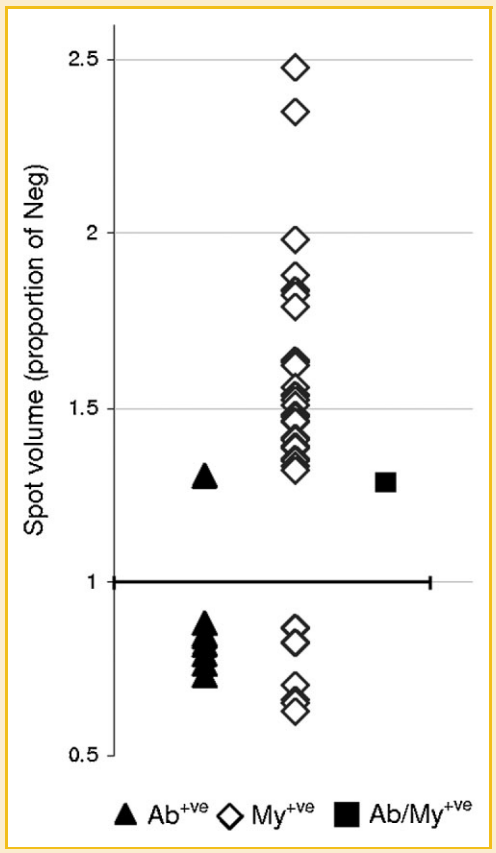

Fig. 1. Differential protein expression in cultures containing antibiotics (Ab+ve), amphotericin B (My+ve) and both (Ab/My+ve) compared with negative controls (Neg). In Ab+ve cultures, 7 out of 8 spots were down-regulated. In contrast, expression levels were generally up-regulated in My+ve cultures, where 7 out of 27 gel spots had an increased normalized volume. The magnitude of the expression changes was greater in My+ve cultures than in Ab+ve cultures.

in Neg and only 1 spot was up-regulated. The My^{+ve} gels were different in that 27 spots were up-regulated compared to 7 that were down-regulated. The fold-change in spot volume was also more extensive in My^{+ve} gels, with dynamic range of 63–247% compared to 73–131% in Ab^{+ve} gels (Fig. 1). The Ab/My^{+ve} spot was 130% the size of that in the Neg gels.

PROTEIN IDENTITIES

Identities were sought for all of the differentially expressed spots in the experiment. Identities were assigned to 7 of the 8 Ab^{+ve} spots (84% of the protein) and 18 of the 33 My^{+ve} spots (53% of the protein) but the Ab/My^{+ve} spot was not identified. Several spots were found to be different isoforms of the same protein (such as β actin, HSP70 and C-1-tetrahydrofolate synthase), so in total, 15 different proteins were identified. These protein identities and their expression levels relative to Neg are shown in Table I. A copy of a representative gel detailing the excised spots of interest is shown as Supplementary Figure 2. The MALDI-MS/MS peptide data with ion scores etc. are in Supplementary Table I. Most of the spots had a single identity but two spots generated two identities: spot 15 (cofilin-1 and a proteasome β2 subunit) and spot 12 (ubiquitin-activating enzyme E1 and alanyl-tRNA synthase). All these identities were generated from at least two MS/MS peptides with statistically significant ion scores. Also, their spot-locations were appropriate for the mass and charge of the full-length proteins. Therefore, we conclude that the double-identities result from both proteins occupying the same coordinates in the gel, and consequently both identities were accepted. However, it is not known whether one or both of the proteins were differentially expressed.

In order to relate the protein changes to biological function, proteins were assigned to one of five functional categories based on their Gene Ontology classification, with a further category for unidentified spots (Fig. 2). In terms of fold-change in the Ab^{+ve} cultures, chaperones were the most affected, with three isoforms of HSP70 together constituting 36% of the total differentially expressed protein in the gels. The chaperone group was followed by the cytoskeletal group, where two isoforms of β actin and the actin-depolymerization protein cofilin 1 together accounted for 30% of the protein. The protein turnover category (alanyl-tRNA synthetase, a proteasomal subunit and an E1 ubiquitin-activating enzyme) made up 18% of the total differentially expressed protein. All of these were down-regulated compared with the Neg gels. The single, up-regulated protein was not identified. In marked contrast, My^{+ve} cultures were characterized by the general up-regulation of a more complex set of proteins that were assigned to five functional categories. As in the Ab^{+ve} gels, chaperones were the most effected (21% of the total), but this time the cytoskeletal category was less significant, with the (two) actin isoforms being up-regulated and no change in cofilin 1 expression.

The protein turnover category accounted for 18% of the altered protein in the My^{+ve} gels (the second most affected category), but a completely different set of proteins was differentially regulated: tetrahydrofolate synthase, transitional endoplasmic reticulum ATPase (*aka* valosin-containing protein), neutral α-glucosidase and NEDD8 chain B (a member of the ubiquitin family of proteins). Uniquely, the My^{+ve} gels included categories for a (down-regulated) signaling protein (acidic leucine-rich nuclear phosphoprotein 32) and an (up-regulated) enzyme that is part of the glycolysis/gluconeogenesis pathways (pyruvate kinase).

DISCUSSION

ANTIBIOTICS IN CULTURE MEDIUM

Since penicillin was discovered in 1928 [Fleming, 1929], a multitude of antibiotics, principally aminoglycosides, β-lactams and fluoroquinolones, have been and continue to be developed to combat the growing number of resistant strains of bacteria. The different classes of antibiotic are defined by their structure, and therefore their modes of action. In cell culture, the routine addition of a mixture of penicillin (a β-lactam) and streptomycin (an aminoglycoside) is commonplace, with the frequent inclusion of gentamicin (another aminoglycoside).

Penicillin and other β-lactams interfere with bacterial cell wall synthesis, causing changes in cell size/shape, the onset of stress responses and ultimately cell lysis [Tomasz, 1979]. They bind to and inhibit the transpeptidases (also known as penicillin-binding

TABLE I. Protein Identities and Their Relative Expression Levels in Cultures Containing Penicillin/Streptomycin (Ab^{+ve}) and Amphotericin B (My^{+ve}) Compared to the Cultures That Were Antimicrobial-Free (Neg). The Spot Numbers are Annotated on the Gel in Supplementary Figure 2.

ID	Ab^{+ve} (% of Neg)	My^{+ve} (% of Neg)	Spot no.
Acidic leucine-rich nuclear phosphoprotein 32		70.3	11
Actin	76.5		17
Actin	82.0	141.2	4
Actin		140.7	5
Alanyl-tRNA synthetase	88.0		12
C-1-tetrahydrofolate synthase		187.1	1
Cofilin-1	73.0		15
HSP10		65.1	16
HSP70	84.4	140.8	7
HSP70		147.4	9
HSP70	84.7	135.4	8
HSP70	79.4	134.9	6
HSP90		155.9	10
Hypoxia up-regulated protein		188.1	2
NEDD8		62.5	14
Neutral alpha-glucosidase		139.1	18
Proteasome beta subunit	73.0		15
Pyruvate kinase		147.9	3
Transitional endoplasmic reticulum ATPase		153.6	13
Ubiquitin-activating enzyme E1	88.0		12

proteins) that cross-link adjacent peptide strands of peptidoglycan units undergoing synthesis [Park and Uehara, 2008]. The peptidoglycan cross-links form a mechanically reinforcing polymer matrix between the inner and outer lipid bilayer of the bacterial cell wall, with the structural integrity of the bacterium directly correlating with the amount of peptidoglycan cross-linking present [Holtje, 1998]. Specifically, the β-lactam cyclic amide ring is an analogue of the peptidoglycan d-alanyl-d-alanine dipeptide, enabling it to act as a substrate for the transpeptidase, blocking its activity [Kohanski et al., 2010].

Aminoglycosides were first used as antibiotics in the 1940s. Their mechanism of action has been extensively studied, and in the 1980s it was established that they work by binding to the 16S subunit of the 30S bacterial ribosome [Moazed and Noller, 1987]. This interferes with translation by preventing ribosomal discrimination between normal and abnormal mRNA-tRNA complexes, leading to

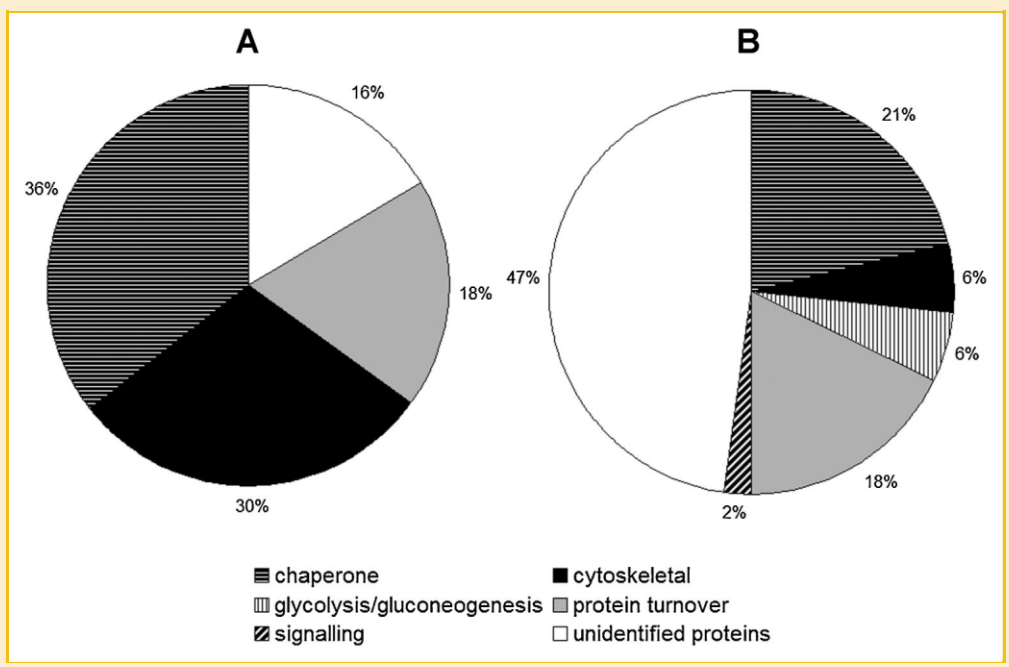

Fig. 2. Altered protein expression expressed by functional category when MCF-7 cells were cultured with antibiotics (A) or amphotericin B (B). A: Chaperones and cytoskeletal proteins accounted for 66% of the altered proteome when a penicillin/streptomycin solution was added to the culture medium. B: A more complex response was generated by the addition of amphotericin B: the chaperone and cytoskeletal categories were less significant, but proteins involved in glycolysis/gluconeogenesis and signaling were now differentially expressed.

the accumulation of non-functional proteins and cell death [Francois et al., 2005]. In the last few years other modes of action have been discovered: aminoglycosides can interfere with the stability of the 70S ribosomal subunit, impairing the translocation of tRNAs [Hirokawa et al., 2007] and inhibiting the acylation of transfer-messenger RNA (tmRNA) [Corvaisier et al., 2003].

Thus, penicillin and streptomycin have very different modes of action and long histories of clinical use. These reasons presumably account for their routine inclusion in culture medium. We have found that typical concentrations of penicillin and streptomycin in cell culture medium alter protein expression in three fundamental cellular processes: maintenance of the cytoskeleton, chaperoning and protein turnover. Cofilin 1 (which regulates assembly and disassembly of actin filaments in non-muscle tissue) and actin were down-regulated in Ab^{+ve} cultures. Although there is no published literature specifically linking actin or cofilin to antibiotics in culture, streptomycin-induced aberrant spindle formation has been reported in murine oocytes, leading to mitotic arrest during metaphase [Lemeire et al., 2007]. Immunocytochemical and ultrastructural techniques have been used to localize actin to the spindle [Gadde and Heald, 2004; O'Connell and Khodjakov, 2007], and actin is also a major integral component of the actomyosin system, where actin filaments associate with spindle microtubules. Furthermore, an actin inhibitor has been shown to induce chromosomes to detach or misalign from the spindle in a marsupial, kidney cell line [Snyder et al., 2009]. Therefore, it is possible that the streptomycin-induced metaphase complications recorded by Lemeire et al. (2007) were in part caused by the down-regulation of actin and cofilin 1 that we have seen.

HSP70 is involved in numerous cellular processes, including mediating the correct folding of newly synthesized proteins, refolding misfolded proteins and preventing protein aggregation [Mayer and Bukau, 2005]. Our finding that three HSP70 isoforms were down-regulated by 16-21% in response to antibiotics is in apparent contrast with a previous study where expression of HSP70 remained unaltered when human lymphocytes were cultured with/without penicillin and streptomycin (at the same concentrations we used) [Rao et al., 1999]. However, Rao et al. assayed over a four-hour period and used pixellation following western blotting as a measure of protein expression. We were culturing over a considerably longer period of time and using a more sensitive method of differential protein expression analysis that is capable of assessing expression levels of individual HSP70 isoforms (2D-DIGE). As such, we would expect to able to detect more subtle changes. In addition, we are using epithelial cells in this study, which may be differentially affected by antimicrobial agents. There is evidence however, that HSP70 activity is impaired by gentamicin, which blocks the HSP70 peptide-binding domain, preventing it interacting with substrate proteins, with some inhibitory effects occurring at 66 μM (the streptomycin concentration in our culture medium) [Yamamoto et al., 2010]. Consequently, there is clearly the potential for a detrimental steptomycin-HSP70 interaction in cultured cells.

The other functional category where protein expression was altered (also down-regulated) in Ab^{+ve} cultures was that of protein turnover. In addition to an E1 ubiquitin-ligase and a proteasomal subunit, both involved in the ubiquitin-proteasome pathway (UPP), we have included alanyl-tRNA synthetase (AlaRS) in this category. AlaRS is a member of the aminoacyl-tRNA synthetase family and it catalyses the acylation reaction that results in the attachment of alanine to its cognate tRNA molecule during translation. In bacteria, this is a process targeted by aminoglycosides via transfer-messenger RNA (tmRNA), a specialized bacterial RNA with dual tRNA and mRNA-like roles that rescues ribosomes that have stalled [Karzai et al., 2000]. In a process called *trans*-translation, AlaRS catalyses the aminoacylation of tmRNA by adding an alanine residue to its 3' end [Komine et al., 1994; Ushida et al., 1994]. The tmRNA-alanine conjugate then enters the stalled ribosome and the alanine is transferred to the nascent peptide's carboxyl-terminus. A tag encoded in the tmRNA's open reading frame is then translated and this is also added to the peptide. The addition of the alanine residue and the tag frees the ribosome, enabling translation to complete. The ribosomal subunits then disassociate for re-use and the problematic, now-tagged protein can be degraded by proteases such as ClpXP and ClpAP [Flynn et al., 2001; Moore and Sauer, 2007; Hayes and Keiler, 2010].

As the tmRNA system is unique to prokaryotes it has attracted the attention of researchers interested in antibiotics, and consequently it was established that each of six tested aminoglycosides inhibited the aminoacylation of tmRNA in a dose-dependent manner [Corvaisier et al., 2003]. Although the mechanism of impairment was not established and streptomycin was not amongst the panel of aminoglycosides tested, it is noteworthy that we found the same protein responsible for the aminoacylation of tmRNA in bacteria (AlaRS) to be down-regulated in Ab^{+ve} cultures. We did not find alterations in expression levels of other aminoacyl-tRNA synthetases, but it is likely that a down-regulation in AlaRS would inhibit translation generally, reducing protein expression, possibly in conjunction with an increase in the translation error rate. However, as an E1 ubiquitin ligase and a proteasomal subunit were also down-regulated in Ab^{+ve} cultures, there is no evidence for a corresponding increase in the components of the UPP. If the reduction in AlaRS expression led to a build-up of incorrectly folded proteins, then an increase rather than a reduction in chaperone expression would also be expected.

AMPHOTERICIN B (FUNGIZONE) IN CULTURE MEDIUM

Amphotericin B (also known as Fungizone) is a very effective polyene antimycotic, which is used clinically to treat deep-seated infections despite its well-characterized side effects, including nephrotoxicity, renal failure and infusion-related toxicity [Arning et al., 1995; Wingard et al., 1999; Rex et al., 2000; Bates et al., 2001]. When incorporated into membranes, amphotericin B molecules aggregate into pores that cause leakage of intracellular ions, leading to cell death. Its specificity is based on the principle that the pore formation is more effective in ergosterol-containing fungal membranes than in the cholesterol-containing membranes of humans [Ghannoum and Rice, 1999]. This paradigm is supported by a recent study in which 2-D PAGE and MALDI-MS were used to describe proteomic changes in *Candida albicans* in response to culture in clinically sub-optimal doses of amphotericin B [Hoehamer et al., 2010]. In the Hoehamer et al., *C. albicans* paper, the authors describe an up-regulation of proteins functionally related to the maintenance of cellular osmolarity, oxidative stress and carbohy-

drate synthesis when amphotericin B was present in the culture medium. We used an amphotericin B concentration that is typical in mammalian cell culture, which nevertheless was nearly 100 times greater than that used in the *C. albicans* study (2.5 versus 0.029 μg/ml). We found a similar percentage of the total number of proteins to be differentially expressed (*c.* 10% of the identified protein) but our identities were different, with protein turnover and cytoskeletal proteins mostly affected. A larger proportion of proteins in our study were also down-regulated. Although some of our different identities could be attributed to the more sensitive techniques we used (DIGE and tandem mass spectrometry), the two studies still highlight the selectivity of amphotericin B in terms of effective dose and demonstrate that amphotericin B affects cellular processes differently in the mammalian proteome vis-à-vis the fungal proteome.

We found chaperones to be the most altered proteins in My^{+ve} cultures, with the greatest up-regulation in hypoxia up-regulated protein 1. Hypoxia is a known side-effect of amphotericin B [Laniado-Laborin and Cabrales-Vargas, 2009], probably resulting from an increase in respiration as the cells generate ATP in an attempt to control the influx and efflux of ions through their leaky plasma membrane. Not only would such an increase in respiration result in higher intracellular concentrations of reactive oxygen intermediaries, but the ionic alterations of the cytosol would also be destabilizing, requiring increased levels of chaperones such as the chaperonin, HSP90 and HSP70 that we found to be up-regulated. It is also probable that some of the increased chaperone expression relates to the recycling of damaged proteins. HSP70 and HSP90 are involved in the UPP, with HSP90 preventing ubiquitinylation when the damage is marginal and HSP70 mediating ubiquitinylation when the damage is more severe [Pratt et al., 2008; Kettern et al., 2010; Pratt et al., 2010]. HSP90 also forms co-chaperoning interactions with valosin-containing protein (VCP), which was also up-regulated in My^{+ve} cultures. The HSP90-VCP complex mediates the removal and destruction of proteins that have become misfolded during their synthesis in the endoplasmic reticulum [Prince et al., 2005; Mimnaugh et al., 2006]. As 30% of new proteins become misfolded during their biogenesis and are degraded via the UPP [Schubert et al., 2000], even a modest increase in translation would drive a corresponding increase in UPP activity. Further evidence of an increase in translation is that three isoforms of cytoplasmic C-1-tetrahydrofolate synthase (which is involved in histidine biosynthesis) and neutral alpha glucosidase (involved in glycoprotein production in the endoplasmic reticulum and Golgi) were also up-regulated. A further protein of note that was identified as being up-regulated in the My^{+ve} cultures is NEDD8 (neural precursor cell expressed developmentally down-regulated protein 8). NEDD8 has homology to ubiquitin (57% identical and 76% similar at the amino acid level) and is involved in a number of similar processes, most notably the activation of E3-ubiquitin ligases that are themselves key regulators of the cell cycle [Gonzalez Fraga et al., 2009]. However, the spot that was larger in the My^{+ve} gels was that of free NEDD, unattached to E3 substrates. Therefore, it is unclear whether the increase in spot volume results from an up-regulation of NEDD expression (equating to an increase in NEDD8 activity) or a down-regulation of the neddylation process (equating to a decrease in NEDD8 activity with a corresponding increase in unbound NEDD).

CULTURES WITH AN ANTIBIOTICS AND AMPHOTERICIN B

The evidence that we present here demonstrates that including antibiotics in cell culture medium induces changes in cultured cells, with a general down-regulation in protein expression. In marked contrast, amphotericin B induces a more dramatic response, characterized by a general increase in protein expression levels. When cells were cultured with both antibiotics and amphotericin B, remarkably, we found the net effect to be minimal, with only one protein, which we could not identify, being up-regulated. We consider it extremely unlikely that at the molecular level the effects of the antibiotics and amphotericin B were offset such that the cells were behaving in the same way as they were in the negative controls. Instead, we think it more likely that on the one hand the cells were dealing with amphotericin B-induced stress, probably caused by a leaking plasma membrane, but that the required increase in protein expression could not be delivered because of the antibiotic-induced down-regulation in translation. The net effect is therefore a population of stressed cells with no changes in protein levels that were measurable using the methods we adopted. Ours is not the first study to report that amphotericin B has enigmatic stimulatory/inhibitory effects depending on whether it is acting in combination with other compounds [Kumar and Chakrabarti, 2000].

Although 2D-DIGE coupled to MALDI-MS/MS is a powerful and sensitive method of differential protein expression analysis, the technique does have inherent weaknesses. A large proportion of the membrane proteome would have been insoluble in the 2D lysis buffer and would therefore fall outside the remit of our study. Also, proteins that are very large sometimes fail to transfer from the IPG strip to the gel and those with isoelectric points outside the pH 3-11 range of the IPG strips will fail to focus. Should these proteins be studied a more comprehensive picture is likely to emerge.

Cell culture-based experiments are valuable because the regulators of cell behavior can be determined in a stringently controlled environment. Our data demonstrates that significant changes in protein expression profiles occur when epithelial cells are exposed to antibiotics and amphotericin B. These effects are not related to antimicrobial-induced alterations to the cell cycle because the proportion of cells in G_1, S and G_2/M phase were similar in all of the cultures, regardless of whether antimicrobials were present in the medium or not. Such changes in a variety of functional protein categories would certainly have effects on cell phenotype, the significance of which would likely depend on cell type, the nature and concentration of the antimicrobial additives and the duration of exposure. Such effects are likely to be even greater in serum-free medium, although this was not investigated in this study. Therefore, routine inclusion of antimicrobials in cell culture should be avoided as they obfuscate the interpretation of in vitro studies and negate comparisons between studies from different laboratories.

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