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Anticancer activity of the liposome-encapsulated cyclic dipeptides, cyclo(His-Gly) and cyclo(His-Ala)

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Cyclic dipeptides have been well characterized for their biological activity, including antimicrobial and anticancer activities. Cyclo(His-Gly) and cyclo(His-Ala) have also recently demonstrated significant anticancer activity against a range of cell lines, however, as a result of their physicochemistry, namely high solubility and low lipophilicity, it can be predicted that cellular permeability would be low, making them ideal candidates for liposome drug delivery. Liposomes were composed of phosphatidylcholine, hydrogenated soy phosphatidylcholine (HSPC), stearylamine, α-tocopherol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000] (PEG-DSPE) or folate-polyethylene glycol-cholesteryl hemisuccinate (F-PEG-CHEMS) using the thin-film hydration method and characterized for size and encapsulation. The cytotoxic activity of the encapsulated cyclic dipeptides was tested against HeLa, low folate HeLa and MCF-7 cells and found to have limited improvement in activity. However, modification of the polyethylene glycol with folic acid to target folate receptors significantly decreased the IC_{50} values recorded in all cells lines tested, particularly HeLa cells cultured in media containing physiological concentrations of folic acid with the lowest IC_{50} being recorded as 0.0962 mM for folate-targeted cyclo(His-Ala). Therefore, hydrophilic cyclic dipeptides are ideal candidates for inclusion into targeted drug delivery systems such as liposomes.

1. Introduction

Of late, much interest has been placed on the biological activity of selective cyclic dipeptides (CDPs). In a study by Milne et al. (1998), it was noted that some of these compounds containing a tryptophan moiety showed significant tumour inhibition. Subsequent studies (Lucietto et al. 2006; van der Merwe et al. 2008) have confirmed these results, identifying cyclic dipeptides as potential chemotherapeutic agents. Other studies (Graz et al. 1999; Rhee 2004) have also outlined the potential therapeutic usefulness of these compounds, exhibiting effects such as ion channel modulation, antibacterial and even antifungal properties. In a more recent study (Brauns et al. 2004), selected cyclic dipeptides were evaluated against a number of cell lines, namely MCF-7 (breast carcinoma), HeLa (cervical carcinoma) and HT-29 (colon carcinoma), indicating that one of the compounds tested, cyclo(Phe-Pro), showed inhibition of more than 50% in some cell lines, and in addition induced apoptosis in the HT-29 cell line. More recently, significant inhibition of the same cell lines was demonstrated with the histidine-containing cyclic dipeptides, cyclo(His-Gly) and cyclo(His-Ala) (Lucietto et al. 2006).

Physicochemical properties of these molecules indicate limitations with respect to their solubility in biological solutions as well as limited cell permeation (Prasad 1995). The potential tumour suppressive properties as well as the physicochemical limitations of this group of molecules make them ideal test candidates in the development of a targeted liposomal drug delivery system.

Liposomes can be characterized as colloidal particles comprising mainly of phospholipids and cholesterol. They form spherical vesicles consisting of a phospholipid bilayer, much like that of normal cell membranes (Malam et al. 2009). These particles have a myriad of possibilities with respect to pharmaceutical applications due to their variation in composition, size and structure (Endruschat and Henschke 2000). Incorporation of many different types of therapeutic molecules such as simple organic drug compounds as well as protein-based and gene therapeutics aim to enhance their actions through the attainment of a number of goals, including the enhancement of their pharmacokinetics, decreased metabolic degradation and improved targeting, thereby enhancing their efficacy and decreasing potential side-effects (Vemuri and Rhodes 1995).

Besides tethering antibodies to liposomes, any molecule that can target proteins that are over-expressed in tumour cells could prove beneficial. One such molecule is folic acid, as folate receptors are often found to be over-expressed in tumours and this approach has been used in assisting with the targeting of liposomes (Gabizon et al. 1999; Lee and Low 1995; Lu and Low 2003).

The aim of this study was therefore to formulate and evaluate liposome-encapsulated cyclic dipeptides that increase the tumour-suppressive actions of the cyclic dipeptides, while showing a high degree of specificity for tumour cells.

Table 1: Summary of IC_{50} cytotoxicity results for free and liposome-encapsulated cyclic dipeptides

Cell Line	Liposome-encapsulated		Free	
	cyclo(His-Gly)	cyclo(His-Ala)	cyclo(His-Gly)	cyclo(His-Ala)
HeLa	1.699 mM	0.353 mM	1.103 mM	0.324 mM
MCF-7	0.358 mM	0.498 mM	0.630 mM	1.258 mM

Figures represent IC_{50} values calculated from dose-response analysis and are in mM. The positive controls, methotrexate and 5-fluorouracil were tested at concentrations of 100 μM to validate the assays and all resulted in inhibitions above 80%.

2. Investigations, results and discussion

2.1. Liposome characterization

The measured encapsulation of the non-targeted liposomes for cyclo(His-Gly) and cyclo(His-Ala) was 0.115 ± 0.012 mg/mg lipid and 0.105 ± 0.005 mg/mg lipid respectively, while folate-targeted liposomes had an encapsulation of 0.1354 ± 0.00739 mg/mg lipid and 0.090623 ± 0.0141 mg/mg lipid for cyclo(His-Gly) and cyclo(His-Ala) respectively.

The mean particle diameter for non-targeted cyclo(His-Gly) and cyclo(His-Ala) liposomes was 134.5 ± 1.082 nm and 145.3 ± 0.20 nm respectively while folate targeted liposomes had a mean particle diameter of 150.0 ± 1.021 nm and 152.5 ± 6.780 nm for cyclo(His-Gly) and cyclo(His-Ala) liposomes respectively.

2.2. Cytotoxicity of free and non-targeted cyclo(His-Gly) and cyclo(His-Ala)

Lucietto et al. (2006) reported that 100 μM of both cyclo(His-Gly) and cyclo(His-Gly) exhibited significant inhibition of, in some cases, greater than 80%. Despite this report, the inhibitory actions of both cyclic dipeptides was found to be relatively limited and IC_{50} values for cyclo(His-Gly) and cyclo(His-Ala) were 1.103 mM and 0.3244 mM respectively for HeLa cells and 0.630 mM and 1.258 mM respectively for MCF-7 cells.

Encapsulation of cyclo(His-Gly) into liposomes showed a slight apparent increase in activity against HeLa cells, although not significant within the concentration range tested. The IC_{50} value for cyclo(His-Gly) liposomes was slightly higher than that of the free drug (1.699 mM as opposed to 1.103 mM) (Table 1). Slower inhibitory response of liposome-encapsulated drug may have been as a result of slower release of the drug from the liposomes, and thus lower levels of free drug available for inhibition. As the liposomes were not targeted to the cells, cellular uptake may have be slower than anticipated. Many studies have shown that drugs encapsulated into non-targeted liposomes display an increase in IC_{50} when compared to the free drug (Lee and Low 1995; Serpe et al. 2004; Zhang et al. 2004) and the benefits should not been viewed in the light of a decrease in IC_{50}, but rather the decreased non-specific cytotoxic effects on free cytotoxic agents that leads to high side-effect profiles. A similar trend was noted for cyclo(His-Ala) liposomes against HeLa cells with an IC_{50} value of 0.3532 mM calculated for liposomal cyclo(His-Ala) as opposed to 0.3244 mM for free cyclo(His-Ala).

The effects of encapsulation were more notable in MCF-7 cells where both encapsulated drugs showed a marked decrease in calculated IC_{50}. IC_{50} for cyclo(His-Gly) liposomes decreased to 0.3584 mM from 0.6296 mM for the free drug, while the IC_{50} for cyclo(His-Ala) liposomes reduced to 0.4978 mM from 1.258 mM of free cyclo(His-Ala). Therefore, for MCF-7 cells, cellular entry of liposome-encapsulated drug could result in poor cellular uptake of the drugs. Encapsulation would therefore

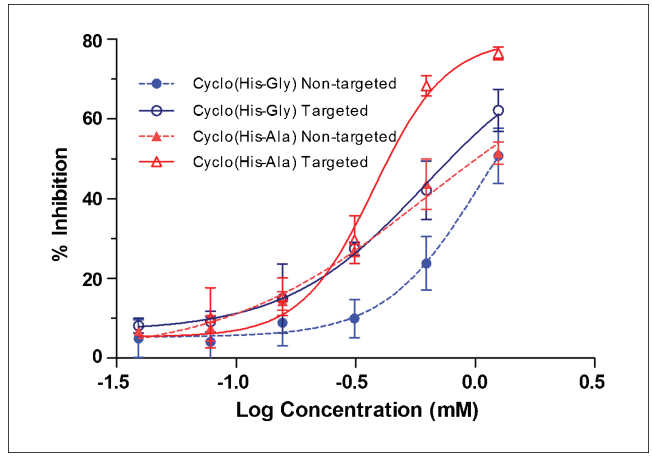

Fig. 1: Cytotoxicity of cyclo(His-Ala) and cyclo(His-Ala) on HeLa cells in standard culture media. The graph depicts the influence of folate receptor targeting on the cytotoxicity of cyclo(His-Gly) and cyclo(His-Ala) on HeLa cells cultured in normal RPMI-1640 medium expressing low folate receptor levels (n = 4 for each point)

enhance cellular entry and promote cytotoxicity. A summary of the results is given in Table 1.

Despite showing no improvement over the inhibitory effect of the cyclic dipeptides against HeLa cells, improvement was noted against MCF-7 cells. The difference in the IC_{50} parameter for liposome cyclic dipeptides was not large for HeLa cells. In their study, Lee and Low (1995) showed that the IC_{50} of free doxorubicin was 0.43 μM as opposed to non-targeted liposomal doxorubicin with an IC_{50} of 25.3 μM. As free doxorubicin shows good permeation into cells, it was concluded by the authors that a reduction in the cytotoxic activity was due to slower release of doxorubicin in liposome formulations. The results obtained in this study are therefore promising as the difference between encapsulated and non-encapsulated drug for HeLa cells was marginal, while an improvement in activity was noted for encapsulated cyclic dipeptides on MCF-7 cells. This further emphasizes the problems faced with drugs exhibiting low permeation due to poorly lipophilic properties. Lee and Low (1995) as well as others (Gabizon et al. 1999; Gabizon et al. 2004; Saul et al. 2003; Xiang et al. 2008; Yoshida et al. 2006; Zhang et al. 2004) have proposed that, while non-targeted liposomes reduce the side-effect profile of cytotoxic drugs, incorporation into liposomes targeted to a particular receptor that over-express in tumour cells, such as the folate receptor, would enhance cellular uptake of the liposome contents through receptor-mediated endocytosis. This strategy was therefore used to improve the cytotoxic actions of cyclo(His-Gly) and cyclo(His-Ala).

2.3. Cytotoxicity of folic acid tethered liposomes

The cytotoxicity of folate-targeted liposomes was assessed against MCF-7, HeLa and HeLa low folate cells and compared to non-targeted liposome-encapsulated cyclic dipeptides. Folate receptor targeting resulted in a significant increase in inhibitory action of both cyclo(His-Gly) and cyclo(His-Ala) when tested against HeLa cells cultured in normal RPMI-1640 as well as folate-free RPMI-1640 media, as well as MCF-7 cells.

IC_{50} values recorded for targeted liposomes against HeLa cells cultured in high folate media were reduced to 0.6153 mM ($r^2 = 0.9503$) for cyclo(His-Gly) and 0.3775 mM ($r^2 = 0.9604$) for cyclo(His-Ala) when compared to non-targeted liposomes (1.029 mM and 0.6194 mM for cyclo(His-Gly) and cyclo(His-Ala) respectively (Fig. 1)). The decrease in IC_{50} was more pronounced in low folate HeLa cells with IC_{50} values of 0.1807 mM ($r^2 = 0.9673$) and 0.09617 mM ($r^2 = 0.9797$)

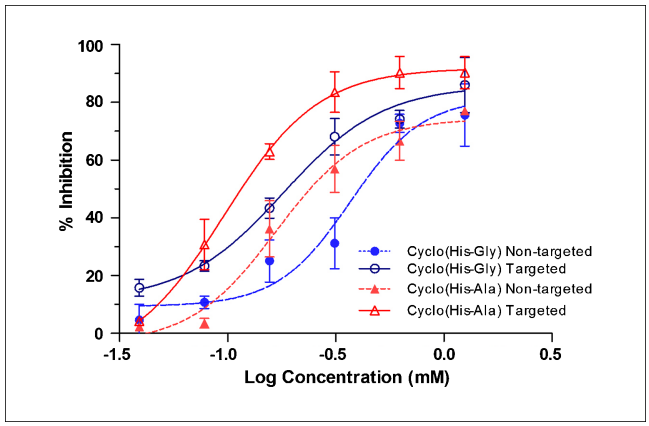

Fig. 2: Cytotoxicity of cyclo(His-Gly) and cyclo(His-Ala) on HeLa cells in low folic acid culture media. The graph depicts the influence of folate receptor targeting on the cytotoxicity of cyclo(His-Gly) and cyclo(His-Ala) on HeLa cells cultured in folic acid-free RPMI-1640 medium expressing high folate receptor levels (n = 4 for each point)

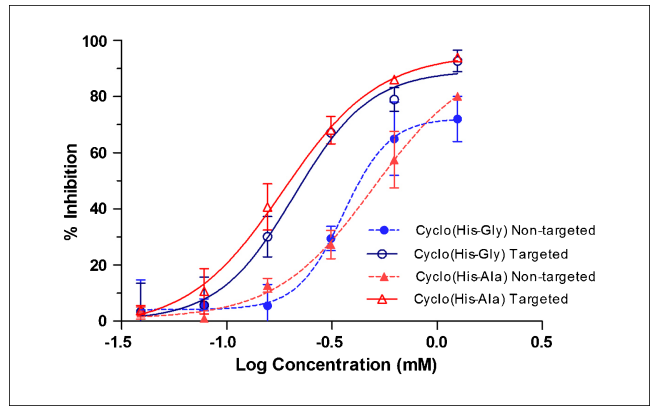

Fig. 3: Cytotoxicity of cyclo(His-Gly) and cyclo(His-Ala) on MCF-7 cells in standard culture media. The graph depicts the influence of folate receptor targeting on the cytotoxicity of cyclo(His-Gly) and cyclo(His-Ala) on MCF-7 cells cultured in normal RPMI-1640 medium expressing low folate receptor levels (n = 4 for each point)

for cyclo(His-Gly) and cyclo(His-Ala) when compared to 0.3692 mM and 0.1680 mM respectively (Fig. 2). The increase in activity of folate-targeted liposomes was most likely due to greater internalization of encapsulated drug as a result of folate-receptor-mediated endocytosis, increasing the intracellular concentrations of the drug as has been shown previously with folate-ligand liposomes (Lee and Low 1995). Increased receptor numbers also played a role in the enhanced activity of liposomal activity with the greatest activity being recorded for cyclo(His-Ala) targeted liposomes against HeLa cells cultured in folic acid-free medium. The overall inhibitory action of the two cyclic dipeptides was more pronounced in these HeLa cells, whether targeted or non-targeted and further highlights the problems with using high folic acid concentrations in cytotoxicity assays as outlined above. Although the cytotoxicity assays were all performed in low folate medium, residual folic acid left on the cell surface during the culturing process could have resulted in competitive binding to folate receptors, preventing cell interactions with targeted liposomes. Despite this potential competition and low folate receptors, increased activity for targeted liposomes was still observed in all cell lines.

An increase in the cytotoxic activity of targeted liposomes was also observed for both cyclic dipeptides against MCF-7 cells. Cyclo(His-Gly) displayed a 42% reduction in IC_{50} decreasing to 0.2094 mM ($r^2 = 0.9674$) from 0.3584 mM ($r^2 = 0.9483$) for the non-targeted liposomes. Cyclo(His-Ala) displayed a substantial decrease of 63% in recorded IC_{50}, decreasing from 0.4987 mM ($r^2 = 0.9811$) for non-targeted liposomes to 0.1856 mM ($r^2 = 0.9818$) (Fig. 3). Table 2 shows a summary of the results.

Encapsulation of cyclo(His-Gly) and cyclo(His-Ala) into folate receptor-targeted liposomes has therefore been shown to increase the cytotoxic activity of the cyclic dipeptides. These are promising results for many other marginally permeable drugs with low Log P and highlight the importance of targeted drug delivery. Problems experienced with long-term stability of the folic acid-tethered liposomes should be addressed and reasons for the instability be elucidated.

Table 2: IC_{50} data for targeted and non-targeted liposomal cyclo(His-Gly) and cyclo(His-Ala)

Cell line	cyclo(His-Gly)		cyclo(His-Ala)	
	Non-targeted	FR Targeted	Non-targeted	FR Targeted
HeLa NF	1.029 mM	0.6153 mM	0.6194 mM	0.3775 mM
HeLa LF	0.3695 mM	0.1807 mM	0.1680 mM	0.0962 mM
MCF-7	0.3584 mM	0.2094 mM	0.4987 mM	0.1856 mM

Values calculated using non-linear dose-response regression analysis. NF = normal folic acid media, LF = low folic acid media, FR = Folate receptor.

2.4. Summary of findings

The anticancer potential of liposomal cyclo(His-Gly) and cyclo(His-Ala) showed no significant improvement against HeLa cells, but reduced the IC_{50} values calculated for both drugs against MCF-7 cells. Improvement in the IC_{50} for non-targeted, encapsulated drugs is not common, particularly for poorly permeable drugs, as escape from the liposome vesicle is slower than immediate exposure to free drug. However, encapsulation into folate receptor targeted liposomes by incorporating F-PEG-CHEMS into the phospholipid bilayer as a ligand showed a significant improvement for all drugs and in all cell lines tested, particularly for HeLa cells grown in low folate media. This indicates that cyclic dipeptides, particularly poorly lipophilic molecules, are ideal candidates for inclusion into targeted drug delivery systems such as liposomes.

3. Experimental

3.1. Materials

Folic acid, PEG-*bis*-amine, dicylcohexylcarbodiimide (DCC), pyridine, cholesteryl hemisuccinate (CHEMS), *N*-hydroxysuccinimide (NHS), α-tocopherol, stearylamine and trypsin were purchased from Sigma-Aldrich (St Louis, USA). L-α-phosphatidylcholine (PC), hydrogenated soy phosphatidylcholine (HSPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, USA). Gibco® RPMI-1640 (standard and folic acid-free) were purchased from Celtic Molecular Diagnostics (Mowbray, South Africa). All other reagents and chemicals were of analytical grade.

3.2. Synthesis of folate-PEG-cholesteryl hemisuccinate

Folate-PEG-cholesteryl hemisuccinate (F-PEG-CHEMS) was used as the targeting molecule in liposome formulations and was synthesized by initial synthesis of its component parts, folate-PEG-amine (F-PEG-NH_2) and *N*-hydroxysuccinimide-cholesteryl hemisuccinate (NHS-CHEMS). F-PEG-NH_2 was synthesized according to the method as adapted from Lee and Low (1995). 40 mg folic acid, 250 mg PEG-*bis*-amine, 23.1 mg dicylcohexylcarbodiimide and 5 µl pyridine were dissolved in 2.5 ml DMSO and stirred overnight, protected from light, at room temperature. NHS-CHEMS was synthesized according to a method adapted from Xiang et al. (2008). 1 g of CHEMS was dissolved in 10 ml tetrahydrofuran (Saarchem, Midrand, South Africa) with 475 mg *N*-hydroxysuccinimide (NHS) and 1.25 g DCC and reacted at room temperature overnight. Tetrahydrofuan was removed under vacuum in a rotary evaporator until dry. For the synthesis of F-PEG-CHEMS,

F-PEG-NH_2 (274 mg, 80 μmol) and NHS-CHEMS (58.4 mg, 100 μmol) were dissolved in 100 ml chloroform ($CHCl_3$) and reacted overnight. The product was dried under vacuum in a rotary evaporator and re-hydrated with 20 ml 50 mM sodium carbonate buffer (pH 7.0) for 1 h at 40 °C to form F-PEG-CHEMS micelles. Low molecular weight contaminants were removed by dialysis of the micelle suspension with reverse osmosis water through a 14 000 MWCO dialysis tube (Snakeskin®, Thermo Fisher Scientific Inc., Rockford, USA). Micelles of F-PEG-CHEMS were lyophilized to a dry powder. Purity and structure were confirmed using TLC and ^{1}H NMR in deuterated-DMSO (Xiang et al. 2008).

3.3. Preparation of liposomes

Liposomes were manufactured using the thin-film hydration method extensively described elsewhere (Berger et al. 2001; Mura et al. 2007; Torchilin and Weissig 2003). Lipid films comprising (in mg) HSPC 13.7, PC 13.5, Cholesterol 5.6, stearylamine 0.07, α-tocopherol 1, and either PEG-DSPE or F-PEG-CHEMS 0.7, were dissolved in 10 ml of a 9:1 mixture of chloroform:methanol and transferred to a 50 ml round bottomed flask. The solvent was then evaporated on a rotary evaporator under vacuum at 50 °C for at least 3 h and the remaining solvent was removed by flushing the film with nitrogen. Lipid films were hydrated at 60 °C with a solution containing cyclic dipeptide in phosphate-buffered saline at pH 7.4 in a shaking water bath for 3 h. Once hydrated, the multilamellar liposomes formed were extruded 13 times through a 100 nm pore polycarbonate membrane filter (Nuclepore) using an extruder (Avanti Polar Lipids, Alabaster, USA).
Just prior to use, liposomes were separated from free drug by size exclusion chromatography using Sephadex G50 loaded mini-spin columns that were equilibrated with PBS. Columns were centrifuged at 5000 rpm for 5 minutes and the eluent, containing only liposomes, was retained for use in experiments.

3.4. Liposome characterization

Cyclo(His-Gly) and cyclo(His-Ala) were quantified by HPLC with Luna NH_2 column (with dimensions of 250 mm × 4.6 mm) with 5 μm diameter packing (Phenomenex, Torrance, USA). The mobile phase comprised of acetonitrile (65%) in 50 mM phosphate buffer (pH 3.0). A flow rate of 1.5 ml/min was used for analysis with an injection volume of 20 μl. Liposome-encapsulated drug was separated from free drug as described above and the liposomes lysed with a 10% Triton X-100 solution before analyzing for cyclic dipeptide content. Phospholipid was quantified according to Stewart (1980) by dispersing 100 μl liposome suspension in 2 ml chloroform and adding 2 ml ammonium ferrothiocyanate solution. The absorbance of the chloroform phase was measured at 485 nm and related to phospholipid content using an external standard. The quantity of cyclic dipeptide encapsulated was therefore calculated as a proportion (in mg) encapsulated per mg phospholipid.

3.5. Cell culture

HeLa cells were maintained in either standard RPMI-1640 medium or folic acid-free RPMI-1640 medium (in order to induce folate receptor upregulation). Cells were conditioned to low folate conditions over a period of three months, by slowly reducing the quantity of folic acid in the media by 5% each time the cells were passaged. Once at the target concentration of folic acid, cells were conditioned to grow in the reduced folic acid content until the doubling time was the same or similar to that of the standard folic acid culture. Reduction of folic acid in growth medium was achieved by a stepwise reduction in the amount of folic acid solution (0.1 mg/ml) in PBS (pH 7.4) that was added to folic acid-free RPMI-1640 medium (Gibco® brand, Celtic Molecular Diagnostics, Mowbray, South Africa).

3.6. Cytotoxicity assay

Cytotoxicity assays were conducted as described previously (Zhang et al. 2004). Cells were lifted by the addition of 1 ml 0.25% trypsin in 0.1% EDTA solution and incubated for 5 min at 37 °C. Cell concentration was determined under a light microscope with a haemocytometer and adjusted to 6×10^4 cells/ml by dilution with culture medium. Wells of a 96-well microtitre tissue culture plate were seeded with 6×10^3 cells per well and incubated at 37 °C with CO_2 humidification for 24 h prior to being exposed to liposome preparations or drug solutions. After incubation, medium was removed by careful aspiration and replaced with 180 μl fresh culture medium. 20 μl drug solution in PBS, sterilized through 0.2 μm cellulose acetate filters, was then added to each well and the cells incubated for 48 h at 37 °C with CO_2 humidification. Methotrexate and 5′-fluorouracil, at concentrations of 100 μM were used as positive controls. Cell viability was determined after incubation for 48 h using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Denizot and Lang 1986; Fotakis and Timbrell 2006). After drug exposure, cell medium was aspirated, washed with 200 μl PBS and replaced with 180 μl fresh culture medium. 20 μl MTT (5 mg/ml in PBS), was added to each well and incubated for a further 5 h at 37 °C. Plates were centrifuged at 3000 rpm for 10 min and culture medium was aspirated and replaced with 100 μl DMSO in order to solubilise the formazan crystals. Absorbance was read at a wavelength of 570 nm. Cell viability was calculated as a percentage of the negative control. All assays were performed in quadruplicate and the assay repeated on separate occasions at least three times.

3.7. Statistical methods

All assays were performed in quadruplicate on three separate occasions. Data was normalized to represent % inhibition relative to a negative control and the dose-response relationship analysed using non-linear regression analysis with GraphPad Prism version 5.03 (GraphPad Software Inc., San Diego, USA).

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