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Anti-MUC1 Aptamer as Carrier Tool of the Potential Radiosensitizer 1,10 Phenanthroline in MCF-7 Breast Cancer Cells

LAÍS N. ALVES[1,2], SOTIRIS MISSAILIDIS[2], CLAUDIA A. S. LAGE[3] and CARLOS EDUARDO B. DE ALMEIDA[1]

[1]*Laboratory of Radiobiology, Division of Medical Physics, Institute of Radioprotection and Dosimetry, Brazilian Nuclear Energy Commission, Rio de Janeiro, Brazil;*
[2]*Institute of Technology in Immunobiologics (Bio-Manguinhos), Oswaldo Cruz Foundation, Rio de Janeiro, Brazil;*
[3]*Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*

Abstract. *Background: Proteins overexpressed in malignant tissues form important targets in the development of targeted therapeutics, and aptamers comprise an important affinity agent for therapy and drug delivery. In this study, aberrantly expressed mucin 1 glycoprotein was investigated as a therapeutic target in a breast cancer model. Materials and Methods: In order to determine the feasibility of using an aptamer against mucin 1 (aptA) as carrier of the cytotoxic compound 1,10-phenanthroline to MCF-7 cells, as a potential radiosensitizer, was studied in experiments using circular dichroism and rhodamine labelling by fluorescent microscopy and flow cytometry. Results: 1,10-Phenanthroline can be intercalated within aptA when complexed with Fe(II) ions, with dissociation constant (Kd) of 30 μM. The complex was subsequently capable of binding to and being internalised in MCF-7 breast cancer cells. Conclusion: aptA can carry 1,10-phenanthroline to cancer cells specifically and this complex represents a potential target-directed anticancer therapy.*

Radiotherapy is widely used for treatment of localized malignancies. About two-thirds of patients with cancer undergo radiation therapy at some stage of the treatment (1, 2). Solid tumours normally present hypoxic areas and areas with low oxygenation. Considering the predominance of the secondary effect of interaction regarding radiotherapy on tissue, the low formation of free radicals can be overcome by the use of staggered doses of ionizing radiation and through administration of compounds capable of sensitizing the malignant tissue (3-5). The use of systemic radiosensitizers usually produces an additive effect on the death of tumour cells during radiotherapy. However, systemic therapy is often limited due to a high level of associated side-effects, which reduces or even opposes the expected success of the treatment (6-10). An ideal condition for systemic antitumour treatment would be the existence of a mechanism capable of delivering therapeutic agents exclusively to tumour cells. Targeted cancer therapy can improve anticancer efficacy, which would consequently be reflected in minimal side-effects and better patient health (3). Overexpression of proteins in malignant tissues, also called biomarkers, is important and of great interest and can offer potential targets for disease-specific treatment (1, 11). This characteristic can be positively explored for the development of therapeutic approaches, with the use of specific agents for tumour tissues (1).

Transmembrane glycoprotein mucin 1 (MUC1) is well-characterized and has been a target for many anticancer therapy studies. Its expression is increased and glycosylation pattern altered in most malignant adenocarcinomas, including breast, lung, and colon cancer (12). Aptamers are oligonucleotide molecules capable of differentiating between tissues by binding with high affinity and specificity to their targets (13-16).

In this study, we explored the premise that a MUC1 aptamer, called aptA, can be used to deliver cytotoxic molecules into breast cancer cells in a tumour-directed manner, in an approach known as drug delivery. This MUC1-targeted aptamer has already been successfully selected and tested for its biodistribution *in vivo*, with and without polyethylene glycol (PEG), constructed in monomeric and multimeric metastable tecnetium-99 (^{99m}Tc)-labeIled forms, compared with another

Correspondence to: Carlos Eduardo B. de Almeida, Laboratório de Radiobiologia, Divisão de Física Médica, Instituto de Radioproteção e Dosimetria, Comissão Nacional de Energia Nuclear, Av. Salvador Allende S/N., Rio de Janeiro, RJ, CEP 22783-127, Brazil. Tel: +55 2121732887, e-mail: cbonacos@ird.gov.br

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antitumour MUC1 aptamer, aptB, a tumour glycosylated MUC1 form (17-22). The delivery of a toxic or radiosensitizing agent specifically to tumour cells, in combination with ionizing radiation, might lead to a selective synergistic cell death effect of tumor cells. The divalent ion chelator 1,10-phenanthroline (Phen) was selected to be carried by the MUC1 aptamer. Interest in the use of Phen combined with different metal ions has increased due to important results suggesting great toxicity against tumour cells (23). Additionally, it was demonstrated that the complex of Phen with Fe^{2+} can induce oxidative damage in DNA when in reaction with hydrogen peroxide, a well-known by-product of water radiolysis (24, 25). This strategy would offer better flexibility in radiotherapy planning and lead to the possibility of using lower doses of radiation to obtain the same level of induced damage, which could preserve the normal tissue adjacent to the tumour.

Materials and Methods

Cell line. The MCF-7 human breast cancer cell line was obtained as courtesy from Dr. Claudia Gallo (Rio de Janeiro State University-UERJ). Cells were cultured in RPMI (Sigma–Aldrich, São Paulo, Brazil) medium supplemented with 10% foetal bovine serum (Sigma–Aldrich) and a mixture of penicillin/streptomycin (Sigma–Aldrich). Cells were grown at 37˚C in a humidified atmosphere with 5% of CO_2. All experiments were performed on cells in the exponential growth phase.

DNA aptamer. The aptamer was purchased from Naxos DNA Services (Rio de Janeiro, Brazil). It was selected against the MUC1 protein core tandem repeat sequence, using a MUC1 synthetic peptide, as previously published (17). This is a single-stranded DNA aptamer, 25 bases long (5'-GCA GTT GAT CCT TTG GAT ACC CTG G-3'). It has an inverted dT at its 5'-terminus to prevent degradation by 5' exonucleases, and an amine group at the 3'-terminus to facilitate coupling of molecules to the aptamer.

Labelling of aptamer with rhodamine-123. The method used as reference was described by Ferreira *et al*. (17). For this procedure, 936.6 μg of aptA were dissolved in 100 μl of bicarbonate-carbonate buffer (600 mM) (pH 9.2), resulting in aptA (1.2 mM). Subsequently, 100 μI of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (1.2 mM; Sigma) was added and allowed to react for 10 min at room temperature. After the stipulated period, 100 μl of rhodamine-123 (12 mM; Sigma) dissolved in dimethyl sulfoxide (DMSO; Sigma) were added. The mixture remained overnight in an oscillator. The compound was separated using Vivaspin 500 centrifugation column (GE Life Sciences, São Paulo, Brazil) with 5 kDa cut-off and free rhodamine washed out form the solution by continuous filtering with water. Following this protocol, a peptide bonding reaction was performed between the amino modification of aptA and the -COOH group of rhodamine-123. The solution was stored at −20˚C.

Circular dichroism (CD). The spectrophotometer used was of the Jasco, model J-815. Two different titrations were performed, the first titrating Phen on DNA and the second titrating Phen with $FeSO_4$ in DNA, in phosphate-buffered saline (PBS) buffer (pH 7.4). All measurements were made using 5 nm of bandwidth and 0.05 cm of optical path. CD spectra were measured in a range of 220-350 nm, according to the method described by Missailidis *et al*. (26). In addition, three accumulations for each spectrum were used, thus three measurements of each sample were made and the data provided by the equipment were the average of the three. The ideal concentration used in all experiments was 30 μM DNA aptamer. For the titrations in the first experiment, 10 μM of Phen was titrated in each measurement, up to a total of 50 μM. For the second experiment, 2.5 μM of Phen plus $FeSO_4$ (3:1) was added in each measurement, up to 37.5 μM.

Fluorescence microscopy. Spherical coverslips with 12 mm diameter were added to the bottom of 24-well plate wells, and 5×10^4 cells/well were seeded onto the coverslips. The plates were incubated in CO_2 incubator at 37˚C for a period of 24 h so that the cells adhered to the coverslips. After incubation, the complete medium was replaced with medium without foetal bovine serum, to avoid non-specific aptA binding. Then MCF-7 cells in medium alone or with different combinations of Phen (1 μM), FeSO4 (330 nM) and rhodamine-labelled aptA (1 μM), namely a) rhodamine-labelled aptA, b) Phen:Fe, or c) Phen:Fe and rhodamine-labelled aptA at a ratio of 1:1, were incubated for 10, 60 and 120 min. After each incubation, the medium was removed, the culture adhered to the coverslips was washed twice with 1X PBS and then fixed at 37˚C with 4% paraformaldehyde (Sigma) for 15 min. At the end of this time, the fixed cultures were washed once with 1X PBS. The coverslips were then removed from the wells with the help of a disposable needle and tweezers, and immediately inverted onto 5 μl of Vectashield mounting medium (Vector Laboratories, Inc. Burlingame, CA, USA) containing 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) on a glass slide, with posterior sealing. The slides were then analysed for the signal of aptA-rhodamine-123 with appropriate filter for excitation wavelength of 511 nm and emission wavelength of 534 nm using an AxioImager A2 epi-fluorescense microscope (Carl Zeiss Microscopy, LLC, New York, NY, USA).

Flow cytometry. For the assays, Guava EasyCyte instrument (Merck Millipore, Hayward, CA, USA) was used. Firstly, 5×10^4 cells/well were plated in 24-well plates, grouped according to the treatment to be performed. The plates were incubated in an incubator with 5% CO_2 at 37˚C for 24 h for cell attachment. At the end of the incubation, the complete medium was replaced with foetal bovine serum-free medium to avoid non-specific aptA binding, and then different combinations of aptA (1 μM) and Phe (1 μM) plus $FeSO_4$ (330 nM) were administered for 10, 60 and 120 min. After each incubation period, the medium was removed, the adhered culture was washed twice with 1X PBS and then suspended using 10 mM EDTA in PBS for 5 min. The suspension was centrifuged at 200 × *g* for 5 min at room temperature. The supernatant was discarded and the precipitate resuspended in 200 μl of 1X PBS. The fluorescence signal of the rhodamine-labelled aptA was collect after different incubation times to track the binding of this molecule to its target in MCF-7 cells and data were analysed using the InCyte application (Guava EasyCyte instrument).

Results

1,10-Phenanthroline intercalates with aptA preferentially bound to $FeSO_4$. When titrating different concentrations of

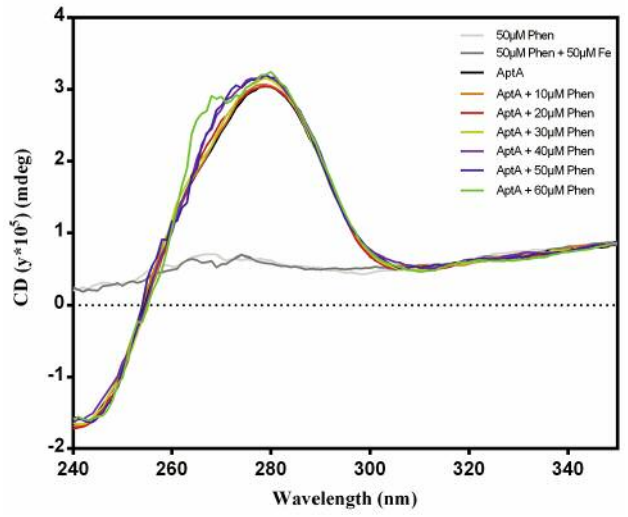


Figure 1. *Circular dichroism (CD) of aptamer-A (aptA) titrated with different concentrations of 1,10-phenanthroline (Phen). The indicated concentrations of Phen were added to 30 µM of aptA. Even though Phen was put in solution with phosphate buffer, no signal alterations related to the buffer were identified for the selected wavelength interval. Phen at 50 µM and iron (Fe) at 50 µM did not present a relevant CD signal. The data provided by the equipment were the average of the three accumulations for each spectrum.*

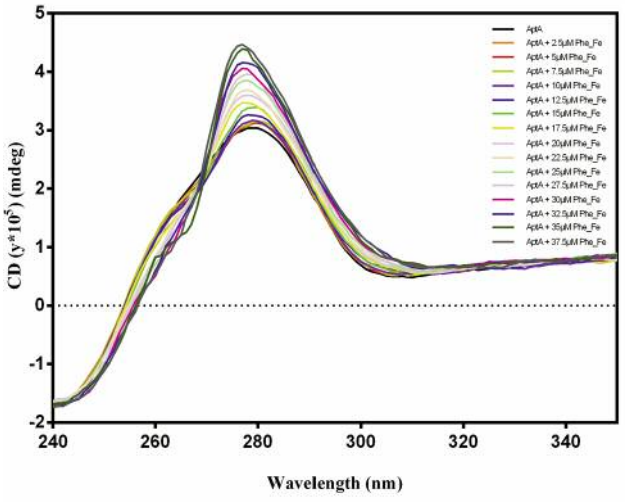


Figure 2. *Circular dichroism (CD) of aptamer-A (aptA) titrated with different concentrations of 3:1 1,10-phenanthroline:iron (Phen_Fe) complex. Indicated concentrations of the complex were added to 30 µM of aptA in PBS. As the double-helix region incorporated the complex, the CD signal increased and the spectra related to each concentration is shown.*

Phen in aptA solution, the CD spectrum obtained showed that there was no binding between molecules. The ellipticity signal of the CD spectrum was related only to the DNA signal, even with the addition of increasing concentrations of Phen (Figure 1). Phen did not show a CD signal when free in solution. There was no change in the DNA signal by the addition of Phen. Ions of Fe were then added to the Phen solution. When titrating different concentrations of the Phen and Fe complex in solution of aptA, this complex was able to intercalate in the double-strand region of aptA (Figure 2), as indicated by changes in the CD spectrum. The ratio of Phen to $FeSO_4$ used was 3:1, as Fe has the ability to coordinate the binding of three Phen molecules (27, 28). Thus, it was evidenced that the planar structure formed after the binding of these two molecules is necessary for intercalation into DNA. As the aptA double-helix region incorporated the Phen:Fe complex, ellipticity increased. This was evaluated through interaction analysis from minimal concentrations of Phen:Fe complex, through equimolarity between aptA and Phen:Fe complex to extrapolated concentrations of this complex. Although Phen and Fe are non-chiral, and thus they do not exhibit a CD spectrum when free in solution (27), they acquired an induced CD signal when complexed with the aptamer. The CD spectrum is induced by the binder–substrate (ligand–host) interaction, and is dependent on position and orientation of the binder to the nitrogenous bases (19, 28).

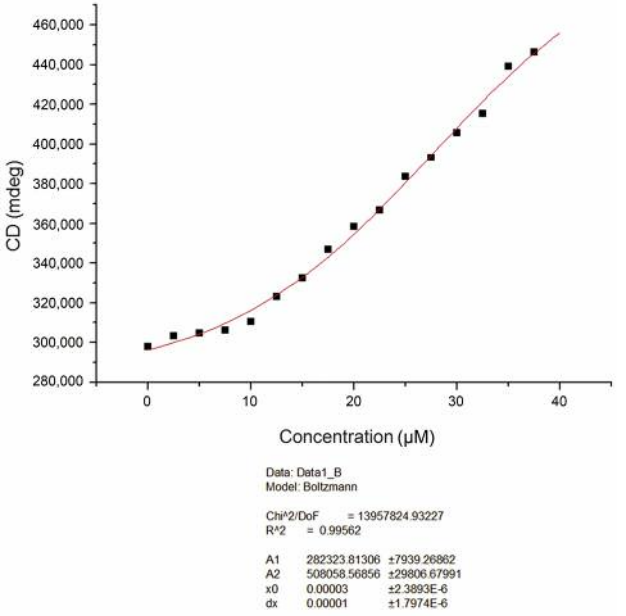
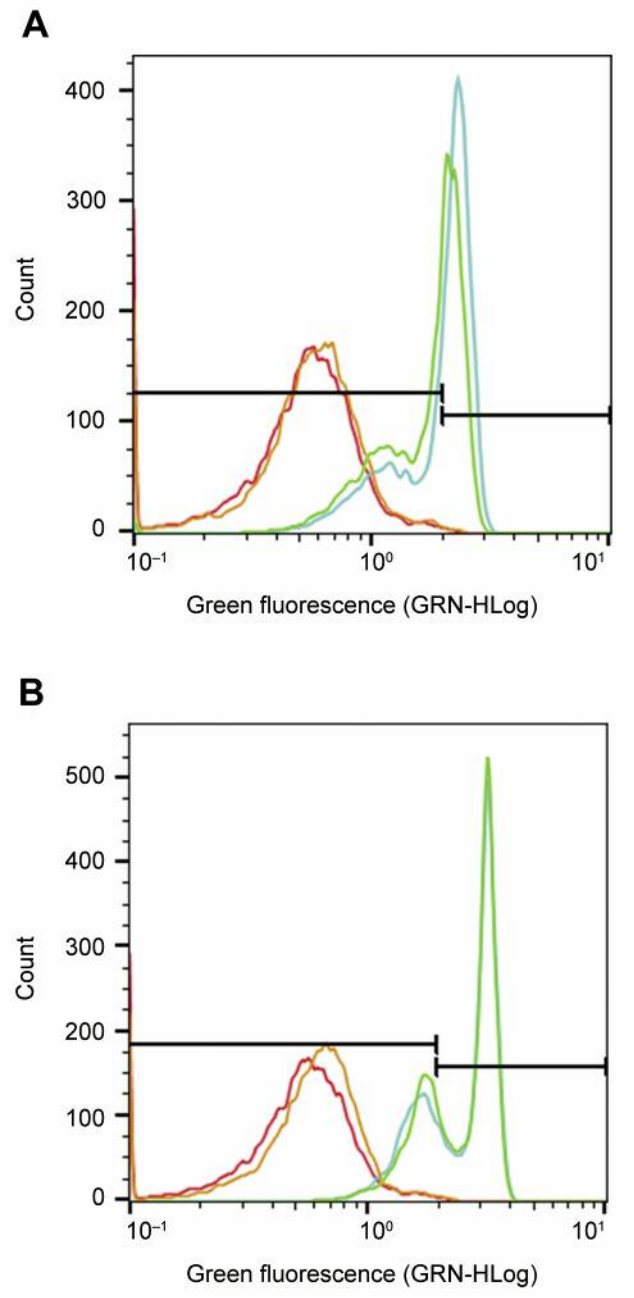


Figure 3. *Sigmoidal curve fitting of the circular dichroism (CD) data with Boltzmann equation. The CD data from the 280 nm wavelength are displayed in graphic form (black squares), with a sigmoidal curve fit (red curve) according to the following equation: y=(A1-A2)/(1+ e^((x-x0)/dx))+A2, where A1 was the initial value of the y-axis, A2 was the final value of the Y axis, x was the experimental values, x0 was the inflection point of the sigmoid, and dx was the constant time.*



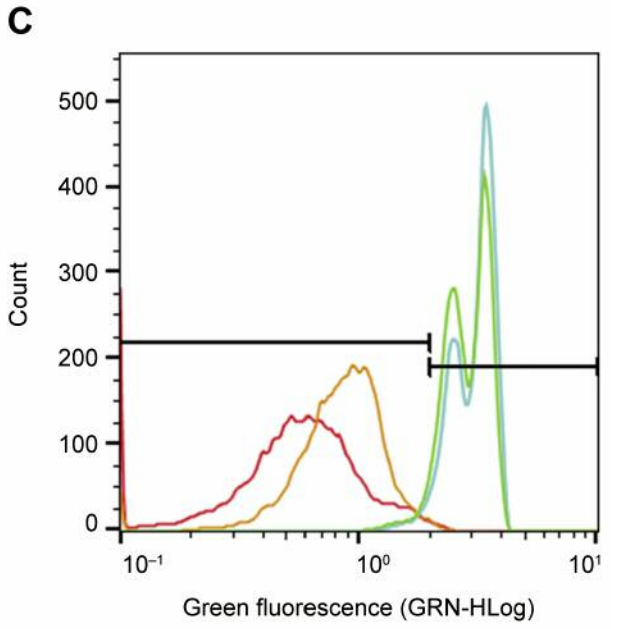


Figure 4. *Flow cytometric analysis of MCF-7 cells treated with different combinations of rhodamine-labelled aptamers (aptA_rho, 1 μM) and 1,10 phenanthroline (Phe) (1 μM):Fe (330 nM) complex for different times. A: 10 min; B: 60 min; C: 120 min. Red: MCF-7 cells only; Blue: MCF-7 + aptA_rho; Orange: MCF-7 + Phen:Fe; Green: MCF-7 + aptA_rho + Phen:Fe.*

The dissociation constant and stoichiometry between the molecules and the aptamer were obtained by plotting the titration data. With the fit of a sigmoidal curve described by the Boltzmann equation, it was found that the 3:1 Phen:Fe complex had a dissociation constant (K_d) of 3×10^{-5} M with DNA, obtained by the equation ($K_d=1/x0$) (Figure 3), which is compatible with other intercalators such as doxorubicin (29). In addition, it appears that more than one 3:1 Phen:Fe complex binds to the same aptamer, which is in agreement with the structure analyzed by nuclear magnetic resonance spectroscopy of the anti-MUC1 aptamer (30). Extrapolating the curve by the same simulation (data not shown), we obtained a concentration of 6×10^{-5} M at the point where complex formation became saturated. This double value of K_d indicates that the association of the Phen:Fe complex with the aptamer occurs at a maximum ratio of 2:1 [two 3:1 Phen:Fe complexes for each aptamer].

AptA maintains affinity with MUC1. When interacting with the double-strand region of aptA, 3:1 Phen:Fe complex has the potential to modify the structure of the aptamer and consequently affect the binding to its target, as structural conformation is an essential feature for aptamer recognition. Therefore, in order to determine if the intercalation of the complex would alter the ability of the aptA to bind a $MUC1^+$ target cell, we evaluated the kinetics of 3:1 Phen:Fe complex binding to $MUC1^+$ cells. The flow cytometric analysis was performed at 10, 60 and 120 min incubation of the cells with aptA and 3:1 Phen:Fe complex, using rhodamine-labelled aptA. The analysis of the results (Figure 4) shows that the aptA maintained its affinity with MUC1, even carrying 3:1 Phen:Fe complex in supposed ratio of two complexes to one aptamer as

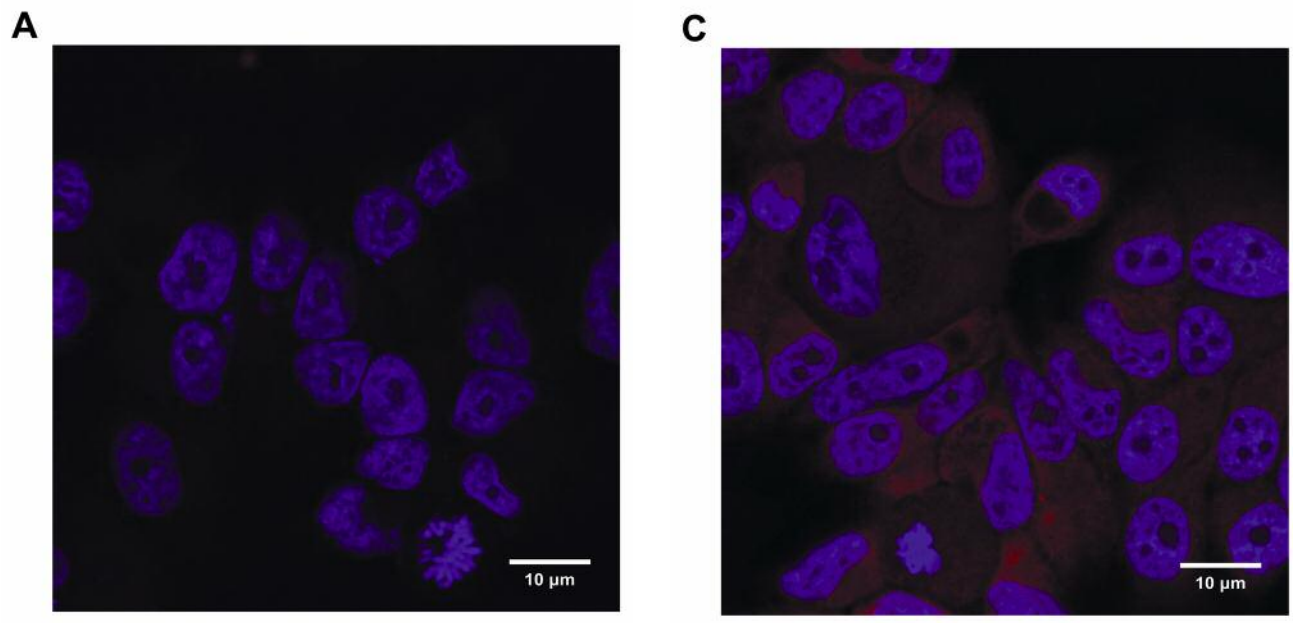


Figure 5. *Kinetics of binding of rhodamine-labelled aptamers to 7MCF-7 cells. Representative fluorescence microscopy images of MCF-7 cells–stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine and treated with rhodamine-labelled aptamers (1 μM) and 3:1 of 1,10-phenanthroline:iron complex at A: 10 min; B: 60 min; and C: 120 min are shown. Stained nuclei are blue, and rhodamine-labelled aptamers are red. The presented images are channel-merged. Original magnification 400×.*

mentioned above. It is possible to perceive a time-dependent fluorescence signal, showing what appears to be the saturation of the binding at 60 min. At 120 min, the fluorescence signal decreased, a fact that may be related to complex processing by the cell, possibly causing extrusion of the fluorophore.

The complex is internalized by MCF-7 cells. As a way to determine the internalization of the complex in $MUC1^+$ cells, fluorescence microscopy analysis was performed using rhodamine fluorophore linked to aptA for the different treatment groups. At 10 min, fluorescence in the rhodamine emission range was undetectable for all treatment groups, indicating that the complex had not yet been internalized by the cells. Fluorescence appeared subsequently for the groups treated with rhodamine-labelled aptA (positive control), and Phen:Fe with rhodamine-labelled aptA (aptamer loaded). Fluorescence signal reached a maximum at 60 min and it was possible to detect diffusion of the fluorophore in the cytoplasm. At 120 min, the fluorescence intensity decreased, suggesting that the complex or fluorophore underwent an extrusion process (Figure 5).

Discussion

The effectiveness of systemic therapy is usually limited by the side-effects of the agents used, which can affect both normal and tumour cells (10, 31). One strategy to reduce side-effects is to use the approach called targeted drug delivery (32). The transmembrane glycoprotein MUC1 is considered a viable target, since it can be found to have altered characteristics and expression in most adenocarcinomas (11, 12). The extracellular portion of MUC1 has a polypeptide chain with laterally

attached oligosaccharides, characterizing glycosylation. In normal tissues, the polypeptide chain of MUC1 is protected by these sugar chains, whereas in tumour cells, the chain is exposed due to a deficiency in glycosylation (12). There is little or complete absence of oligosaccharides, causing loss of adhesive properties and contributing to the aggressive nature of tumours that express this protein (12, 17).

In this work, we evaluated the complex formed between an aptamer that specifically binds to the MUC1 core at the tandem repeat sequence, aptA, and the Phen molecule bound to Fe ions (3:1 Phen:Fe complex). The use of Fe is necessary due to the planar structure formed with Phen, at the ratio of 3 Phen:1 Fe (27). Cells exposed to Phen in its monomeric form, as well as forms complexed to ions such as copper(II), had shown increased oxygen uptake which enhanced free radical damage (33-35). In general, it is assumed that Phen may influence mitochondrial function and decoupling of the respiratory chain, but the biological effect of the molecule depends on the ion to which it is complexed (33, 36-39). The antitumour activity of Phen in cell culture has been evidenced when complexed with palladium(II) and vanadium ions, in a cisplatin-like complex. However, the studied complexes showed low efficiency for the proposed aims (23, 38, 40).

Here we showed that the 3:1 Phen:Fe complex binds to the MUC1 aptamer with a K_d of 30 μM, which is compatible with the K_d of other DNA-intercalating molecules, such as doxorubicin (29). Doxorubicin is a chemoradiotherapeutic compound widely used for the treatment of several cancer types, and has been covalently or non-covalently conjugated to aptamers in many studies. In general, the complexes increased the therapeutic efficacy of the drug, considerably reducing its associated side-effects. For example, doxurubicin conjugated to aptamers against epithelial cell adhesion molecule (EpDT3), human epidermal growth factor receptor-type 2 (HER2), MUC1, prostate-specific membrane antigen (PSMA) and HepG2 cells were successfully used in the treatment of retinoblastoma (29), and breast (41), lung (42), prostate (43) and liver (44) cancer, respectively.

Many studies on the application of aptamer-guided therapy report the use of nanoparticles for the transport of toxic agents in sophisticated constructions (16, 45-52). However, it has also been reported that isolated aptamers can efficiently deliver toxic agents or siRNA molecules to tumour cells *in vivo* (53-56). Studies indicate that the delivery of ligands is made more agile when there is no covalent binding, compared to the covalently bound complexes (57). In addition, it is well established that aptamers tend to interact with planar molecules through intercalation, without altering aptamer affinity for the target, and release the drug in low pH environments, such as lysosomes (43). Without the use of nanoparticles, a complex with lower molecular weight is generated, which facilitates the elimination of the compound, usually through the urine (21).

From the data obtained in the development of this work, it was possible to determine that each aptA has the capacity to load two 3:1 Phen:Fe complex structures. Maintenance of the loaded aptamer specificity for MUC1 was confirmed by flow cytometry, which also pointed to an apparent saturation at 60 min and possible extrusion or photobleaching of the complex at 120 min. These data were compatible with fluorescence images that showed the same dynamics of time-dependent interaction. Hu *et al*. described the selection of an anti-MUC1 aptamer and evaluation of its ability to deliver doxorubicin specifically to tumour cells, also through non-covalent interaction. In their work, the MUC1 aptamer, called MA3, had minimal reactivity with albumin, which is the most abundant protein in the blood. Thus, MA3 would have good availability in human serum to bind to its target. In *in vitro* models of lung and breast cancer, the complex was able to selectively deliver the drug into the tumour cells and had greater therapeutic efficiency when compared to treatment with doxorubicin alone (42).

AptA is a powerful tool with high specificity and affinity for MUC1. It has stability *in vivo* and is able to accumulate in the tumour mass (21). Further studies would need to be performed to improve the biological half-life of the molecule *in vivo* for clinical use (21, 22), but currently available data characterize aptA as an ideal molecule for replacement of other compounds in tumour imaging and target-directed drug delivery in breast cancer (17, 20, 21).

The approach proposed here highlights that this aptamer can be loaded with a radiosensitizer and offer the potential to have synergistic or additive lethal effect in combination with ionizing radiation. During the radiotherapy treatment of breast tumours, part of the cardiorespiratory system lies in the path of the radiation beam and is often affected by undesirable side-effects (58). In a scenario that allows the use of specific radio-sensitizing agents, radiotherapy planning would more effectively preserve adjacent healthy tissues. This would occur by reducing the dose of ionizing radiation that reaches healthy tissues surrounding the target tissue that should be irradiated, while maintaining the desired level of radiobiological damage to the tumour volume. In the breast cancer model, such advancement to new protocols with greater radiotherapeutic efficiency and lower doses of radiation, would imply less debility from radiotherapy-related cardiotoxicity in surviving patients. These results may contribute to a novel drug-delivery approach for use in chemoradiotherapy.

Our results demonstrate that the 3:1 Phen:Fe complex can react with the anti-MUC1 aptamer AptA and the complex thereby formed can discriminate breast cancer cells that express the MUC1 tumour marker and be internalized into such cells through receptor-mediated endocytosis, thus carrying Phen with it into cancer cells. This suggests that aptA can find application as a carrier of molecules such as Phen and other radiosensitizers with the potential to create a target-directed

approach to increase the efficacy of radiotherapy. Future work may focus on testing this complex for *in vitro* radiosensitization potential under various forms of use as adjuvant, neo-adjuvant or concomitant with irradiation, and in the evaluation of *in vivo* pharmacokinetics for potential therapeutic use.

Conflicts of Interest

The Authors confirm that there are no conflicts of interest in regard to this study.

Authors' contributions

Laís N. Alves (MSc student) performed the majority of the experimental work described in this article and participated in its writing. Sotiris Missailidis was responsible for the selection of the MUC1 aptamer and the performance and analysis of the CD work and article writing. Claudia A.S. Lage was the principal investigator of the project and supervisor of the MSc work, responsible for the conception of the radiosensitizing project and article writing. Carlos Eduardo B. de Almeida was co-supervisor of the project and responsible for orientation and performance of the cell work (flow cytometry and fluorescence microscopy) as well as article writing.

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