2015

In Vitro Evaluation of the Cytotoxicity of a Folate-modified β-cyclodextrin as a New Anti-cancer Drug Delivery System

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Recommended Citation

Tofzikovskays, Z., Casey, A., Howe, O., O'Connor, C. & McNamara, M. (2015). In vitro evaluation of the cytotoxicity of a folate-modified β-cyclodextrin as a new anti-cancer drug delivery system. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*,81(1-2), pp. 85-94. doi:10.1007/s10847-014-0436-0

In vitro evaluation of the cytotoxicity of a folate-modified β-cyclodextrin as a new anti-cancer drug delivery system.

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Abstract

Many chemotherapeutic drugs are therapeutically non-selective and do not distinguish between healthy cells and tumour cells which can result in severe side effects and toxicity. Drug delivery systems can be used to target specific cells and therefore may eliminate many of the side effects, increasing drug efficiency and efficacy, and controlling drug release. One possible strategy for targeted drug delivery is to use unique molecular markers such as folate receptors in cancer cells. In this work the cytotoxicity of a novel cyclodextrin-folate conjugate, 6-deoxy-6-[(1-(-2amino)ethylamino)folate-β-cyclodextrin (CDEnFA) was studied using the MTT assay and the MCF-7 (Breast), HeLa (Cervical), A549 (Lung cancer) and BEAS-2B (normal Lung) cell lines.

The MTT assay showed that the drug delivery vehicle CDEnFA is not cytotoxic towards the cell lines studied even towards the normal BEAS-2B cell line and therefore it is expected that it is safe for medical use. The inclusion complex CDEnFA:MTX has superior cytotoxic activity towards all of the cancer cell lines studied compared to the drug MTX alone and CDEnFA:MTX is four times less cytotoxic than the drug towards the normal cell line. The observed toxicity is attributed solely to MTX since CDEnFA did

not exhibit significant cytotoxicity. These results also suggest that the drug remains bioactive even after inclusion in the CD cavity. The cytotoxicity trend observed for CDEnFA:MTX in this study is MCF-7 (Breast) > A549 (Lung) > HeLa (Cervical) > BEAS-2B (normal Lung).

Keywords: Cyclodextrin, Cytotoxicity, Drug delivery, Folate.

Introduction

Many chemotherapeutic drugs are therapeutically non-selective and do not distinguish between healthy cells and tumour cells which can result in severe side effects and toxicity. This toxicity can limit the dose of drug which can be used. Chemotherapeutics can also have a narrow tumour spectrum being effective against some cancers and ineffective against others and therefore one of the goals of medicinal chemistry is to direct an effective therapeutic dose to the required site of action. Drug delivery systems are molecular tools which can be used to target specific sites without interaction at other sites [1]. In this way targeted delivery systems can eliminate some of the above drawbacks, increasing drug efficiency and efficacy, controlling drug release and minimising harmful side effects [2]. For chemotherapy these systems must be capable of recognising cancer cells, specifically targeting these cells and delivering an effective therapeutic dose. One possible strategy for targeted drug delivery is to use unique molecular markers in cancer cells that are not expressed or have low expression in normal

cells. Recently there has been interest in exploiting folate receptors for tumour specific targeted delivery of therapeutics.

Folate is essential for the synthesis of amino acids such as serine, glycine and methionine which occurs in the cytosol and mitochondria. The human body cannot synthesise folate and it is therefore obtained *via* the daily diet from leafy vegetables, citrus fruits, beans and whole grains. Most human cells contain folate receptor (FR) sites located on the cellular membrane but cancer cell growth requires a lot more folate in comparison with normal cells and therefore cancer cells can express over 500 times more FRs [3]. FRs provide a route for internalisation of folates into the cytosol of cells and there are at least four forms, known as α, β, δ and γ/γ'. Most work to date has concentrated on the first three as potential disease markers [4]. These FRs have similar amino acid sequences but differ in their expression patterns. FR-α, β and δ are comprised of glycosylphosphatidylinositol-linked proteins which are expressed in many carcinomas but are not expressed or have low expression in normal cells [5]. FR-α is expressed in several cancers including ovarian, uterine, testicular and to a lesser extent in breast, colon and renal cancers [3]. It is also expressed in some normal epithelial cells but access from the bloodstream is limited. FR-β is expressed in chronic and acute myelogenous leukaemia, macrophages associated with rheumatoid arthritis and other inflammatory diseases [6]. FR-δ is expressed in regulatory T-cells [7]. These differences in expression of FRs can be exploited for chemotherapy and the FRs provide ideal targets for effective targeted drug delivery.

Several drug delivery systems based on folate derivatives are currently in development. For example Chen *et al.* have developed liposomes comprised of folate (FA) conjugated

with distearoylphosphatidylethanolamine (DSPE) derivatised with polyethyleneglycol (PEG) [8]. The FA-PEG-DSPE delivery system was loaded with arsenic trioxide (As_2O_3) which has potential anticancer applications and the resultant drug delivery system demonstrated higher anticancer activity than As_2O_3 alone against solid tumour cells. Pinhassi *et al.* have conjugated the natural polysaccharide arabinogalactan to folate and the drug methotrexate through the peptide Gly-Phe-Leu-Gly and have reported that the conjugate system shows increased cytotoxicity towards Chinese hamster ovary cancer cells which over-express FRs in comparison to normal cells which do not express FRs [9]. Due to their low toxicity and inclusion properties cyclodextrins are proposed here as suitable drug carriers for use in drug delivery systems.

The partial hydrolysis of starch using glucosyltransferase gives cyclic sugars containing six to eight α-(1-4)-linked D-glucopyranose units [10]. The cyclodextrins (CDs) produced can be distinguished on the basis of the number of α-D-glucose units i.e. α-cyclodextrin (six glucose units), β-cyclodextrin (seven glucose units), and γ-cyclodextrin (eight glucose units) (Figure 1).

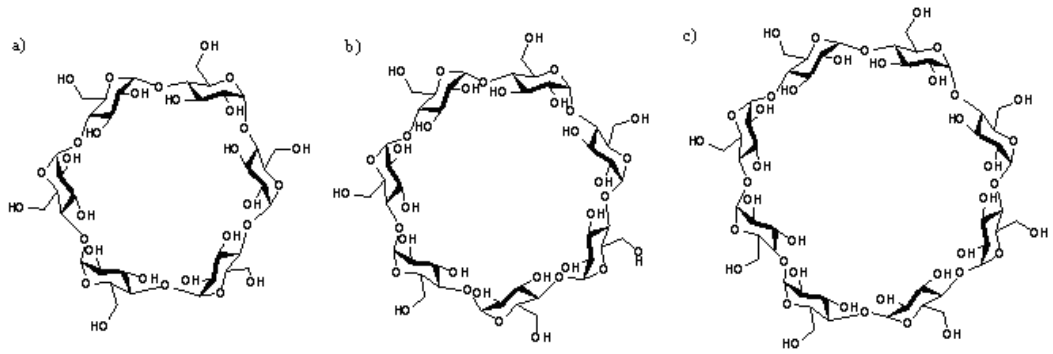


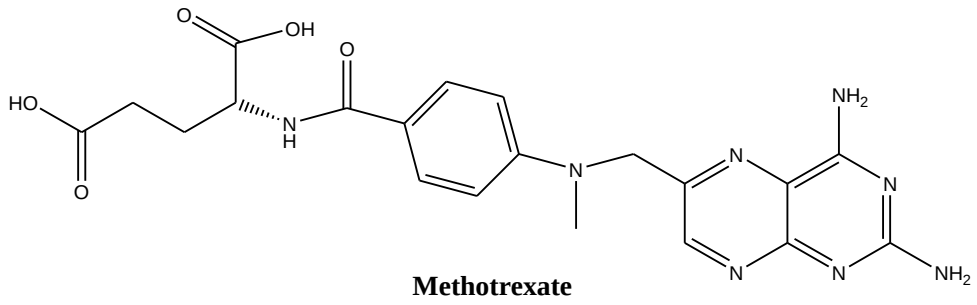
Figure 1. Structure of a) α–cyclodextrin, b) β–cyclodextrin and c) γ-cyclodextrin

The toxicological properties of cyclodextrins have been reviewed and in general they are classified as non-toxic materials which may cause some eye or skin irritation [11, 12]. This has led to the use of CDs in the cosmetic, food, flavour and pharmaceutical industries [11]. As well as improving solubility, β-CD is used to protect several compounds against various degradation processes such as photo-degradation, thermal degradation and hydrolysis [13-18]. The hydrophobic cavity can include many guest molecules while the multitude of hydroxyl groups can be modified to give a wide range of derivatives. Caliceti *et al.* reported a C6 monosubstituted CD folate conjugate with a PEG spacer as a potential drug delivery system [19]. However because of the side-chain the conjugate is flexible which allows self-inclusion of the folate group in the CD cavity. This hinders inclusion of drugs in the cavity and prevents use of the conjugate as a drug delivery system. Previously we reported the synthesis and stability of a folate-modified β-cyclodextrin without a spacer group [20]. The γ isomer of the mono-substituted derivative 6-deoxy-6-[(1-(2-amino)ethylamino)folate]-β-cyclodextrin (CDEnFA) was synthesised in high yield and was shown to be considerably more photo-stable than free folic acid in both the solid state and aqueous solution. It is now proposed that CDEnFA can be used as a drug delivery system *via* drug inclusion in the CD cavity. Internalisation of the drug in cells can be accomplished *via* receptor-mediated endocytosis.

Methotrexate (MTX), is the model drug used in this work and is an example of an antifolate. It was one of the first chemotherapeutic drugs developed in the 1950s and is used to treat various types of cancer including breast, bladder and bone marrow cancer as well as leukaemia and rheumatoid arthritis. MTX is thought to act as a chemotherapeutic due to the fact that it has a structure very similar to the structure of folates and folic acid

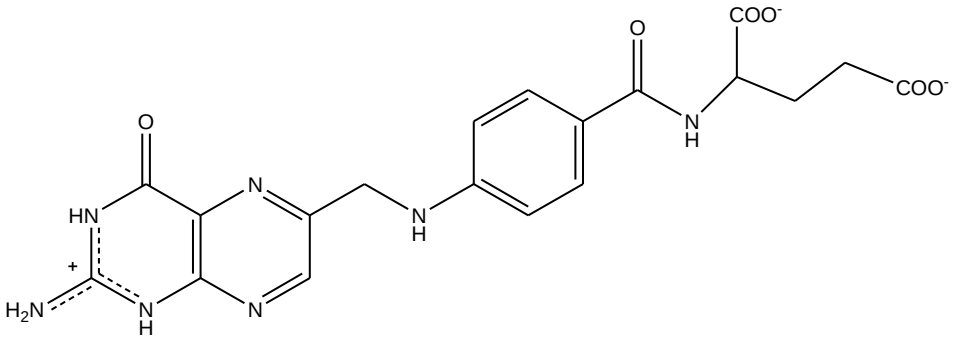
(Figure 2). Therefore MTX is a competitive inhibitor since it prevents folic acid metabolism by preferentially binding to enzymes.

(a)



Methotrexate

(b)



Folate

Figure 2. Chemical structure of (a) methotrexate and (b) folate.

There is debate as to the exact mechanism of action of MTX but there are some reports that show that after entering the cell, methotrexate is polyglutamated by the enzyme folylpolyglutamate synthase. MTX and its polyglutamates then bind to and inhibit the enzyme dihydrofolate reductase (DHFR) which is required to catalyse the conversion of dihydrofolate (DHF) to tetrahydrofolate (THF) during the synthesis of DNA [21]. As tetrahydrofolate stores are depleted, synthesis of thymidine monophosphate (dTMP) is

reduced, which in turn reduces the synthesis of amino acids such as methionine and ultimately inhibits DNA synthesis (Figure 3) [24]. Single and double strand breaks occur in the DNA helix because DNA synthesis is interrupted while it is still in progress and because repair mechanisms remove the damaged DNA. Cell death then occurs *via* necrosis or apoptosis [23].

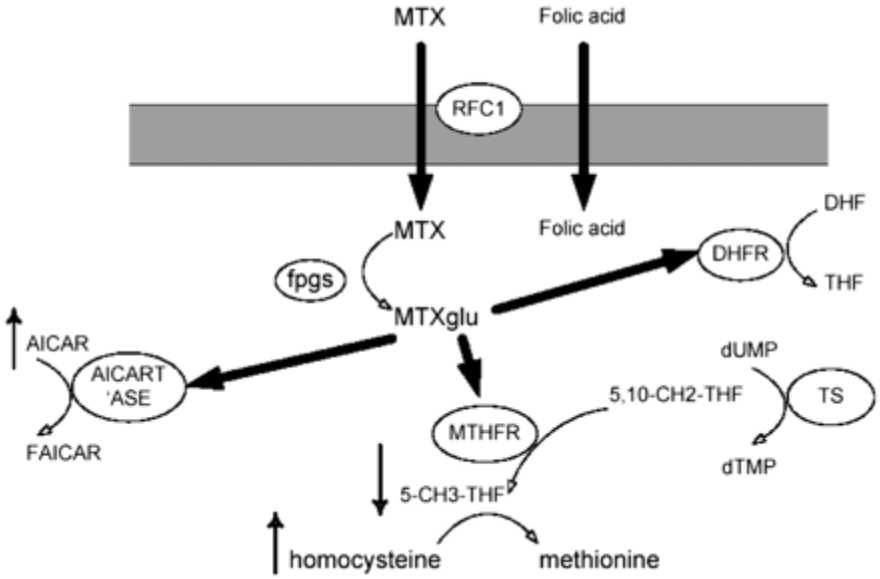


Figure 3. The folate pathway where MTX = methotrexate; MTXglu = methotrexate polyglutamate; RFC1 = reduced folate carrier; FPGS = folylpolyglutamate synthase; TS = thymidylate synthase; 5,10-CH_2-THF = 5,10-methylene tetrahydrofolate; MTHFR = methylene tetrahydrofolate reductase; 5-CH_3-THF = 5-methyl tetrahydrofolate; AICAR = 5-aminoimidazole-4-carboxamide-ribonucleotide; AICART'ASE = AICAR transformylase; FAICAR = formyl-AICAR, dUMP = deoxyuridine monophosphate, dTMP = thymidine monophosphate [24].

However to achieve high efficacy, high doses of MTX are required for several reasons. To be effective MTX must be internalised into cells. But MTX is anionic and cannot permeate negatively charged cell membranes due to repulsion, unless a high concentration is used. Also the enzyme DHFR has a greater affinity for folate than for MTX unless the concentration of MTX is high [24]. MTX also has a number of other disadvantages. MTX has a narrow tumour spectrum and also does not cross the blood brain barrier and therefore cannot be used for solid tumours and brain tumours. Resistance to MTX is also possible. A system for the delivery of MTX to the required site of action may avoid some of these disadvantages and therefore MTX was chosen as the model drug for this work.

There are two different systems used by cells for the uptake of folates and folate-like molecules such as MTX. The first are folate receptors (FR) that internalise folates by receptor-mediated endocytosis which is cellular uptake of a substance by invagination of the membrane. The second system is a reduced-folate carrier (RFC) which uses an anion exchange transport mechanism to internalise folates. RFC is present in all cells and is responsible for the majority of folate transport across cell membranes. The binding affinity for folates and MTX differ for the two systems. FRs have a high binding affinity for folates but a low affinity for MTX while the reverse is true for the RFC [25].

We now report an *in vitro* evaluation of the cytotoxicity of CDEnFA in various cell lines and its inclusion of and effect on the bioavailability of methotrexate. In accordance with the EU policy of Reduction, Replacement and Refinement (RRR) an *in vitro* rather than an *in vivo* model is used to assess the cytotoxic response of the system using the MTT assay [26].

Materials and methods

β-cyclodextrin was obtained from Wacker Chemie (Munich, Germany) and 6-deoxy-6-[(1-(2-amino)ethylamino)folate]-β-cyclodextrin (CDEnFA) was synthesised as previously reported [20]. The inclusion complex, CDEnFA:MTX, was prepared by the paste method by combining MTX and CDEnFA in a 1:1 molar ratio. Briefly CDEnFA was stirred with a minimum volume of water to obtain a homogeneous paste. Then, MTX powder was slowly added and the mixture was stirred for 45 min. During the process a few drops of water were introduced to maintain a suitable consistency. The resulting paste was dried in an oven at 45 °C for 48 h and the solid was finally ground and stored in the dark. The inclusion complex CDEnFA:MTX was analysed by ^{1}H NMR where shifts of protons H3 and H5 of cyclodextrin were observed indicating successful complexation. Previously it was reported that MTX forms an inclusion complex with β-cyclodextrin *via* the methyl group of MTX included inside the CD cavity [27].

All cell culture reagents and media, *cis*platin, methotrexate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Aldrich Ltd. (Dublin, Ireland). Cell lines were purchased from the American Type Culture Collection. HeLa is a cervical cancer cell line taken from patient Henrietta Lacks in 1951 while MCF-7 is a breast cancer cell line isolated from a 69 year old Caucasian woman in 1970. A549 is a lung cancer cell line first developed in 1972 and BEAS-2B is a normal lung cell line.

Cell culture

The MCF-7 cell line (passages 5-15) was grown at 37 ^{o}C in a humidified atmosphere with 5 % CO_2 in MEM medium containing Foetal Calf Serum (FBS, 10 %), L-glutamine (2 mM), penicillin/streptomycin solution (100 U/ml penicillin and 100 μg/ml streptomycin), and sodium pyruvate solution (1 % of 100 mM). The other cell lines, A549 (passages 5-15), BEAS-2B (passages 5-15) and HeLa (passages 5-15), were grown at 37 ^{o}C in a humidified atmosphere with 5 % CO_2 in RPMI-1640 medium containing Foetal Calf Serum (FBS, 10 %), L-glutamine (2 mM) and penicillin/streptomycin solution (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were grown to confluence and subcultured after being trypsinised with protease enzyme trypsin.

Preparation of test solutions

Test solutions were prepared in deionised water and diluted with cell culture medium, under laminar flow and sterile conditions. *Cis*platin was used as a standard and both DMSO and cell culture medium as positive and negative controls respectively. The concentration range used for the cytotoxicity assays was 0.5 μM – 50 μM for all samples, standard and controls.

Cytotoxicity assay

Cell viability was monitored using the MTT assay by measurement of absorption at 550 nm in a 96 well micro-plate. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a soluble tetrazolium salt yielding a yellow solution in phosphate buffered saline (PBS pH 7.4) and is converted to purple 3,5-diphenyl-1-(4,5-dimethyl-2-thiazolyl)formazane (formazan) by enzymes in living cells. Each cell line was seeded at a

density of 2000 cells per well per 100 µl medium and incubated at 37 °C in a 5% CO_2 humidified atmosphere. After 24 hr, the cell culture medium in each well was replaced with 100 µl medium containing the test compounds. Nine replicate wells were used for each control and test solution per micro-plate. After 24 hr of exposure, the control medium or test solution was removed. The cells were washed with PBS (100 µl of 0.1 M, pH 7.4) and 100 µl of freshly prepared MTT (5 mg/ml of MTT in un-supplemented medium) was added to each well. After a 4 hr incubation, the medium was discarded and the cells were rinsed with PBS (100 µl of 0.1 M, pH 7.4) and DMSO (100 µl) was added to each well to extract the dye. The plates were shaken at 240 rpm for 5 min and the absorbance was measured at 550 nm in a Varioscan plate reader.

Statistics

Cytotoxicity data were expressed as mean percentage cell viability relative to control (100%) ± standard deviation (SD). Statistical analyses were carried out using one-way analyses of variance (ANOVA) followed by a post-ANOVA Dunnett's test and statistical significance was accepted at $p \leq 0.05$ at 95 % confidence levels for all tests. An IC_{50} value was calculated and is the concentration of compound required to give 50 % cell viability.

Results and discussion of cytotoxicity study

*Cis*platin was used as a standard in the MTT assay and IC_{50} values of 106.49 ± 10.44 and 0.9 ± 0.05 µM were obtained for the A549 cell line after 24 and 96 h incubation respectively. Values of 305.95 ± 26.14 and 3.20 ± 0.48 µM were obtained for the HeLa

cell line after 24 and 96 h incubation respectively. These values compare favourably with Kellett *et al.* [28].

The results of the MTT assay obtained for the model drug MTX are shown in figure 4 for each cell line.

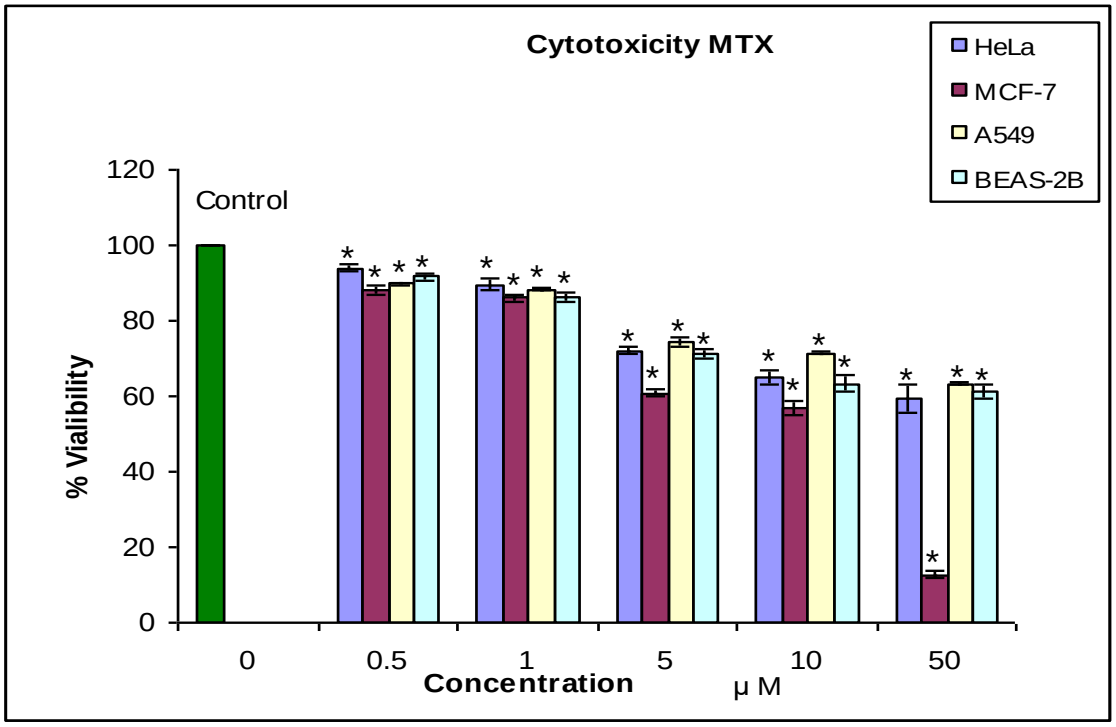


Figure 4. Effect of MTX on the viability of HeLa, MCF-7, A549 and BEAS-2B cells following a 24 hr incubation at concentrations of 0.5, 1, 5, 10 and 50 μM MTX. (* results which are significantly different to the control ($p<0.05$ at 95% confidence))

It can be seen from figure 4 that MTX demonstrates a statistically significant cytotoxicity towards all cell lines studied even at a low concentration of 0.5 μM MTX. However there is some selectivity demonstrated by these results. MTX shows the greatest cytotoxicity

towards the MCF-7 cell line while HeLa and A549 cells show a reasonable viability. This explains the use of MTX for the treatment of breast cancer and these results also show that the drug may have a narrow tumour spectrum. As mentioned earlier there are two different systems used by cells for the uptake of folate-like molecules: folate receptors (FR) which internalise folates by receptor-mediated endocytosis and the reduced-folate carrier (RFC) which uses an anion exchange transport mechanism. FRs have a low affinity for MTX and therefore the drug is normally internalised *via* RFC which is present in all cells. The HeLa, A549 and BEAS-2B cell lines behave in a similar manner and show cell viabilities in the range 60-63 % after 24 hr incubation with 50 μM MTX. These results compare favourably with Patel who studied the cytoxicity of MTX towards these cell lines. An exact comparison cannot be made since these authors used a 72 hr incubation period [29]. It has been reported that HeLa cells have high expression of FR while A549 cells have low FR expression [8, 25]. Since there was no great difference observed in the viabilities of HeLa and A549 cells towards MTX it suggests that both of these cell lines use the RFC system to internalise the drug. It is interesting to note that MTX showed similar cytotoxicity towards the normal lung and lung cancer lines which demonstrates the necessity for a MTX delivery system which would target the tumour cells only.

The MCF-7 line behaves very differently to the others and shows a viability of only 12 % when exposed to 50 μM MTX for 24 hours. The low viability of MCF-7 cells could be explained on the basis that these cells use a different route for internalisation of MTX such as the FR pathway. However MCF-7 cells are known to have low expression of FR [8, 25]. It may be suggested that the enhanced cytotoxicity of MTX towards MCF-7 cells

results from the use of a combination of the FR and RFC routes by these cells for uptake of the drug. At the lower concentrations of MTX similar trends were observed. But all of the cell lines show viabilities in the range 94-86 % after 24 hr incubation with both 0.5 and 1 μM MTX. On this basis MTX has been shown to be an effective chemotherapeutic towards the cell lines studied but only at high concentrations in excess of 5 μM.

The results of the cytotoxicity assay obtained for the CDEnFA drug delivery system are shown in figure 5 for each cell line.

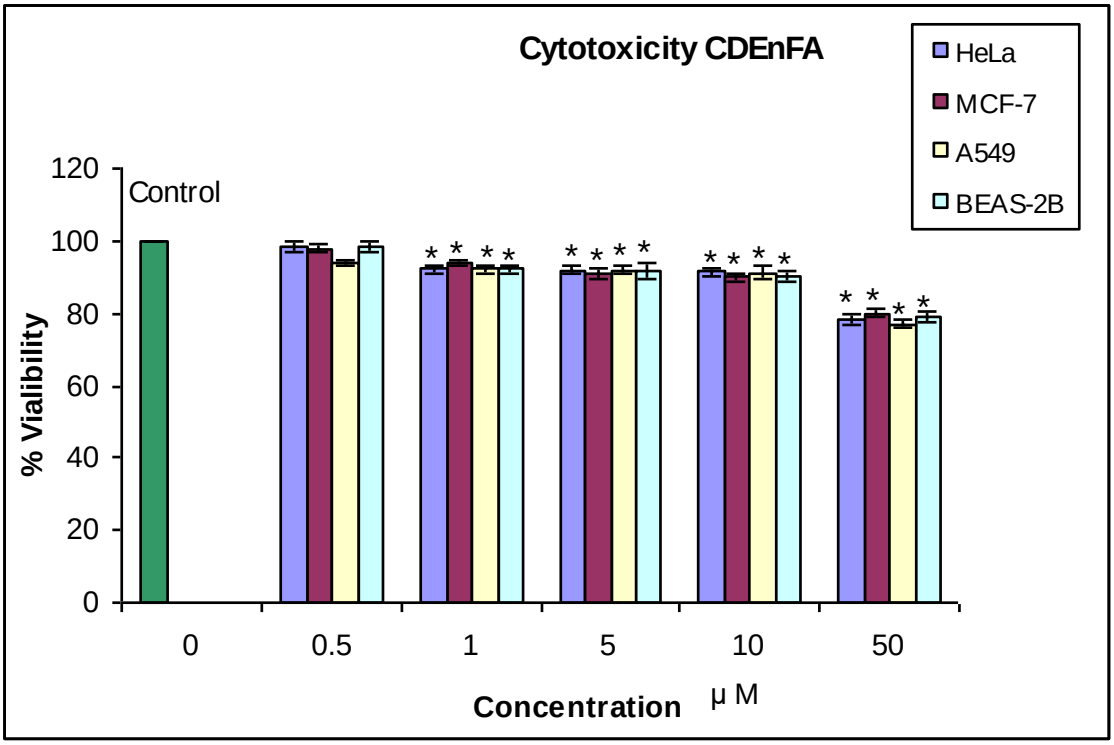


Figure 5. Effect of CDEnFA on the viability of HeLa, MCF-7, A549 and BEAS-2B cells following a 24 hr incubation at concentrations of 0.5, 1, 5, 10 and 50 μM CDEnFA. (* results which are significantly different to the control ($p<0.05$ at 95% confidence))

It can be seen from figure 5 that the drug delivery system CDEnFA does not show a significant cytotoxicity at a concentration of 0.5 μM towards any of the cell lines studied. In particular it should be noted that CDEnFA at this concentration is not cytotoxic towards the normal cell line BEAS-2B. This is a very exciting result and shows that the material is biocompatible at this concentration. Even though cell viability is statistically significantly different to the control at concentrations of 1, 5 and 10 μM CDEnFA the viability remains in the range 90-94% for all cell lines. Zhang *et al.* and Oh *et al.* have suggested that drug delivery systems are biocompatible when cell viabilities remain greater than 90 % [30, 31]. This is the first reported study of the cytotoxicity of CDEnFA and the results compare very favourably with other proposed drug delivery systems. For example Zhang *et al.* showed cell viabilities of 93.3 % and 97.6 % for HeLa and fibroblast (FB) cells respectively towards folic acid-glutathione-gold nanoparticles [30]. Oh *et al.* obtained viabilities in excess of 90 % for the osteosarcoma Saos-2 and MG-63 cells lines when exposed to a layered double hydroxide (LDH) $Mg_2Al(OH)_6$ which they have proposed as a new system for delivery of MTX [31]. The results obtained for CDEnFA provide some evidence that it has potential applications as a drug delivery system.

The results of the cytotoxicity assay obtained for the inclusion complex CDEnFA:MTX are shown in figure 6 for each cell line.

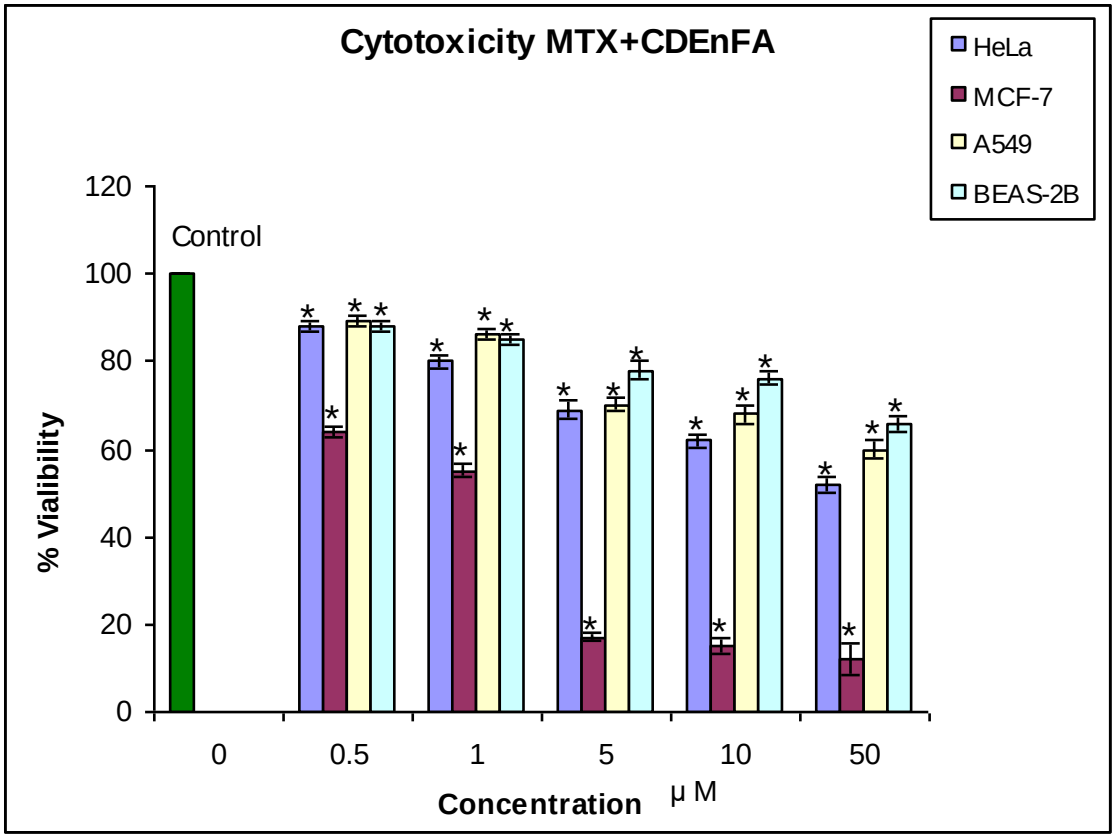


Figure 6. Effect of CDEnFA:MTX on the viability of HeLa, MCF-7, A549 and BEAS-2B cells following a 24 hr incubation at concentrations of 0.5, 1, 5, 10 and 50 µM CDEnFA:MTX. (* results which are significantly different to the control ($p<0.05$ at 95% confidence))

The results obtained from the MTT assay for CDEnFA:MTX have a similar profile to those obtained for the drug alone. Therefore it can be seen from figure 6 that CDEnFA:MTX demonstrates a significant cytotoxicity towards all cell lines studied at concentrations of 0.5, 1, 5, 10, 50 µM. However there are obvious differences in cell viability in the presence of CDEnFA:MTX in comparison to MTX. HeLa and A549 cell lines behave in a similar manner and show cell viabilities in the range 52-60 % after 24 hr

incubation with 50 μM CDEnFA:MTX. The biggest differences between MTX and CDEnFA:MTX can be seen from the cell viabilities of the BEAS-2B and MCF-7 cell lines. Figures 7 and 8 compare the results obtained for the CDEnFA host, MTX and the inclusion complex CDEmFA:MTX, with these cell lines.

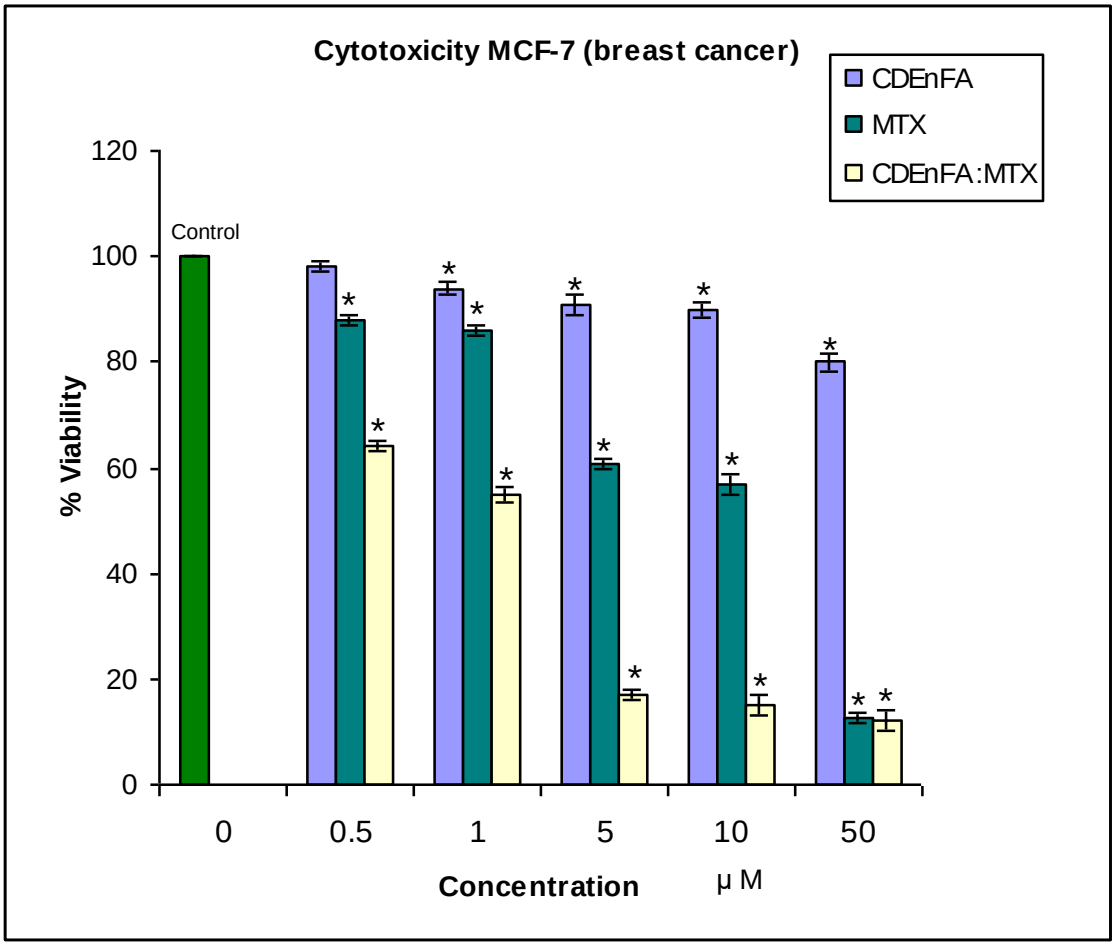


Figure 7. Effect of CDEnFa, MTX and CDEnFA:MTX on the viability of MCF-7 cells following a 24 hr incubation at concentrations of 0.5, 1, 5, 10 and 50 μM. (* results which are significantly different to the control ($p<0.05$ at 95% confidence))

It is obvious from figure 7 that inclusion of MTX in the cyclodextrin host has enhanced the efficacy of the drug. Even at the lowest concentration of 0.5 μM used the cell viability has decreased from 88 % for MTX to 64 % for CDEnFA:MTX. There are no comparable reports of a cyclodextrin-based drug delivery system for MTX. However comparisons can be made with other targeted drug delivery systems. Oh *et al.* reported Saos-2 cell viabilities of ~95 and ~55 % after 24 hr incubation with MTX and MTX-LDH [31]. However Wang *et al.* reported no significant difference in MCF-7 cell viability when treated with the anticancer drug paclitaxel (PTX) and PTX-loaded micelles composed of deoxycholic acid-*O*-carboxymethylated chitosan-folic acid [32].

Figure 8 compares the results obtained for the CDEnFA host, MTX and the inclusion complex CDEnFA:MTX towards the BEAS-2B normal cell line.

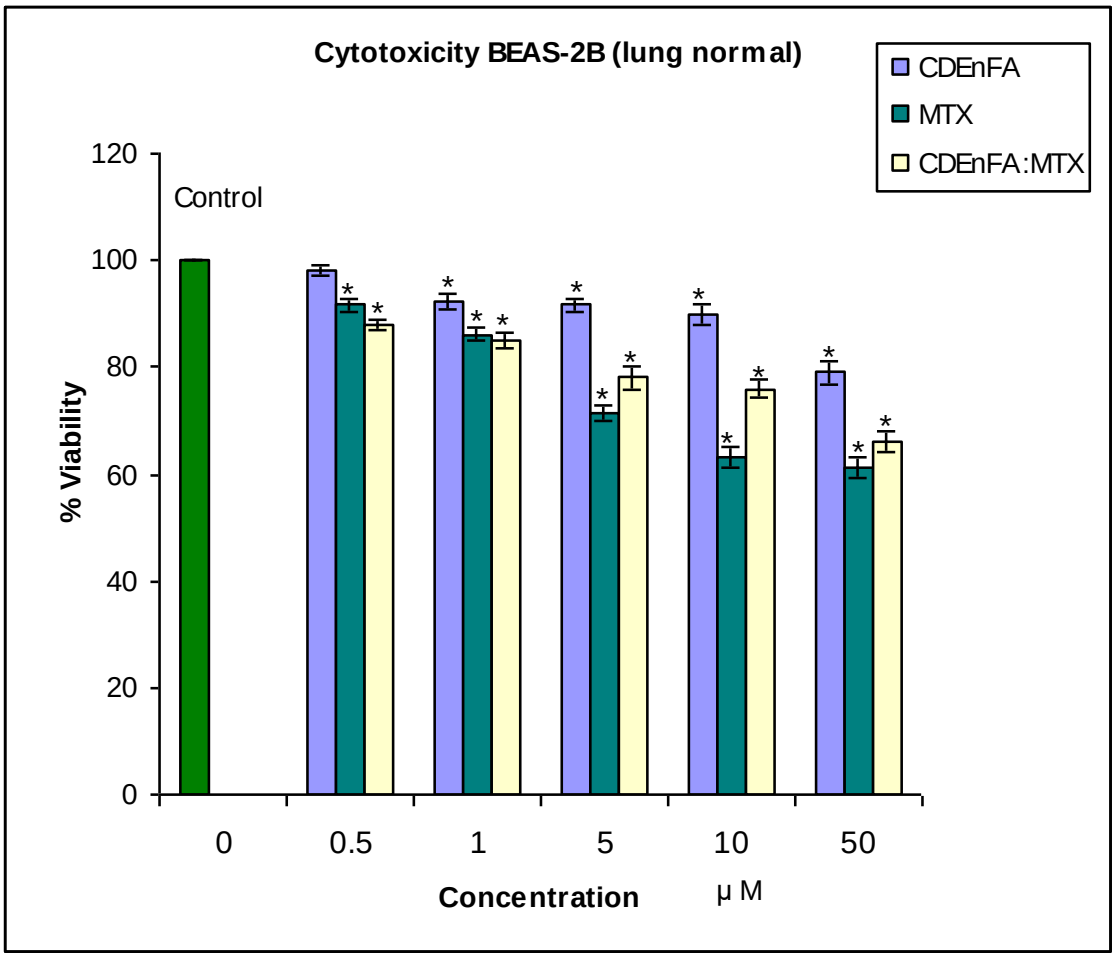


Figure 8. Effect of CDEnFA, MTX and CDEnFA:MTX on the viability of BEAS-2B cells following a 24 hr incubation at concentrations of 0.5, 1, 5, 10 and 50 µM. (* results which are significantly different to the control ($p<0.05$ at 95% confidence))

The BEAS-2B line shows similar viability at concentrations of 0.5 and 1 µM MTX and CDEnFA:MTX. The results shown in figure 8 however show that the viability of the normal cell line BEAS-2B improved when MTX is included in the cyclodextrin host in comparison to the drug alone. Although the difference is moderate these results suggest that the new drug delivery system may have some improved selectivity towards cancer cell lines compared to normal cell lines.

Conclusion

An IC_{50} value shown below in Table 1 was calculated for each test compound.

Table 1. IC_{50} values for CDEnFA, MTX and the Inclusion Complex CDEnFA:MTX

Cell line	**CDEnFA** (Conc µM)	**MTX** (Conc µM)	**CDEnFA:MTX** (Conc µM)
HeLa	1290.6±657	82.4±4.4	49.89±7.7
MCF-7	1316.4±759	9.4±1.5	1.08±1.1
A549	1119±557	153.5±16.5	117.2±11.2
BEAS-2B	1245.9±623	106.3±10.7	545.1±62.9

The MTT assay showed that the drug delivery vehicle CDEnFA is not cytotoxic towards the cell lines studied even towards the normal BEAS-2B cell line and therefore it is expected that it is safe for medical use. The inclusion complex CDEnFA:MTX has superior cytotoxic activity towards all of the cancer cell lines studied compared to the drug MTX alone. And CDEnFA:MTX is four times less cytotoxic towards the normal cell line than the drug alone. The observed toxicity is attributed solely to MTX since CDEnFA did not exhibit significant cytotoxicity. These results also suggest that the drug remains bioactive even after inclusion in the CD cavity. The cytotoxicity trend observed for CDEnFA:MTX in our study is MCF-7 (Breast) > A549 (Lung) > HeLa (Cervical) > BEAS-2B (normal Lung).

The effectiveness of many anticancer agents such as MTX depends on their uptake by cells and their ability to bind to DNA or cleave DNA. Although only a small number of

cell lines have been studied the cytotoxic properties of the inclusion complex CDEnFA:MTX presented here suggest that the complex is internalised in cells. The folate moiety in CDEnFA may play an important role in enhancing the activity of MTX *via* increased cellular uptake through receptor-mediated endocytosis and therefore increased internalisation of the drug. Further work is now required in order to explain the cytotoxic profiles and to investigate if indeed the complex is taken up by cells and to determine the localisation of the material in cells.

Acknowledgements

The authors would like to thank the Focas Research Institute, Dublin Institute of Technology and the Technological Sector Research: Postgraduate R&D Skills Programme for funding and support.

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