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Histidine Modifications in Peptides: Impact on Anticancer Activities

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Table of Contents

Declaration	4
Acknowledgements.....	5
Abstract.....	6
1. Introduction and background	8
<i>Conventional Cancer Treatments</i>	<i>10</i>
Chemotherapy	10
Radiotherapy	10
The drawbacks of conventional therapies	11
<i>Anticancer peptides</i>	<i>12</i>
Why are anticancer peptides superior?.....	12
Dual action antimicrobial/anticancer peptides.....	14
Mechanisms of action	15
The G3 Peptide.....	19
HHC10 and HHC36	25
Magainin II	27
Penetratin	28
<i>Current/further research and aims</i>	<i>29</i>
2. Materials and Methods	35
2.1 – Cell culture	35
2.1.1 – Cell lines.....	35
2.1.2 – Sterilisation	36
2.1.3 – Passaging.....	36
2.1.4 – Cryopreservation	37
2.1.5 – Thawing protocol	37
2.2 – Peptides.....	38
2.2.1 – Peptide dissolution	38
2.2.2 – AlphaFold2.....	38
2.3 – Microscale Thermophoresis (MST)	39
2.3.1 – Peptide and siRNA preparation	39
2.3.2 – MST experiment.....	40
2.4 – Cell proliferation assay.....	40
2.4.1 – Seeding multi-well plates	41
2.4.2 – Fixing and staining procedure	41
2.4.3 – High Content Screening	42
2.4.4 – High Content Analysis.....	42
2.5 – Transfection	43
2.5.1 – Plate preparation	43
2.5.2 – Peptides and siGLO Red Transfection Indicator	43
2.5.3 – High Content Screening	44
2.5.4 – High Content Analysis.....	45
2.6 – Statistical Analysis.....	45
Results	46
1. MST	46
2. Cell Proliferation	52

HCT116	54
Caco-2	61
HFF	66
3. <i>Transfection</i>	72
HCT116	74
Caco-2	80
HFF-1	85
Discussion.....	91
<i>MST</i>	94
<i>Cell proliferation</i>	97
<i>Transfection</i>	101
Conclusion/future work.....	106
References.....	109

Declaration

I, the author, confirm that the thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not previously been presented for an award at this, or any other, university. The introduction of this thesis was previously included in my first-year confirmation report, where it served as a literature review and is expected to constitute a substantial portion of the introduction section.

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Abstract

Peptide-based therapeutics have emerged as a promising avenue in the development of novel anticancer strategies, offering potential for high specificity and reduced toxicity compared to conventional treatments. This study explored the anticancer potential of a set of rationally designed peptides, focusing on the impact of specific amino acid modifications. Human cancer and non-cancer cell lines were used to assess biological activity through a range of biochemical and imaging-based techniques. Preliminary findings suggest that certain modifications may influence cellular uptake and selective cytotoxicity. These results lay the groundwork for further investigations into peptide-mediated drug delivery systems and their potential application in cancer therapy.

Certain details have been redacted to protect intellectual property and future publication opportunities.

1. Introduction and background

Cancer is a collection of diseases marked by uncontrolled growth due to a sequence of DNA mutations. It ranks as the second most prevalent cause of death worldwide, after cardiovascular disease (Arnold et al., 2015).

The ability of tumours to support angiogenesis—the creation of new, localised blood vessels that adjoin pre-existing ones in the body to obtain the nutrients and oxygen needed for ongoing growth—is a crucial characteristic of cancer. A key hallmark of cancer is the fact that tumours can sustain angiogenesis, which is the production of localised blood vessels that adjoin to the body's existing blood vessels in order to get the oxygen and nutrition required for continued growth (Nishida et al., 2006).

Additionally, these tumours can infiltrate neighbouring cells and organs, forming tumour masses (Anderson and Simon, 2020). A major element of today's molecular understanding of cancer is that it develops over time, as a protracted and complicated cascade of genetic mutations. The effects of each change assist precancerous cells in acquiring some of the features essential for cancer cells to progress to the malignant stage of development.

Two different types of genes play a crucial role in the initiation of cancer. These genes control the cell cycle, which is the mechanism by which cells multiply, in their original form. The first category of these genes, known as tumour suppressor genes, function as inhibitors of cell proliferation (Hinds and Weinberg, 1994). Proto-oncogenes are a type of gene that stimulate cell division. Proto-oncogenes and tumour suppressor

genes cooperate to regulate cell growth and ensure that each cell, tissue, and organ in the body maintains the correct size and shape for its function. Proto-oncogenes, when mutated, transform into oncogenes, which are genes that stimulate excessive cell growth (Adamson, 1987). Mutations in tumour suppressor genes cause them to become inactive, leading to loss of control over cell division and facilitating uncontrolled growth. Combined mutations in these two gene classes account for most uncontrolled proliferation of cells seen in malignant cases.

The most often diagnosed kinds of cancer globally are colorectal, lung, prostate, and breast cancers (Domalaon et al., 2016). The search for anticancer treatments with minimum side effects and toxicity has become an important priority due to cancer being the second largest cause of death worldwide.

In cancer research, the primary goal has been about successfully killing cancerous cells whilst protecting the surrounding healthy cells and tissue. Cheok highlighted in 2012 (Cheok, 2012) that a great deal of work has been conducted to protect normal, healthy cells from the harmful effects of chemotherapy. Recent advancements in oncological research have led to the development of various aggressive chemotherapies and radiotherapies (Earle et al., 2008). However, these treatments are costly, can cause adverse reactions, and could potentially increase the likelihood of developing secondary cancers.

Conventional Cancer Treatments

Chemotherapy

Chemotherapy is the treatment of cancers with cytotoxic drugs. These drugs eliminate cancerous cells along with a substantial number of cells that are healthy, however the healthy cells may be restored following therapy (Zhang et al., 2017a). Cytotoxic medications, such as alkylating agents, operate by attaching an alkyl group to DNA proteins, causing disruption to the double helix strands' ability to bind together. This causes the DNA strands to fragment and inhibits cancer cells from multiplying, ultimately resulting in the death of the cancer cell (Colvin, 2002). Alkylating agents such as thiotepa (Wilson and Weber, 2013) and mechlorethamine (Zhou et al., 2011) are utilised in contemporary medicine to treat different types of cancers. Topoisomerase inhibitors are a family of cytotoxic drugs used in chemotherapy to combat cancer. DNA topoisomerases are enzymes essential for preserving the structure of DNA during transcription, replication, and recombination processes to ensure the integrity of the cell's genetic information (Champoux, 2001). Topoisomerase inhibitors decrease the activity of these enzymes, leading to the suppression of DNA replication in cancer cells and ultimately killing the tumour. Irinotecan and teniposide are two examples of topoisomerase inhibitors (Long, 1992).

Radiotherapy

Radiotherapy is another therapeutic approach which utilises ionising radiation to treat cancer. High-energy rays are used to target and destroy cancer cells irreparably, preventing their growth and division, thus stopping the tumour or cancer from progressing (Baskar et al., 2012). Radiation therapy can either cause direct damage

to the DNA of cancer cells or produce free radicals that can harm the DNA (Yoshimura et al., 2013). This treatment strategy is localised, specifically targeting the cancer cells undergoing treatment. The procedure can be administered externally via a machine or internally by brachytherapy, which includes the placement of a radioactive implant near the tumour (Chargari et al., 2019). External radiation therapy, also known as external beam radiation therapy, is commonly administered using x-rays or gamma radiation, with gamma radiation being the more potent method (Welsh et al., 2006).

The drawbacks of conventional therapies

Acute myeloid leukaemia can develop as a result of chemotherapeutic treatments involving topoisomerase inhibitors (Ratain and Rowley, 1992). These inhibitors modify the DNA of cancerous cells but can also damage nearby normal tissue, potentially leading to the formation of a secondary cancer in a different part of the body (Pui and Relling, 2000). Scientists also found that patients with acute myeloid leukaemia had an increased risk of developing therapy-related secondary malignancies following chemotherapy for a previous disease (Morton et al., 2013).

Cancer treatments have historically given rise to many adverse effects, such as damage to nearby cells, tissues, and sometimes surrounding organs (O'Connor, 2015). Chemotherapy can lead to therapy-induced secondary cancer through numerous mechanisms. One method involves carcinogenic chemicals that alter DNA in nearby tissues (Poirier, 2004). These compounds undergo a chemical reaction with the nitrogen and oxygen atoms in DNA, changing typical base-pairing patterns and causing a mutation.

Researchers investigated the probability of secondary treatment-induced malignancies in breast cancer patients who received radiation therapy compared to a control group of patients who did not undergo treatment (Burt et al., 2017). The study's primary findings were that 13% of patients who had received radiation treatment developed a secondary cancer and the likelihood of patients developing a second cancer was significantly greater than that of the control group. A therapy-induced secondary cancer often arises when cancer cells detach from the primary tumour and establish a new tumour in another region of the body (Seyfried and Huysentruyt, 2013). The characteristics of therapy-induced secondary malignancies and their incidence rate in a patient post-cancer treatment are determined by the potency and efficacy of the cytotoxic drugs administered for the primary cancer and the level of exposure to particular chemicals (Godley and Larson, 2008). Chemical compounds designed to target rapidly proliferating cancer cells have the potential to also harm normal, healthy cells due to their mechanism of action. Due to the non-selective targeting of cells from the bone marrow, gastrointestinal tract, and hair follicles, myelosuppression, thrombocytopenia, mucositis, and alopecia are quite common (Riedl et al., 2011).

Anticancer peptides

Why are anticancer peptides superior?

Recent oncological research suggests that peptides are a rapidly growing area of study for further investigation, and the outlook for using peptides in cancer treatment has been quite positive (Boohaker et al., 2012). Conventional treatments are declining in popularity due to concerns about long-term adverse effects from primary cancer

treatments. Additionally, many chemotherapy drugs are unable to effectively differentiate between healthy and cancerous cells and tissues and consequently, there is an urgent requirement to create a novel approach to combat cancer.

One of the key advantages of anticancer peptides is their selective targeting of cancer cells due to differential membrane composition. Unlike normal mammalian cells, which contain mostly zwitterionic phospholipids, cancer cell membranes exhibit higher levels of anionic lipids such as phosphatidylserine and heparan sulfate proteoglycans. This negative charge attracts cationic anticancer peptides, which electrostatically interact with the cancer cell membrane, leading to membrane disruption and increased permeability (Deslouches and Di, 2017, Hoskin and Ramamoorthy, 2008). Anticancer peptides can act via pore formation, leading to immediate cytolysis, or by penetrating the membrane to interfere with intracellular targets, such as mitochondria or nucleic acids, disrupting essential cancer cell functions (Gaspar et al., 2013), mechanisms that offer a significant advantage over chemotherapy by minimising the risk of resistance development, a common issue where cancer cells mutate drug targets or increase efflux mechanisms.

Peptide-based oncological research has been somewhat limited, with most studies concentrating on peptides that possess both antibacterial and anticancer effects. Anticancer peptides are a compelling area of study due to their biocompatibility, varied cell penetration mechanisms, smaller size compared to proteins and antibodies, and ease of synthesis due to their short amino acid chains (Gaspar et al., 2013). Peptides can be used to deliver therapeutic drugs and substances into cells owing to their wide range of chemical and biological properties, and their potential for modification to

enhance target selectivity (Craik et al., 2013). Quick and rapid interactions with the cell membrane and internal components help minimise the likelihood of resistance to these peptides emerging by targeting conserved or essential components of cancer cell membranes and intracellular pathways, providing therapeutic effects in ways that are less vulnerable to adaptation by cancer cells (Xie et al., 2020). Several challenges hinder the advancement of these peptides as conventional pharmaceutical treatments, however, such as the significantly higher synthesis costs compared to organic antibiotic molecules, which can also be used to treat some cancers in the body (Isidro-Llobet et al., 2019, Wang et al., 2022). Consequently, recent peptide design has focused on shortening the primary structure to lower production expenses and enable simple adjustment of physicochemical properties, which is crucial for their antimicrobial and anticancer abilities (Domalaon et al., 2016).

Dual action antimicrobial/anticancer peptides

Chronic infections are a major cause of cancer globally, since they induce cancer cells to proliferate extensively, as patients with chronic infections have a compromised immune system and are therefore more inclined and prone to tumour mass development (Rolston, 2001). Antimicrobial peptides have recently demonstrated the capacity to work against cancer cells in addition to microbial pathogens in the body, earning them a reputation for being prospective anticancer agents. These peptides have previously shown mechanisms of interaction with cancer cell membranes, but can also excel at the intracellular level (Le et al., 2017) already demonstrating a significant advantage over more targeted anticancer therapies. When electrostatically interacting with cancer cell membranes, the secondary structure of these peptides plays a critical role in how the mechanisms of action target cancer cells, generating α -

helical or β -sheet structures (Eiríksdóttir et al., 2010). Furthermore, the electrostatic interactions between the positively charged antimicrobial peptides and the negatively charged components of cancer cell membranes facilitate strong binding and selective DNA breakdown, resulting in apoptosis (Tornesello et al., 2020). Antimicrobial peptides can also induce apoptosis in cancer cells by mitochondrial disruption, which enhances their anticancer efficacy (Huang et al., 2011). These intricate mechanisms allow antimicrobial peptides to overcome certain limitations related to highly specific cancer therapies, such as the emergence of resistance (Deslouches and Di, 2017).

Although antimicrobial peptides were once thought to be membrane-active peptides, it has been shown over time and research that they may also target several pathogenic processes – including metabolism, cell growth and the immune system (Hancock et al., 2016). These characteristics were also investigated for anticancer peptides, detecting necrosis and apoptosis, the former being the most frequently used anticancer targeting strategy, with electrostatic interactions inducing membrane rupture (Sahni et al., 2020). Anticancer peptides may exhibit additional properties following their impact at the membrane level, including directly targeting cell proteins, preventing angiogenesis, and targeting malignant cells by mobilising immune cells (Wu et al., 2014). HNP-1 has also been demonstrated to be a peptide that suppresses the process of angiogenesis which is a key part of tumour development and growth (Xu et al., 2008).

Mechanisms of action

While not all antimicrobial peptides are anticancer peptides, the similarities in their methods of action against cellular membranes are noteworthy and may be applied to

both classes of peptides in the majority of instances. Previous studies have summarised the mechanisms by which cationic antimicrobial peptides, tumour-targeting peptides, and cell penetrating peptides interact (Hoskin and Ramamoorthy, 2008). These interactions often commence with the peptides accumulating on the cell surface, as seen in the initial step of the carpet model. This is generally followed by the membrane disintegration stage, or alternatively, the barrel-stave model and the toroidal pore wormhole model, which form pores, or alternatively the detergent-type membrane lytic process. (summarised in figure 1).

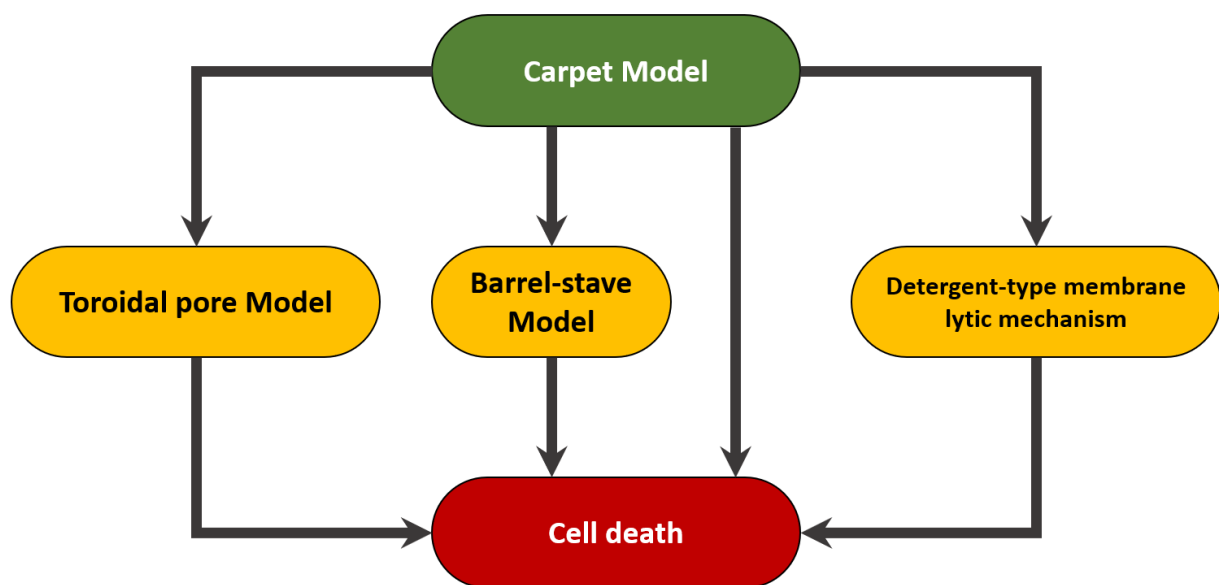


Figure 1: A schematic illustrating how cationic antimicrobial peptide interactions initiate with the carpet model and conclude with apoptosis (Hoskin and Ramamoorthy, 2008, Kumar et al., 2018).

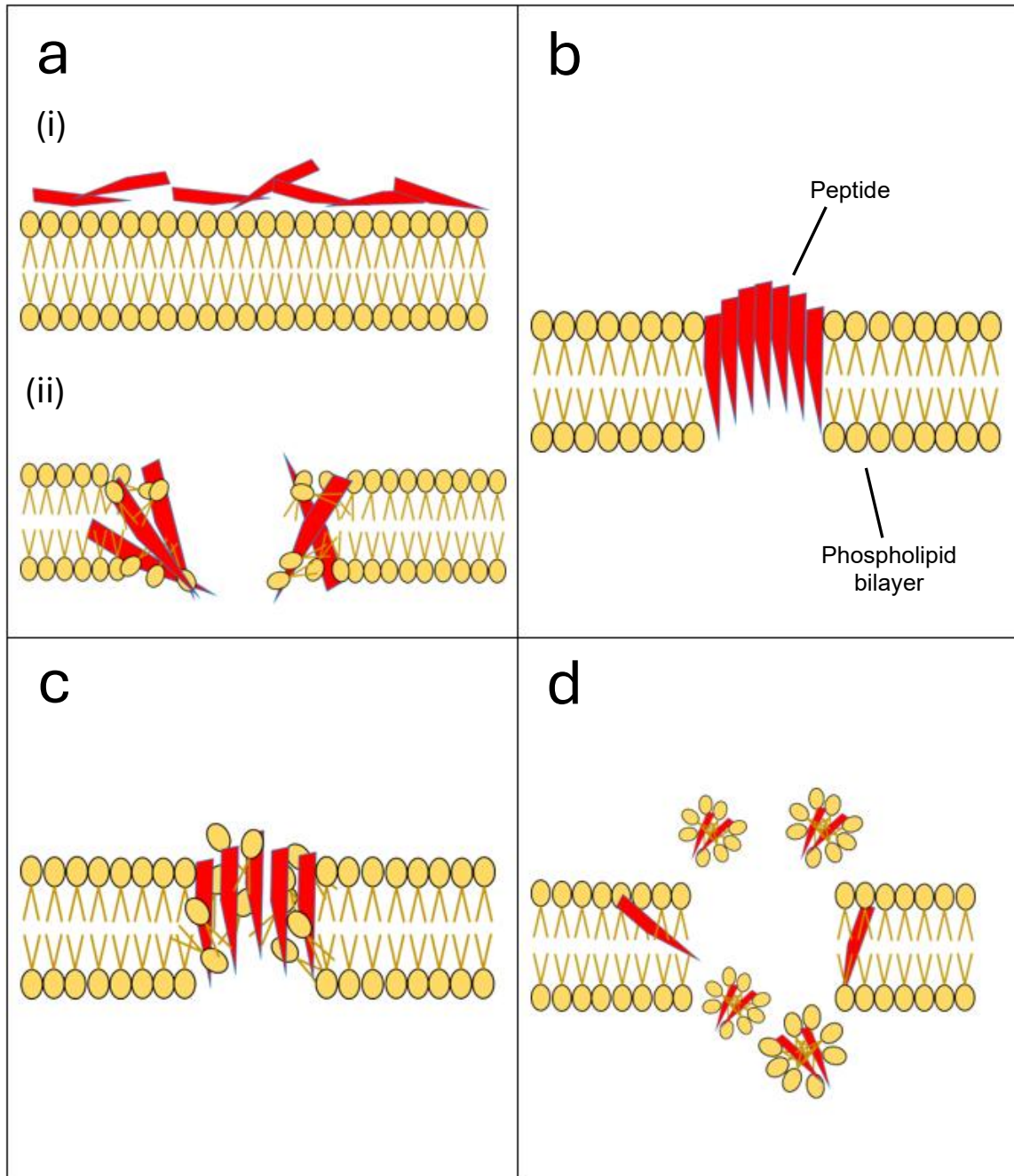


Figure 2: — a: (i) the initial step of the carpet model (ii) the second stage of the carpet model; — b: the barrel-stave model; c: the toroidal pore wormhole model; d: the detergent-type membrane lytic process.

Carpet Model

This mechanism enables the peptides to induce cell lysis via penetration after aggregation on the cell membrane surface after a carpet-like formation (figure 2a(i)).

The cationic poles of the peptides disrupt the membrane by electrostatic interactions with the anionic poles of the phospholipid bilayer of the cell, thus forming pores which enable the peptide to permeate through (figure 2a(ii)).

Barrel-Stave Model

The α -helical structure of the antimicrobial peptides is embedded into the membrane itself, allowing the peptides to establish multiple ion-channel pores in the phospholipid bilayer. However, these are only formed by a limited number of antimicrobial peptides (figure 2b) (Kumar et al., 2018).

Toroidal Pore Model

As shown in figure 2c, the toroidal pore model is similar to the carpet model in that the peptides are aligned to the lipid bilayer surface and are adjacent to the polar head area, with the hydrophobic region of the helix entrenched in the membrane's hydrophobic region, forming pores upon the aggregation of the peptides (Kumar et al., 2018).

Detergent-type membrane lytic mechanism

As demonstrated in figure 2d, the membrane fragmentation occurs when the high concentration of aggregated peptides on the outer side of the phospholipid membrane causes destabilisation (Kumar et al., 2018).

The G3 Peptide

The base G3 sequence

Despite the large number of different peptide structures synthesised and evaluated in recent years, α -helical anticancer peptides have emerged as the frontrunner and display the most anticancer efficacy in comparison to β -sheet peptides due to their flexibility for more enhanced membrane disruption and cellular uptake via direct penetration (Sun et al., 2016) – whereas β -sheet peptides often require endocytosis (Baxter et al., 2017). Hu et al. (2011) published one of the earliest reports on the class of peptides with the sequence $G(IKK)_nI-NH_2$ ($n= 1-4$), in which they investigated the antimicrobial and anticancer activity of this series of designed peptides, with an emphasis on optimising short cationic amphiphilic peptides; as previously stated, a shorter peptide sequence results in a lower synthesis cost. They examined several combinations of amphiphilic peptides and discovered that the IKK sequence produced the best results.

These sequences (G1, G2, G3, and G4, where the number of IKK repetitions denotes the number adjacent to G) had Glycine at the N-terminus and were amidated at the C-terminus, which is very favourable for α -helical hydrogen bonding. Hu et al. synthesised these peptides originally using solid-phase peptide synthesis on a Rink amide MBHA resin and then refined them to >95% percent purity. The structure and folding of these peptides in aqueous solution were then determined using circular dichroism spectroscopy. They discovered that while the peptides remained unfolded in aqueous solution, when placed in anionic DPPG (dipalmitoylphosphatidylglycerol) SUVs (small unilamellar vesicles) - which emulated (to an extent) the negatively charged cancer cell and bacterial cell membrane environments - every peptide except

G1 formed an α -helical structure, almost immediately rendering G1 ineffective since it indicated that it lacked antimicrobial/anticancer activity against bacterial and cancer cell lines.

The same experiment was performed on DPPC (dipalmitoylphosphatidylcholine) SUVs (which emulated the structure of normal, healthy mammalian cell membranes), and the researchers discovered that none of the peptides produced an α -helical shape. DPPC and DPPG were used because such synthetic lipids provided controlled and reproducible model systems to analyse membrane interactions, free of the complexities associated with living cells, allowing isolation and examination of the membrane-targeting characteristics of peptides with high precision, providing simplified, standardised, and cost-effective models that circumvent the biological variability and complications associated with live cell cultures (Morrow et al., 2007). The antimicrobial activity of the sequence of peptides was determined against Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis*, with G1 exhibiting the weakest activity against both bacteria, even at considerably greater doses. Additionally, it was discovered that the greater the number of IKK repetitions, the greater the antibacterial activity; hence, G3 and G4 exhibited the greatest levels of antimicrobial activity, with minimum inhibitory doses ranging from 0.5 to 8 μ M. Furthermore, the researchers determined that these MIC values rendered the peptides as potent as a variety of commercial antibiotics. These peptide sequences were also evaluated against HeLa and HL60 cancer cell lines and demonstrated a similar pattern to the antibacterial results, with G1 exhibiting weak anticancer activity and G3 and G4 exhibiting strong anticancer activity with IC₅₀ values ranging from 4 to 25 μ M, making

them as potent as cisplatin, a broadly used chemotherapeutic agent (Takara et al., 2006).

However, the haemolytic activity against human erythrocytes was the penultimate test for these peptide sequences. This experiment demonstrated that the G3 peptide induced less than 10% haemolysis against *E. coli* at an 80 μM concentration, compared to the G4 peptide, which generated 25% haemolysis at a mere 20 μM concentration. This indicated that the G3 peptide was the most optimal peptide in this sequence; although G4 was more powerful, it displayed much more toxicity against human red blood cells than the balanced G3.

To further validate the cytotoxicity of these peptides, the researchers synthesised the G3 peptide but labelled it with fluorescein isothiocyanate (FITC) and added it to two co-culture systems: one containing both aforementioned bacteria and another containing HL60 cancer cells in co-culture with NIH 3T3 model host mammalian cells. The green fluorescence of the FITC-labelled G3 was imaged and demonstrated that it congregated within the HL60 cells and bacteria but not within the mammalian host cells, indicating that the G3 demonstrated rapid responses and effective recognition of the bacteria and tumour cells in the co-culture environment. As previously stated, the researchers observed that the G2 and G3 peptides created α -helical structures in the DPPG SUVs (which emulate bacterial cell membranes) but did not form any structures in the DPPC SUVs through circular dichroism (imitating normal, healthy cell membranes).

Three years later, the same research team (Chen et al., 2014) investigated the G3 peptide's high cell selectivity and low level of antibacterial resistance further, as it had previously been suggested that G3 was the ideal peptide to use from the G(IKK)_n-NH₂ (n= 1-4) series due to its high potency in comparison to G1 and G2, and significantly lower haemolytic activity in comparison to G4. However, rather than the mouse host cells employed in the previous study (NIH 3T3), adult human dermal fibroblast cells (HDFa) were used in this study. The procedures used to synthesise the G3 and FITC-G3 peptides remained consistent with those used in the previous study. Circular dichroism spectroscopy studies confirmed that the G3 peptide developed random coil structures in water but formed α -helical coil structures in an environment emulating bacterial cell membranes. The research demonstrated that the G3 peptide indicated significant antibacterial efficacy against multidrug-resistant *Escherichia coli* at clinically acceptable concentrations, with no damage towards human HDFa cells. This highlighted its relative selectivity, showing its ability to successfully target bacterial cells while preserving healthy human cells.

Due to the peptide's positive charge in comparison to the anionic bacterial cell membrane, its antibacterial activity revealed that it acted largely via disrupting the membrane. Importantly, the researchers discovered that numerous treatments with the peptide did not result in antimicrobial resistance in *E. coli*, which was a critical finding in terms of the peptide's future potential.

Sequential G3 Modifications

Apart from its significant antibacterial and anticancer properties, Zhang et al. recently demonstrated in 2021 that the designed G3 peptide may also be utilised as an

antifungal treatment (Zhang et al., 2022). The group removed the glycine from the N-terminus of G(IKK)₃I-NH₂ and replaced it with isoleucine at the C-terminus and evaluated them against *Candida* strains and *Cryptococcus neoformans*; they discovered that these peptide sequences were particularly effective against planktonic microbes and fungal biofilms, while exhibiting very low toxicity to host cells at clinically practical concentrations. (IKK)₃II-NH₂ exhibited the greatest antifungal efficacy and showed a high therapeutic index, indicating an enhanced capacity to selectively target fungal cells while inflicting minimal damage to mammalian cells, whilst on the contrary, (IKK)₃-NH₂ exhibited the least antifungal efficacy and demonstrated a lower therapeutic index, indicating that the addition of an isoleucine group at the C-terminus enhanced hydrophobicity and increased both potency and selectivity. The implications of this work were substantial, implying that future alterations to the G3 peptide (and hence to any current α -helical peptide sequences) may exhibit distinct and varied degrees of activity, most notably in the area of cancer.

Hadianamrei et al. investigated a similar sequence of peptides to Hu et al. in 2011 but included cysteine rather than glycine at the N-terminus (Hadianamrei et al., 2022, Hu et al., 2011). The general base sequence was altered, and variants of these peptides in the series included the addition of more isoleucine residues to the C-terminus, the substitution of cysteine for LC-propargylglycine (LC-G) at the N-terminus, and the substitution of isoleucine and lysine for leucine and arginine, respectively. The series of peptides was evaluated using a variety of assays and techniques, including circular dichroism spectroscopy, LC-mass spectroscopy, surface pressure measurements using via a Langmuir trough, and cytotoxicity assays such as MTT assays and cell proliferation assays against HCT116 (colorectal cancer cell line), HeLa (cervical

cancer cell line), and HDF (human dermal fibroblasts) cell lines. The list of peptide sequences tested in this study are listed in table 1 (Hadianamrei et al., 2022).

<i>Table 1: The sequences of the designed cationic amphiphilic peptides used by Hadianamrei et al.</i>	
Peptide	Sequence
IK-13	C IIKK IIKK IIKK - NH ₂
IR-13	C IIRR IIRR IIRR - NH ₂
LK-13	C LLKK LLKK LLKK - NH ₂
LR-13	C LLRR LLRR LLRR - NH ₂
CI-15	C IIKK IIKK IIKK II - NH ₂
GI-15	LC-Propargyl-G IIKK IIKK IIKK II - NH ₂

Some of the peptides (IK-13, LK-13 and CI-15) showed enhanced effectiveness in targeting colorectal and cervical cancer cells (IC₅₀ values of 29 ± 6 , 23 ± 2 and 7.7 ± 0.2 respectively), while also displaying decreased toxicity to normal HDF cells (IC₅₀ values of >100 for both IK-13 and LK-13 and 83 ± 5 for CI-15) compared to the G3 peptide and its modifications (Chen et al., 2014, Hu et al., 2011, Zhang et al., 2022). It was also important to note that the peptide concentrations used in the transfection for the colorectal and cervical cancer cell lines were over 1000 times lower (5–25 nM) than the concentrations used for the HDF cells, thus, the peptides that exhibited significant toxicity against HDF cells would remain nontoxic had the same concentrations been utilised across all peptides and cell lines.

The scientists also noted that the peptides caused degradation to the mitochondrial membrane of the cancer cells, leading to apoptosis, in line with previous research (Chen et al., 2021). It was shown that enhancing the peptide's anticancer activity while

maintaining low damage to normal cells may be achieved by adding isoleucine to the C-terminus and substituting glycine with cysteine at the N-terminus.

HHC10 and HHC36

Cherkasov's research group first identified HHC10 (KRWWKWIRW) and HHC-36 (KRWWKWWRR) as two of the most potent new antimicrobial peptides, with MX-226 serving as a control peptide, and then tested them against a commonly used mouse model of invasive *Staphylococcus aureus* (Cherkasov et al., 2009). HHC10 and HHC-36 were found to be significantly more potent than MX-226. In this study, substantial doses of the peptides (200µg per mouse) were delivered, and the antibacterial activity of the HHC-10 and HHC-36 peptides was shown to be significant; total eradication of the bacteria was recorded in some test subjects 24 hours after infection. The MX-226 peptide, on the other hand, had no bactericidal effect, and many of the MX-226 test animals died as a result.

Since the full potential of HHC10 was not thoroughly examined until 2013, the available research at the time indicated that it was effective in killing *Staphylococcus aureus* in vivo. Llamas-Gonzalez et al. conducted further study on the HHC-10 peptide's modes of action and novel bacterial targets, namely *Mycobacterium bovis*. Interferon, TNF- α , IL-4, and IL-10 mRNA transcript levels were evaluated and compared between treated and untreated control mice (Llamas-González et al., 2013). After 24 hours of incubation, it was observed that HHC10 reduced colony forming units (CFUs) by 69% at 50 µg/ml and by 88% at 100 µg/ml, suggesting that HHC-10 could theoretically be used as a therapeutic agent against tuberculosis in the future.

Nano-encapsulation of α -helical peptides for the purpose of killing bacteria inside cells may also be a valuable approach. The host defence peptide HHC-10 with the sequence KRWWKWWRR (KRWWKWIRW was used by the researchers) was evaluated using *Escherichia coli* (Sharma et al., 2018b), since this peptide had previously shown antibacterial activity against a variety of Gram-positive and Gram-negative bacterial strains. The purpose of this work was to create delivery mechanisms for the HHC-10 peptide that would specifically target bacteria inside macrophage cells. The peptide was originally synthesised on Rink amide and then purified using RP-HPLC. The peptide was subsequently nano-encapsulated, and the nanoparticles' uptake into *E. coli* was determined using FITC and a CFU assay. The research team discovered that the peptide's bactericidal effect is mediated by a multimodal process of bacterial cell death including membrane lysis. Additionally, encapsulation of the HHC10 peptide increased peptide distribution inside macrophage cells, improved the toxicity profile, and boosted the host-mediated innate response to infection whilst decreasing macrophage cytotoxicity. Although this peptide was tested on an *E. coli* strain, it is possible that the same or comparable techniques might be utilised to target multiple cancer cell lines owing to the HHC10 peptide's cell membrane lytic mode of action inducing the apoptotic death pathway.

Antimicrobial peptides HHC36 (KRWWKWWRR) and Tet213 (KRWWKWWRRRC) were loaded into calcium phosphate coated Ti implants in a rabbit model to further explore in vitro drug release, antimicrobial performance, cytotoxicity, and in vivo osteogenesis (Kazemzadeh-Narbat et al., 2012). Initially, the two peptides were examined, and it was observed that HHC-36 was much less cytotoxic to MG-23 osteoblast-like cells than Tet213, its cysteinated equivalent. As a result of this finding,

the study team mostly selected HHC36 as the antimicrobial peptide coating rather than Tet213. The study's primary finding was that CaP-HHC36 samples were capable of completely killing *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria in less than 150 minutes after administration. Recent research and experimentation on HHC36 resulted in the development of smarter drug delivery systems capable of delivering antimicrobial peptides on-demand via titanium implants, demonstrating that titanium implants could potentially be used in a variety of other applications, including future cancer treatments (Chen et al., 2020).

Magainin II

Several decades ago, naturally derived antimicrobial peptides were examined, with Magainin II (a peptide obtained from the African clawed frog - *Xenopus laevis*) being a significant example. It was previously known that when these peptides interacted with anionic lipid bilayers, they produced an α -helical coil, similar to the peptides discussed earlier. Additionally, evidence suggested that the interaction with the lipid membranes triggered apoptotic pathways, resulting in cell death (Baker et al., 1993). It was observed that although Magainin II (GIGKFLHSAKKFGKAFVGEIMNS) had a lower efficacy than its two analogues – MSI-136 and MSI-238 (GIGKFLKKAKKFAKAFVKIINN and D-GIGKFLKKAKKFAKAFVKIINN, respectively) – the two analogues exhibited greater cytotoxicity towards both cancerous and non-cancerous cells, indicating lower specificity for targeting cancerous cells. This research indicated that the MSI-238 counterpart was more successful and may hold promise for therapeutic development if its specificity for cancer cells can be further optimised to reduce off-target effects.

Subsequent research examined the anticancer effect of the base Magainin II sequence in more detail (Lehmann et al., 2006). Magainin II has previously been studied and analysed against a variety of cancer cell lines (Baker et al., 1993), but this research examined the anti-proliferative efficacy of the peptide against bladder tumour cells and normal human fibroblasts. Normal fibroblasts were also compared using the same techniques, and the cytotoxicity detection test comprised mass spectrometry and monitoring of LDH (lactate dehydrogenase) activity. Magainin II decreased cell proliferation and DNA synthesis in bladder cancer cells at doses far lower than those required to inhibit normal fibroblasts, with an average IC_{50} value of 75.2 μM (range, 31.0–135.3 μM) across the three cancer cell lines with little to no effect to fibroblasts, while also causing apoptosis by generating ion-permeable pores in the cell membrane. There was no evidence of LDH release, and normal fibroblast cells did not undergo cell lysis. The researchers concluded that more study was necessary to understand if high-grade tumours are more sensitive to Magainin II's effects.

Penetratin

Anticancer peptides derived from p53 were synthesised and linked to Penetratin, a peptide sequence with cell membrane penetrating properties. These peptides were discovered by mistake while being synthesized to block the binding of the tumour suppressor protein p53 to the HDM-2 in malignant cells. However, the researchers discovered that PNC-27 and PNC-28 caused cell death in cells without p53 through tumour cell necrosis (Bowne et al., 2008).

The research by Sharma et al. (Sharma et al., 2013) showed the anticancer potential of Penetratin, a 16 amino acid long peptide. The researchers examined how Penetratin

may be employed to transport anticancer agents over the brain endothelial membrane in comparison to other carriers. Additionally, the researchers evaluated the effect of several cell penetrating peptides (Tat, Penetratin, and Mastoparan) on doxorubicin's ability to traverse the blood brain barrier. Moreover, a cytotoxicity experiment utilising various phospholipid concentrations was performed.

The findings of this investigation suggested that the inclusion of Tat and Penetratin on the surface of liposomes resulted in an increase in the efficiency of doxorubicin translocation through brain endothelial barriers; nevertheless, cell survival reduced as the phospholipid content rose but Mastoparan, on the other hand, had a greater cytotoxicity, suggesting that *in vivo* usage should be limited and controlled. This study makes a significant contribution to the development of more efficient, low-toxicity drug delivery systems but also implies that the applications of these anticancer peptides are not limited to cancer therapy but can also be applied to other parts of contemporary medical research.

Current/further research and aims

Histidine is an amino acid that has received limited attention in anticancer literature, resulting in a lack of research on its potential anticancer properties compared to other amino acids in the extensive body of anticancer peptide literature. It was primarily chosen for incorporation into these anticancer peptide chains because of its distinctive biochemical features that set it apart from other amino acids. The imidazole side chain of histidine facilitates pH-responsive behaviour, an essential characteristic for specifically targeting the acidic tumour microenvironment – with the side-chain having

a pKa of 6.0, allowing it to become protonated in acidic environments (such as the bacterial or cancer microenvironments). In contrast to lysine or arginine, histidine is capable of switching between neutral and positively charged states based on pH, facilitating more precise activation in cancer cells (Dharmayanti et al., 2021). Moreover, histidine's capacity to bind to metal ions provides a way of enhancing oxidative stress and induce mitochondrial disruption in cancer cells, mechanisms often unachievable with most other amino acids (Vera-Aviles et al., 2018). Despite being extensively studied for its role in enzymatic activities and antimicrobial peptides, histidine's potential in the design of anticancer peptides is still insufficiently explored. In the realm of anticancer research, histidine has predominantly been used as a physical food supplement or in conjunction with other compounds which function as carriers, rather than as a standalone anticancer medication (Moro et al., 2020). This research project aims to fill this gap through harnessing histidine's distinctive chemical reactivity and environmental adaptability to develop novel peptide therapies for cancer by building upon previous literature.

A series of rationally designed peptides, building upon prior literature (Hu et al., 2011, Chen et al., 2014, Hadianamrei et al., 2022), were produced and subjected to a range of experiments for evaluation purposes. [Table 2 partially redacted due to embargo — peptide sequences and molecular weights of novel peptides withheld to protect future publication.]

Table 2: The sequences of the peptides used in this study. *Peptide names, sequences, and molecular weights for novel peptides have been redacted to protect future publication.				
Group	Peptide	Sequence	Molecular weight (g/mol)	Reference
	G3 (parent peptide)	GIKKIIKKIIKKI - NH ₂	1635.22	(Hu et al., 2011, Chen et al., 2014, Hadianamrei et al., 2022)
<i>Redacted – Group 1</i>	CI-13	CIKKIIKKIIKKI - NH ₂	1568.16	(Hadianamrei et al., 2022)
	<i>Redacted</i>	<i>Redacted</i>	<i>Redacted</i>	
<i>Redacted – Group 2</i>	<i>Redacted</i>	<i>Redacted</i>	<i>Redacted</i>	
	<i>Redacted</i>	<i>Redacted</i>	<i>Redacted</i>	
	<i>Redacted</i>	<i>Redacted</i>	<i>Redacted</i>	
<i>Redacted – Group 3</i>	HHC10 (parent peptide)	KRWWKWIRW - NH ₂	1443.74	(Sharma et al., 2018a, Sharma et al., 2018b)
	<i>Redacted</i>	<i>Redacted</i>	<i>Redacted</i>	
	<i>Redacted</i>	<i>Redacted</i>	<i>Redacted</i>	
	<i>Redacted</i>	<i>Redacted</i>	<i>Redacted</i>	

As mentioned previously, another rationale for selecting this collection of peptides stems from a lack of academic research that addresses the field of anticancer activity of histidine. The peptides are categorised into three main groups, with one previously published peptide serving as a reference model. A subset of modified peptides, derived from a parent sequence reported in literature, was also designed for this study. [Details of these modified peptides have been redacted to protect future publication.]

The secondary structure of these peptides was predicted using predictive computational methods (Varadi et al., 2022, Jumper et al., 2021), specifically AlphaFold2 with a single sequence MSA mode. AlphaFold2 produces a confidence measure called pLDDT, which represents the projected score of the model on the LDDT-C α metric. Regions with a pLDDT value greater than 90 are predicted to be accurately modelled, while values between 70 and 90 suggest a generally favourable backbone prediction. Regions with pLDDT values between 50 and 70 indicate a low degree of confidence and should be interpreted with caution, and values below 50 indicate potential disorder, implying that the region is either unstructured under normal physiological conditions or only structured as a component of a complex. The secondary structure of the peptides was predicted to be α -helical. [Figure 3 redacted due to embargo — 3D visualisations of novel peptides withheld to protect future publication.]

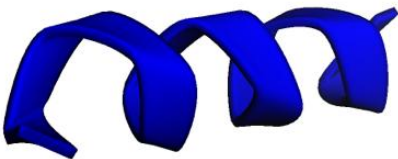
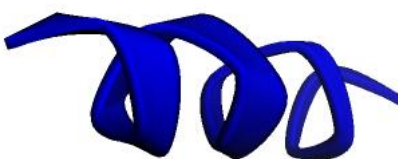
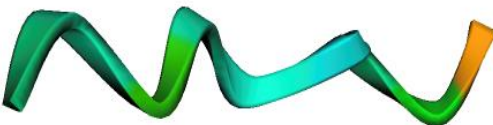
a		b	
c	REDACTED PEPTIDE	d	REDACTED PEPTIDE
e	REDACTED PEPTIDE	f	REDACTED PEPTIDE
g		h	REDACTED PEPTIDE
i	REDACTED PEPTIDE	j	REDACTED PEPTIDE

Figure 3: – a: G3; b: CI-13; g: HHC10; panels c: d: e: f: h: i: j redacted to withhold novel peptide structures for future publication.

Based on prior research that has demonstrated the potential impact of histidine on peptide stability and cellular uptake (Iwasaki et al., 2015, Saurabh et al., 2022), our hypothesis proposes that peptides containing histidine, particularly those with a higher number of histidine residues, may exhibit enhanced efficacy in anticancer applications when compared to peptides lacking histidine or containing fewer histidine residues in

the sequence, with the effects also being dependent on residue positioning within the sequence. In order to evaluate this hypothesis, the cellular uptake of histidine-containing peptides will be assessed through the addition of siGLO, a fluorescently labelled siRNA, to the peptides. The siGLO will allow the tracking and imaging of peptides within cells, offering insights into the extent and efficacy of uptake. Alongside evaluating the cellular uptake, the siRNAs can also quantify the peptides' drug-delivery potential, enabling the determination of the ability to effectively transport therapeutic drugs into target cells. Fluorescence microscopy will be used to observe the uptake of peptide-siRNA complexes into cancer cells and will allow us to determine if peptides with higher histidine content improve cellular uptake and augment the peptides' potential as drug-delivery vehicles for anticancer treatments.

It is also important to note that whilst the anticancer efficacy of histidine is under evaluation, the incorporation of excessive histidine residues may present several potential challenges. The interactions between the imidazole groups on each residue may be insignificant at standard quantities; but at excessive quantities, they could compromise the stability of the peptides (Saurabh et al., 2022) in addition to reducing the selectivity of the peptide sequence, leading to non-specific interactions due to specificity of the peptide partly stemming from the variance and composition of amino acid residues in its sequence (Lointier et al., 2021).

2. Materials and Methods

2.1 – Cell culture

2.1.1 – Cell lines

This study utilised three distinct, immortalised cell lines: a HCT116 (human colorectal carcinoma) cell line, a Caco-2 (human colorectal adenocarcinoma) cell line, and an immortalised HFF-1 (human foreskin fibroblast) cell line. The HCT116 cells (#CCL-247) and Caco-2 cells (#HTB-37) were purchased from the American Type Culture Collection (ATCC) and the non-cancerous HFF-1 cell line was acquired from Mr. Yazeed Almukhlifi from the University of Sheffield.

The HCT116 cell line was chosen for its well-defined genetic profile featuring a mutation in KRAS and wild-type TP53, which are indicative of colorectal carcinoma and make it a valuable model for studying anticancer treatments (Guo et al., 2011). The Caco-2 cell line is commonly used for researching colorectal cancer and is recognised for its ability to differentiate into enterocyte-like cells, so simulating the intestinal epithelium (Meunier et al., 1995, Pinto et al., 1983). These two cancer cell lines provide complementary models for assessing peptide efficacy in colorectal cancer. In order to evaluate the peptides' cytotoxicity and selectivity on normal human cells and to ensure that their therapeutic potential is not accompanied by notable adverse reactions, the HFF-1 cell line—a non-cancerous fibroblast model—was selected.

2.1.2 – Sterilisation

The Esco Airstream Gen 3 Vertical Laminar Flow Cabinet was used for all sterile procedures, including cell culture and peptide preparation. Prior to each use, the hoods underwent a 20-minute exposure to UV radiation, followed by sterilisation using 70% ethanol.

2.1.3 – Passaging

The medium utilised for both cancerous cell lines (HCT116 and Caco-2) was identical. The composition of the medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) with high glucose and pyruvate (GIBCO, ThermoFisher Scientific, #41966029), 10% heat inactivated Fetal Bovine Serum (FBS) (GIBCO, ThermoFisher Scientific, #10082147), and 1% Penicillin-Streptomycin (GIBCO, ThermoFisher Scientific, #15140122). The HFF-1 cell line was cultured in a medium consisting of Dulbecco's Modified Eagle's Medium with high glucose (SigmaAldrich, #D5796), 10% heat inactivated Fetal Bovine Serum (FBS) (GIBCO, ThermoFisher Scientific, #10082147), and 2.5% HEPES solution (SigmaAldrich, #H3537). The cells were maintained in an incubator (Sanyo, #MCO-20AIC) at 37°C, 95% humidity and a CO₂ concentration of 5%. The cells were always passaged at a maximum confluency of 80-90% and this was done by removing the media from the flasks, washing the cells once with 1X phosphate buffered saline (PBS) (GIBCO, ThermoFisher Scientific, #20012019), and then adding 2ml of Trypsin-EDTA (GIBCO, ThermoFisher Scientific, #25300096) for 4 minutes for HCT116 cells and 10 minutes for Caco-2 cells in the incubator at 37°C. Once the designated time had elapsed, the flasks were removed from the incubator and the Trypsin was deactivated with the addition of 6ml of DMEM

media. The cells were then split into new T-75 flasks (Nunc, ThermoFisher Scientific, #156499), typically at 1:12 dilutions and placed in the incubator. The remaining cells were used in experiments.

2.1.4 – Cryopreservation

Multiple batches of HCT116, Caco-2, and HFF-1 cells were also frozen and stored at a concentration of 1×10^6 cells/ml. The cells were trypsinised as per the passaging protocol and subsequently centrifuged at a speed of 135rpm for a duration of 10 minutes. The cells were then collected into a 15ml tube (BD Falcon, #352096). The supernatant was then discarded, and the remaining pellet of cells was mixed with 1ml of freezing media, which consisted of 7% sterile DMSO (Santa Cruz, #sc358801) added to FBS. Following resuspension, the solution was then transferred into cryovials (Starlab, #E31106112) for the purpose of storage. Subsequently, the cryovials were placed in a Mr. Frosty Freezing container (ThermoFisher Scientific, #51000001) and stored at a temperature of -80°C for a duration of 48 hours. Following this, they were transferred into liquid nitrogen tanks with a temperature of -180°C for long-term preservation.

2.1.5 – Thawing protocol

The frozen cell samples were defrosted at a temperature of 37°C in a water bath for the purpose of cell culture. An aseptic technique was used to fill a 15ml tube with 4ml of DMEM media that had been pre-warmed. The cells that were then mixed with the warmed DMEM media and the solution was subsequently centrifuged at a speed of 135rpm for a duration of 10 minutes. The remaining cell pellet was then mixed in 6ml

of DMEM media and transferred into T-75 flasks. Once the cell culture reached 70% confluency, it was split into dilutions of 1:3 and 1:6.

2.2 – Peptides

2.2.1 – Peptide dissolution

The peptides were obtained from GLBiochem (Shanghai, China) and were dissolved in filtered and deionised H₂O. The water used for filtration was passed through a 0.2µM filter (SigmaAldrich, #SLGV033RS) before use. For microscale thermophoresis work, the peptides were dissolved in a buffer made up of 1X PBS and 0.1% Tween 20 (SigmaAldrich, #P1379).

2.2.2 – AlphaFold2

The peptide sequences underwent AlphaFold2 analysis, using a combination of ColabFold and AlphaFold techniques, to predict the secondary structure of the peptides and generate 3D models of each peptide sequence (figure 3). After inputting the peptide sequence into the program, a single-sequence MSA approach was employed, along with an unpaired-paired pair mode, and subsequently a greedy pairing strategy was also applied. The generated data includes 3D images and pLDDT rankings for each sequence. The colour of each coil indicates the pLDDT, which, as mentioned previously, is a confidence measure created by AlphaFold2. pLDDT represents the expected score of the model on the LDDT-Cα metric (Mariani et al., 2013).

2.3 – Microscale Thermophoresis (MST)

An MST experiment was conducted to assess the peptide-siRNA interactions. This is a relatively novel approach that is not widely prevalent in literature, thus requiring several months of dedicated effort to develop an appropriate assay with the optimum levels of reagents. MST is a highly effective biophysical method used to quantify the motion and interactions of molecules within glass capillary tubes at a multitude of dilutions through a temperature gradient. A new batch of peptide and ligand was created for every repetition of the experiment, all experiments were performed in triplicate.

2.3.1 – Peptide and siRNA preparation

The peptides were dissolved in a buffer consisting of 1X PBS and 0.1% Tween 20 to prepare stock solutions of [concentration redacted] for each peptide. The siGLO Red Transfection Indicator (Dharmacon, #D0016300220), a fluorescent ligand, was used in the experiment and was dissolved in the same PBS-Tween buffer as the peptides, but its stock concentration was adjusted to [concentration redacted].

Following this, every Eppendorf tube (ThermoFisher Scientific, #10398031) containing peptide underwent centrifugation at a speed of [rpm redacted] to remove peptide aggregates. A series of [number redacted] dilutions was then prepared in 0.2ml PCR tubes (ThermoFisher Scientific, #12134102), with each tube, with the exception of the first one, containing [volume redacted] of buffer solution. First, [volume redacted] of peptide stock was added to the first PCR tube. Following that, the serial dilution procedure was initiated by transferring [volume redacted] of the solution from each

tube and adding it to the following tube. Then, [volume redacted] of siGLO Red Transfection indicator was added to each PCR tube, thoroughly mixed, and then kept covered at room temperature for approximately [time redacted] to facilitate the interactions between the peptide molecules and the siGLO.

2.3.2 – MST experiment

After a period of [time redacted], a glass capillary tube (NanoTemper, #MO-Z022) was inserted into each of the PCR tubes. The capillary tube absorbed the liquid through capillary action and was then placed into the Monolith microscale thermophoresis machine (NanoTemper, Monolith X). The experimental conditions applied were expert mode in a nano-blue channel, with a consistent temperature setting of [temperature redacted] and a dilution ratio of [ratio redacted] starting at a concentration of [concentration range redacted]. The results (including the K_D values, where obtainable) were then collected through MO Affinity Analysis (version 2.3) and subsequently analysed through GraphPad Prism (version 10.1).

2.4 – Cell proliferation assay

An assay was developed to assess the antiproliferative ability of each peptide towards the three cell lines tested during this research. In contrast to the MST experiment, the peptides in this experiment were dissolved in filtered, deionised water with an initial stock concentration of [concentration redacted]. All experiments were performed with four technical replicates and three biological replicates.

2.4.1 – Seeding multi-well plates

The cells were dispensed into clear-bottom 384-well plates (ThermoFisher Scientific, #10184221) using a microplate dispenser (ThermoFisher Scientific, Multidrop Combi). The seeding concentrations were selected based on prior conducted trial experiments and concluded to be around [cell count range redacted] cells per well, with a volume of [volume redacted] in each well.

The quantification of cell counts per millilitre was conducted using the Countess II Automated Cell Counter (ThermoFisher Scientific). In order to achieve this, the counting slide (ThermoFisher Scientific, # 12157220) was loaded with [volume redacted] of a 1:1 mixture of cell suspension and Trypan Blue Solution (ThermoFisher Scientific, #10702404) and then inserted into the cell counter. Once the cell solution was appropriately diluted according to the initial cell count, it was used to seed the 384-well plates. The plates were then incubated overnight at a temperature of 37°C. Following a 24-hour period, peptides were added at the following final concentrations: [concentration range redacted], with the last column of wells being used for deionised water. The plates were then left to incubate at 37°C for [time redacted].

2.4.2 – Fixing and staining procedure

The media in each well was removed using a microplate washer (BioTek, ELx405). Afterwards, [volume redacted] of a fixative solution was added to each well using a microplate dispenser, consisting of [volume/concentration redacted] formaldehyde (SigmaAldrich, #252549-1L), [volume redacted] 1X PBS, and [volume/concentration redacted] Hoechst 333420 (ThermoFisher Scientific, #62249) with the volume then

being increased to [volume redacted] by adding filtered, deionised water. The plate was then left to incubate for [time redacted] at room temperature whilst being shielded from direct light to prevent photobleaching.

Previous experimental trials revealed that the microplate washer did not remove or detach the cells from the bottom of the wells. Therefore, it was deemed safe to utilise the washer to aspirate the fixative solution after the [time redacted] incubation period. The plates were subsequently rinsed twice with [volume redacted] of 1X PBS in each well, followed by incubation of each well in [volume redacted] of 1X PBS for a minimum of [time redacted] prior to imaging.

2.4.3 – High Content Screening

After the cells had been fixed and the required minimum period of [time redacted] had passed, the plates were now ready for High Content Screening. This was accomplished by using a High Content Microscope (ImageXpress Micro, Molecular Devices) together with the MetaXpress software (version 6.1.0.2071, Molecular Devices). Each well was scanned from one site using a 2X objective, which captured the whole well. The DAPI (4',6-diamidino-2-phenylindole) channel was selected for the wavelength, and the exposure time was accurately determined based on the specific cell line and plate, varying between [range redacted].

2.4.4 – High Content Analysis

Once the images were obtained, they underwent processing using the MetaXpress software. Custom modules were then created to measure the number of cells in each well of every plate using the Multi Wavelength Cell Scoring algorithm.

The fluorescence value of the background was recorded and then subtracted from the obtained data and in order to eliminate unwanted hairs and fibres present in the wells, algorithms were created using the Custom Module Editor (version 2.0.33.3, Molecular Devices). These algorithms recognised and removed fibres from the calculations by adjusting the maximum size threshold for detected objects.

2.5 – Transfection

To assess the cellular uptake of the peptide, a specialised assay was created to quantify the interactions of the peptides with the cells and to visualise this process using the fluorescent siGLO Red Transfection Indicator. Unlike the MST experiments, the siGLO was diluted with filtered deionised water instead of PBS/Tween buffer.

2.5.1 – Plate preparation

In similar fashion to the previous cell toxicity experiment, the cells were dispensed into clear-bottom 384-well plates using a microplate dispenser. The seeding concentrations were selected at around [cell count range redacted] cells per well, after being counted and diluted to the appropriate concentration before seeding and left to incubate overnight at 37°C.

2.5.2 – Peptides and siGLO Red Transfection Indicator

After a 24-hour incubation period, the peptides were added. The concentrations of the peptides were selected based on the evaluation of the results of the cell toxicity

experiment and the selected final concentrations were [concentration range redacted]. Additionally, a control group was established where only [volume redacted] of siGLO Red Transfection Indicator was added to the cells without any peptide.

The peptides were dissolved in filtered and deionised water to achieve the desired stock concentrations. In addition, siGLO Red Transfection Indicator was added to the peptides at a concentration of [concentration redacted], maintaining a 1:1 ratio. After mixing and a period of incubation of [time redacted] at room temperature, [volume redacted] of the peptide and siGLO mixture was added to each well. The plates were then left to incubate for a period of [time redacted], similar to the cell toxicity experiment, and subsequently fixed using the Hoechst fixative solution.

2.5.3 – High Content Screening

After the cells had been fixed and the required minimum period of one hour had passed, the plates were prepared for High Content Screening through the High Content Microscope and the MetaXpress software.

Four sites with a 10X objective were selected near the edges of each well in the HCT116 and HFF cell line plates for imaging. However, nine sites were chosen from the Caco-2 plates due to the cells within these plates being clustered together and growing somewhat slower compared to the HCT116 and HFF-1 cell line plates. The DAPI channel was chosen for observing the cells stained with Hoechst, while the CY3 (cyanine3) channel was chosen to observe the siGLO-labelled peptides. The exposure time for each peptide differed once again, ranging from [range redacted] for the DAPI channel and [range redacted] for the CY3 channel.

2.5.4 – High Content Analysis

Once the images had been acquired for each cell line, they were processed through the MetaXpress software and analysed accordingly. Unlike the cell toxicity experiment, this experiment involved superimposing images acquired from both wavelengths and then establishing new parameters and the acquisition of an additional set of measurements. These were the "positive W2" and "% positive W2" values which indicated the number of total cells that exhibited the siGLO-detecting CY3 wavelength (referred to as W2 – wavelength 2 – in the algorithm) and the percentage of these cells relative to the cells stained with Hoechst, respectively.

2.6 – Statistical Analysis

The quantitative data was graphed and evaluated by statistical analysis using GraphPad Prism (version 10.1). The mean and standard deviation (SD) values were obtained for all assays where possible. In order to make statistical comparisons, a two-way ANOVA test was selected to assess the effects of the peptides on the three cell lines to be observed in this research (HCT116, Caco-2 and HFF-1), with a confidence interval of 95% ($\alpha = 0.05$).

Results

1. MST

Although there is limited existing research on the utilisation of microscale thermophoresis (MST) research (Zimmermann et al., 2022, Scheuermann et al., 2016), this innovative study differed from that literature by using peptides in combination with siGLO Red Transfection Indicator to explore their potential for use in future cellular experiments. The siGLO Red Transfection Indicator was chosen as a fluorescent marker to facilitate the tracking and imaging of peptide uptake and distribution within cells. This methodology contrasts with current MST studies, which often investigate protein-ligand interactions rather than peptide-siRNA conjugates. By conjugating siGLO to the peptides, this study aimed to quantify the binding affinity of each peptide to the siGLO and allowed the assessment and evaluation of their effectiveness as drug-delivery vehicles.

The MST experiment utilised in this analysis was improved and developed over several months to ensure that it aligned with the research goals and after multiple unsuccessful attempts and tests in which lower concentrations of both siGLO and peptide led to a lack of viable or weak data, it was concluded that an initial concentration of [redacted] for the peptide and [redacted] for the siRNA (siGLO Red Transfection Indicator) was the most effective for this specific research involving peptides and siGLO. Through this study, we demonstrated an innovative and novel approach to MST by focusing on peptide-siRNA conjugation, distinguishing it from other publications in the literature.

After the completion of the MST experiments, the data was collected and processed, and the EC_{50} (half maximal effective concentration) and K_D (dissociation constant) values for each peptide were recorded. The MST traces were also recorded (a sample of a trace shown in figure 4), which is data typically collected to illustrate the fluctuations in normalised fluorescence signal after the peptide and ligand molecule (siGLO Red Transfection Indicator in this experiment) interact. However, it is unsuitable to draw any inhibitory or stimulatory implications solely based on this data.

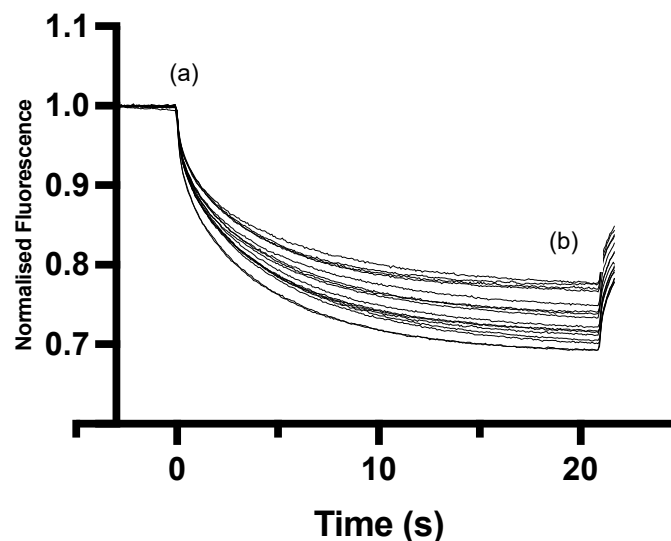


Figure 4: a sample MST trace graph, (a) IR (infrared laser) is turned on, (b) IR laser is turned off. This figure demonstrates a three-second delay before the timer begins at 0, indicating that the signal is in equilibrium and represents a measurement recorded at room temperature. This lag occurs before the temperature gradient is applied through the infrared laser. Traces with higher normalised fluorescence generally suggest a greater degree of binding to siGLO and are frequently associated with higher peptide concentrations. On the contrary, lower peptide concentrations indicate less binding and thus result in lower levels of fluorescence.

The MST trace data obtained from the assessed peptides exhibited different amounts of interactions with the siGLO (figure 5 – redacted). [Specific observations about individual peptides have been redacted.]

Table 3: The list of peptides tested in the MST experiment and their EC ₅₀ and K _D values (calculated in a log[agonist] vs. response [three parameters] format). All values, as well as names of unpublished peptides, have been redacted due to embargo and pending publication.		
Peptide	EC ₅₀ (μM)	K _D (μM)
G3	[redacted]	[redacted]
CI-13	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
HHC10	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

Figure 5

Redacted – Data Withheld for Confidentiality

Figure 5: The recorded MST trace graphs for the tested peptide series, all data is conducted in replicate and the means are shown in these graphs. Data is withheld for confidentiality.

Figure 6

Redacted – Data Withheld for Confidentiality

The binding affinity (K_D) and EC_{50} values of the peptides were evaluated to determine their capacity to bind siGLO and release it under suitable cellular conditions (table 3). All numerical EC_{50} and K_D values have been redacted.]

[Specific comparative performance of individual peptides, including references to strongest and weakest binding, has been redacted due to embargo.]

The fluorescence dose response curves (figure 6) [data redacted]. [Details about fluorescence trends, concentration effects, and aggregation behaviour have been removed to preserve publication potential.]

[Peptide-specific observations, such as self-quenching or differences between MST trace and dose-response curves, have been redacted.]

Overall, the results from the MST experiment demonstrated that the siGLO successfully bound to the majority of the peptides. This indicated that fluorescent labelling of peptides with siGLO in subsequent cell research experiments would be suitable. [Specific findings regarding individual peptides have been redacted.]

2. Cell Proliferation

The figures illustrate the antiproliferative effect of the three groups of peptides on normalised cell proliferation (%) in the three cell lines under investigation (HCT116, Caco-2, and HFF-1). The peptides were categorised into three groups to allow for

comparison within each group and to enhance visual clarity, and the G3 peptide was included in all the graphs for comparison with the other peptides, as it had been previously studied and served as a reference.

**REDACTED –
Experimental cell
proliferation data for
Group 1 peptides.**

Figure 7a: The graph shows the normalised cell count (%) of HCT116 cells following treatment with peptides from Group 1 (G3, CI-13 and redacted peptide) at increasing concentrations.

**REDACTED –
Experimental cell
proliferation data for
Group 2 peptides.**

Figure 7b: The graph shows the normalised cell count (%) of HCT116 cells following treatment with peptides from Group 2 (G3 and redacted peptides) at increasing concentrations.

REDACTED – Experimental cell proliferation data for Group 3 peptides.

Figure 7c: The graph shows the normalised cell count (%) of HCT116 cells following treatment with peptides from Group 3 (G3, HHC10, and redacted peptides) at increasing concentrations.

As previously described in the Materials and Methods section, the HCT116 cell line was treated with ten peptides across a range of [redacted concentrations], including a deionised water control. After incubation, the cells were fixed and imaged using high-content microscopy. Figures 7a–c [redacted] illustrate the normalised cell count (%) for each peptide group: Group 1 (Figure 7a), Group 2 (Figure 7b), and Group 3 (Figure 7c).

All peptides at the highest tested concentration exhibited a substantial reduction in HCT116 cell counts, indicating marked cytotoxic effects. It is important to note that such high concentrations necessary for almost complete cytotoxic efficacy may pose challenges for in vivo applications, particularly regarding bioavailability, peptide stability, and delivery – points that are further explored in the Discussion section. Notably, two redacted peptides and G3 demonstrated the strongest cytotoxic

responses at this concentration, whereas one redacted peptide appeared less effective, despite its strong binding affinity seen in the MST experiments. In contrast, at lower to intermediate concentrations, cell viability remained relatively unaffected for most peptides.

The cell count data was normalised to the untreated control (deionised water), which was set to 100%, to reduce inter-replicate variability. Results are presented as mean $\pm$ standard deviation, derived from three biological replicates ($n = 3$), each comprising four technical repeats. The original figures included error bars representing the standard deviation across replicates, as well as statistical significance markers (p-values). [All error bar values, p-values, and pairwise comparison details have been redacted due to embargo.]

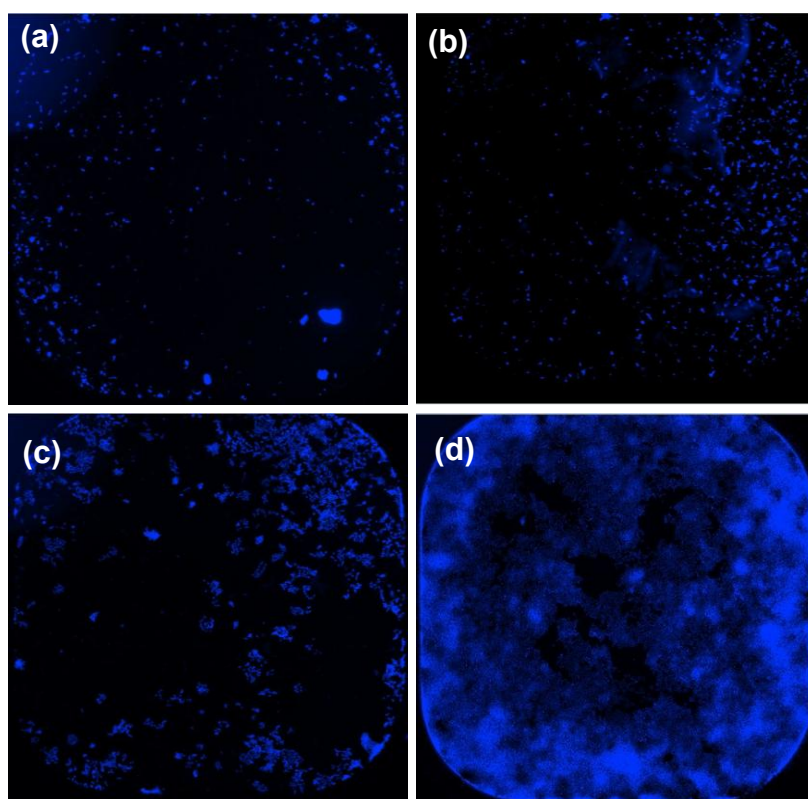


Figure 8: A sample set of images acquired under a 2× objective lens depicting the phenotype of the HCT116 cells after being treated with (a–d) peptides at redacted concentrations.

The images show the HCT116 cells under peptides at various redacted concentrations (figure 8a–d), where differences in observable proliferation were noted. This effect was observed under all peptides, though with slight variations.

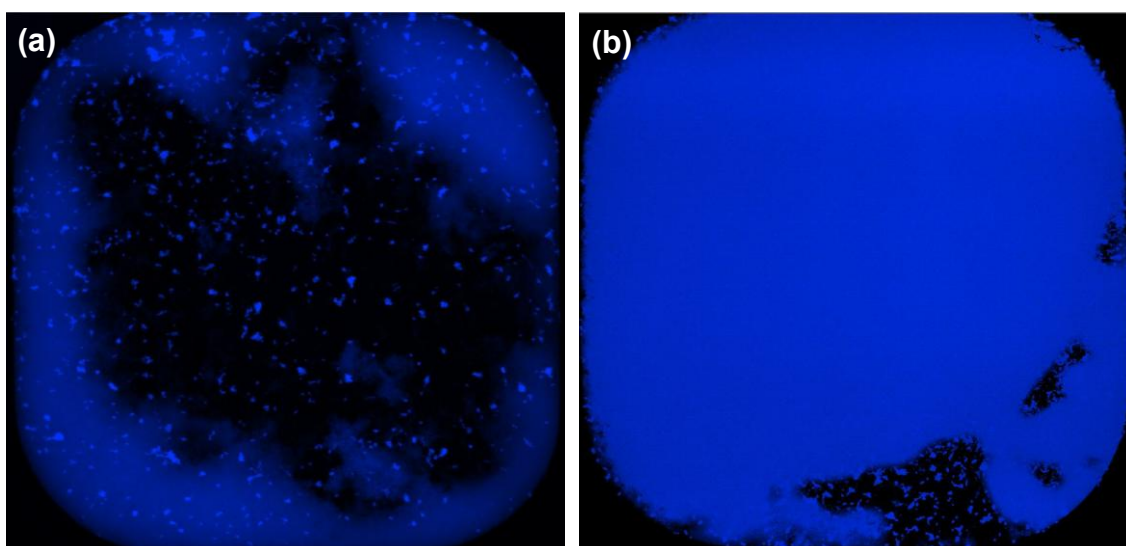


Figure 9: a sample set of images acquired under a 2x objective lens depicting the wells under heavy peptide aggregation and precipitation, where (a) the data can be obtained through increasing the fluorescence threshold, and (b) the well is omitted from the calculations entirely.

At the highest concentration of peptide, particularly for the [REDACTED PEPTIDES], there was a noticeable formation of aggregates and intense staining, which made it difficult to distinguish the cells clearly. To address this, the detection threshold of the applied algorithm in MetaXpress was increased, enabling the identification and counting of cells that exhibited fluorescence levels beyond those of the peptide, as depicted in figure 9a. Wells showing similar characteristics to figure 9b indicated significant amounts of peptide aggregation and staining. [REDACTED: Observations on phenotype, cytotoxicity, and morphological assessment.]

Table 4: the IC ₅₀ values obtained from the addition of the peptides to the HCT116 cells	
Peptide	IC ₅₀ (μM)
G3	Redacted result
CI-13	Redacted result
Redacted Peptide	Redacted result
HHC10	Redacted result
Redacted Peptide	Redacted result
Redacted Peptide	Redacted result
Redacted Peptide	Redacted result
Redacted Peptide	Redacted result
Redacted Peptide	Redacted result
Redacted Peptide	Redacted result

The IC₅₀ values in table 4 were determined for the series of peptides against the HCT116 cell line, using GraphPad Prism with a "log(inhibitor) vs. response (three parameters)" model fitted to a sigmoidal curve, where 'parameters' refers to the Hill slope (gradient of the curve) and the top and bottom responses (maximum and minimum, respectively). The data from the control cells that had been treated with deionised water was normalised to 100%, and the highest concentration used in this assay ([redacted]) was designated as the maximal inhibition. The IC₅₀ values were then calculated from the concentration of each peptide that corresponded to 50% of the distance between the minimum and maximum response values. [All IC₅₀ values and comparative statements have been redacted due to embargo.]

As previously mentioned, a two-way ANOVA test was performed to assess differences across peptide concentrations and between peptide types against the HCT116 cells. [The statistical outcomes and interpretations of these comparisons have been redacted due to embargo.]

**REDACTED –
Experimental cell
proliferation data for
Group 1 peptides.**

Figure 10a: The graph shows the normalised cell count (%) of Caco-2 cells following treatment with peptides from Group 1 (G3, CI-13 and redacted peptide) at increasing concentrations.

**REDACTED –
Experimental cell
proliferation data for
Group 2 peptides.**

Figure 10b: The graph shows the normalised cell count (%) of Caco-2 cells following treatment with peptides from Group 2 (G3 and redacted peptides) at increasing concentrations.

REDACTED – Experimental cell proliferation data for Group 3 peptides.

Figure 10c: The graph shows the normalised cell count (%) of Caco-2 cells following treatment with peptides from Group 3 (G3, HHC10, and redacted peptides) at increasing concentrations.

As described in the methodology, Caco-2 cells were treated with ten peptides across [redacted concentration range] and incubated for 72 hours before fixation and imaging using high-content microscopy. Figures 10a–c present the normalised cell count data for the three peptide groups: Group 1 (Figure 10a), Group 2 (Figure 10b), and Group 3 (Figure 10c). Values were normalised to the deionised water control (set to 100%) and are presented as mean $\pm$ standard deviation from three biological replicates, each containing four technical replicates.

As seen with the HCT116 cells, the highest concentration of peptides produced a strong antiproliferative effect across nearly all peptides. [Details regarding specific

concentration thresholds and comparative sensitivity between cell lines have been redacted.]

[Detailed comparisons of peptide efficacy within Groups 1–3 (Figures 10a–c), including specific peptide names and their relative performance, have been redacted due to embargo.] Statistical analysis using two-way ANOVA and Tukey’s post hoc test was performed to evaluate the influence of peptide identity and concentration on cell count. [All statistical outcomes and pairwise comparisons have been redacted.]

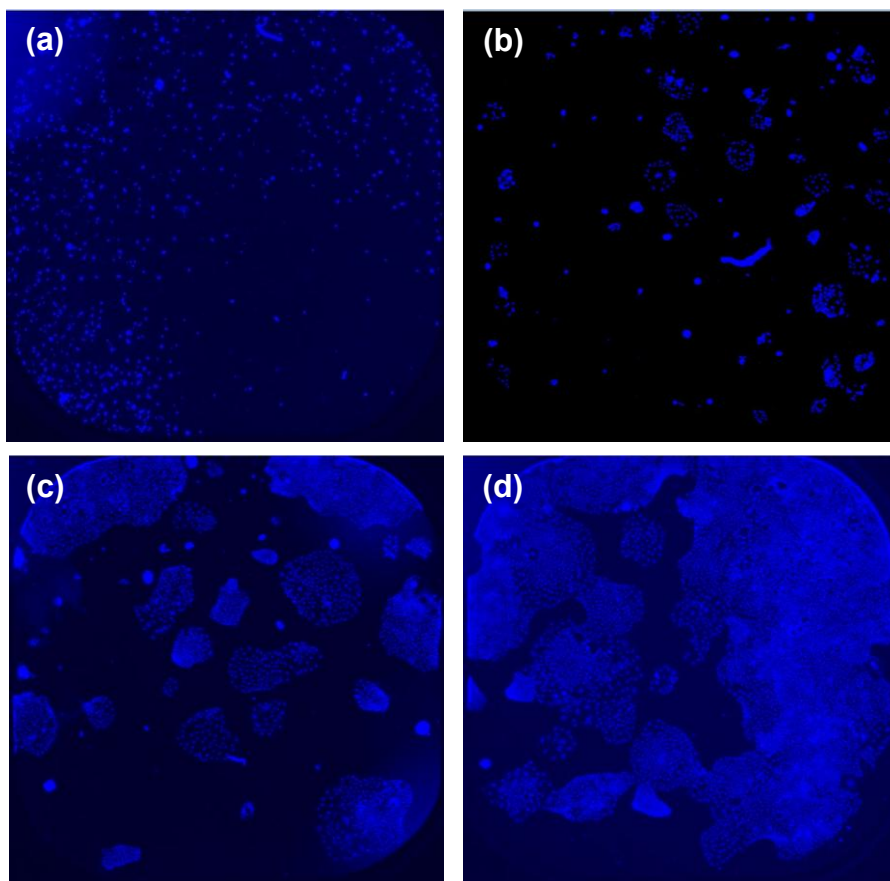


Figure 11: A sample set of images acquired under a 2× objective lens depicting the phenotype of the Caco-2 cells after being treated with (a–d) peptides at redacted concentrations.

The images show Caco-2 cells observed under varying [redacted] peptide concentrations (figure 11a–d). [Observations regarding growth patterns and phenotypical changes have been redacted due to embargo.]

Table 5: the IC ₅₀ values obtained from the addition of the peptides to the Caco-2 cells	
Peptide	IC ₅₀ (μM)
G3	<i>Redacted result</i>
CI-13	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
HHC10	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>

The IC₅₀ values were also obtained for the Caco-2 cell line (table 5) using the same method as for the HCT116 cells. [All IC₅₀ values, peptide comparisons, and interpretations regarding histidine-modified peptide efficacy have been redacted due to embargo.]

**REDACTED –
Experimental cell
proliferation data for
Group 1 peptides.**

Figure 12a: The graph shows the normalised cell count (%) of HFF cells following treatment with peptides from Group 1 (G3, CI-13 and redacted peptide) at increasing concentrations.

**REDACTED –
Experimental cell
proliferation data for
Group 2 peptides.**

Figure 12b: The graph shows the normalised cell count (%) of HFF cells following treatment with peptides from Group 2 (G3 and redacted peptides) at increasing concentrations.

REDACTED – Experimental cell proliferation data for Group 3 peptides.

Figure 12c: The graph shows the normalised cell count (%) of HFF cells following treatment with peptides from Group 3 (G3, HHC10, and redacted peptides) at increasing concentrations.

The HFF-1 cells were treated under the same experimental conditions as the HCT116 and Caco-2 cell lines, with each peptide tested across [redacted concentration range] alongside a deionised water control. As shown in figures 12a–c, the specific responses of individual peptides and their comparative effects have been redacted due to embargo. Overall, the normal cell line demonstrated markedly different trends compared to the cancer cell lines, but the precise outcomes and observations are withheld.

Details on statistical comparisons between peptides, dose-dependent effects, and group-level differences have also been redacted. However, the analysis approach and evaluation of replicate consistency remain unchanged from the previously described methodology. Although normalisation artefacts and error bar variations were noted during data review, all related interpretations and specific findings have been redacted to protect unpublished data.

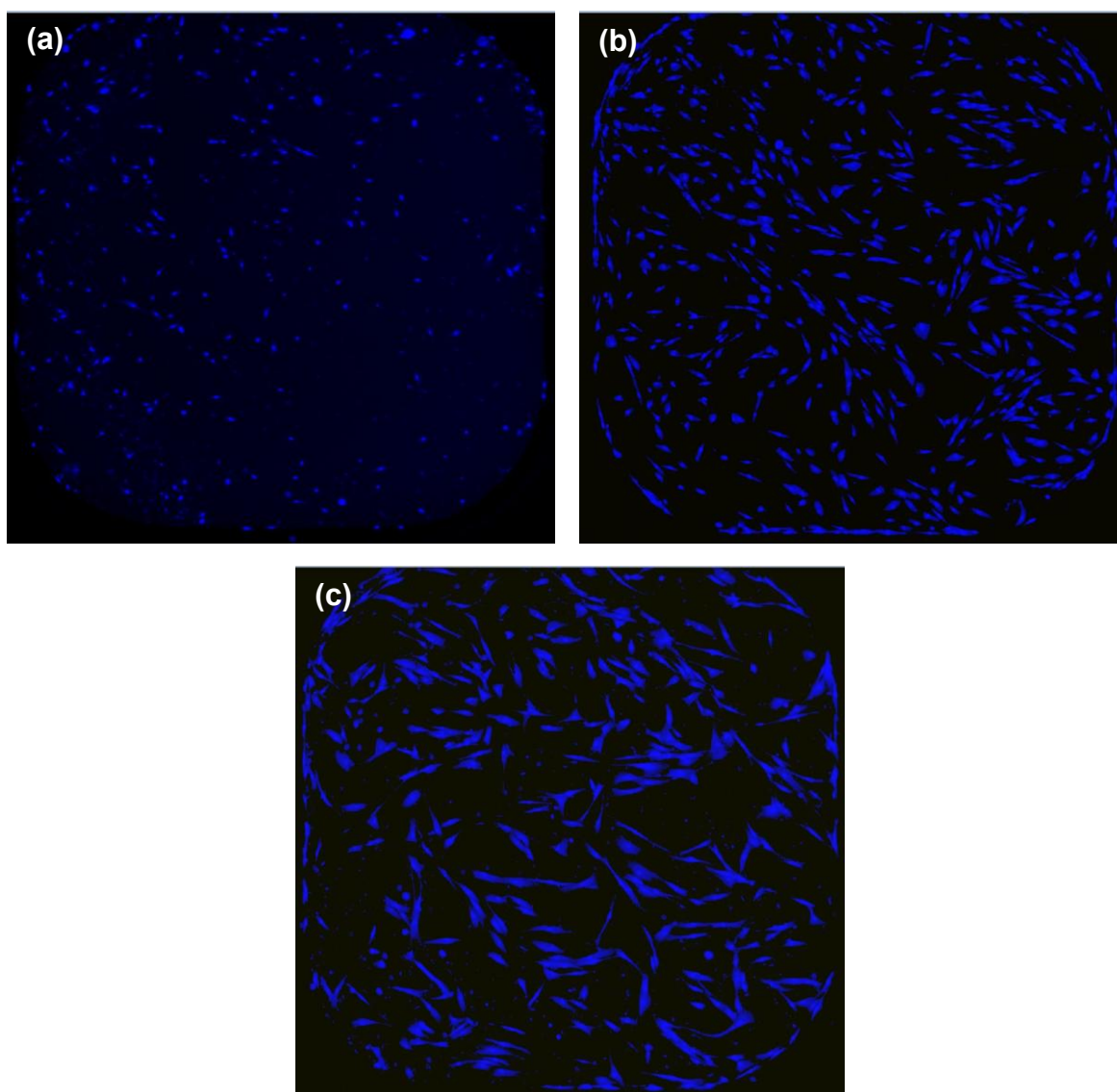


Figure 13: A sample set of images acquired under a 2× objective lens depicting the phenotype of the HFF cells after being treated with (a) peptides at the highest tested concentration, (b) a higher intermediate concentration, (c) a moderate concentration, and (d) a low concentration. Exact concentrations have been redacted.

The images in figure 13 show representative wells from the HFF-1 experiments at various peptide concentrations (redacted). While these images illustrate general experimental conditions, all detailed findings regarding cell count, growth, or peptide-related effects have been redacted due to the embargo. No specific interpretation of cell density, proliferation, or morphology is included here to protect unpublished results.

The purpose of including these images is solely to demonstrate the visual workflow used for microscopy within this study. Detailed quantitative and qualitative observations from the HFF-1 assays have been withheld, though the methodology remains consistent with the previously described high-content imaging and analysis approaches. Future work may include a full morphological assessment to complement the results, but these findings are not disclosed at this time.

Table 6: the IC ₅₀ values obtained from the addition of the peptides to the HFF-1 cells	
Peptide	IC ₅₀ (μM)
G3	<i>Redacted result</i>
CI-13	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
HHC10	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>
<i>Redacted peptide</i>	<i>Redacted result</i>

The IC₅₀ data for the HFF-1 cell line (table 6) has been redacted due to the embargo, along with detailed comparisons of peptide potency. Specific trends relating to the inhibitory response of individual peptides are not disclosed here.

While statistical analyses were performed to evaluate the effects of peptide type and concentration, the outcomes of these tests have also been withheld. Only a general note is provided that differences between peptides and concentrations were assessed, but no quantitative values or conclusions are presented to protect unpublished findings.

3. Transfection

The transfection experiments were designed to evaluate the cellular uptake of the series of peptides after conjugation with siGLO Red Transfection Indicator. The primary aim was to visualise and quantify the peptide-mediated delivery of the siGLO into the three different cell lines used thus far in this study and to evaluate the peptide's potential as delivery vectors for therapeutic agents. We hypothesised that peptides containing histidine, particularly those with a higher number of histidine residues, may exhibit enhanced efficacy in siGLO delivery into cancer cells when compared to peptides lacking histidine or containing fewer histidine residues in the sequence.

After completion of the transfection experiments, the 384-well plates for the HCT116, Caco-2 and HFF-1 cell lines underwent analysis using the MetaXpress software. The transfection assay differed from the cell proliferation assay in that it required superimposing images obtained from both DAPI and CY3 wavelengths. It involved analysing a new set of measurements, specifically the "positive W2" and "% positive W2" values. These values indicated the total number of cells that showed fluorescence from the siGLO-detecting CY3 wavelength (referred to as W2 in the algorithm) and the percentage of these cells relative to the cells stained with Hoechst (the wavelength for this stain is referred to as DAPI). The "positive W2" data indicated the total number of transfected cells, whereas "% positive W2" denoted the percentage of positive cells relative to the overall cell count in each well. This variable was prioritised in the study since it provided a clear assessment of siGLO delivery efficiency in relation to the overall cell count, hence the "positive W2" data was excluded from analyses, having already been incorporated into the calculation of "% positive W2."

The images observed and analysed in this assay were a superimposition of the two wavelengths tested, DAPI to show the cells and CY3 to show the siGLO molecules, and an example of the separated images and superimposed images is shown in figure 14.

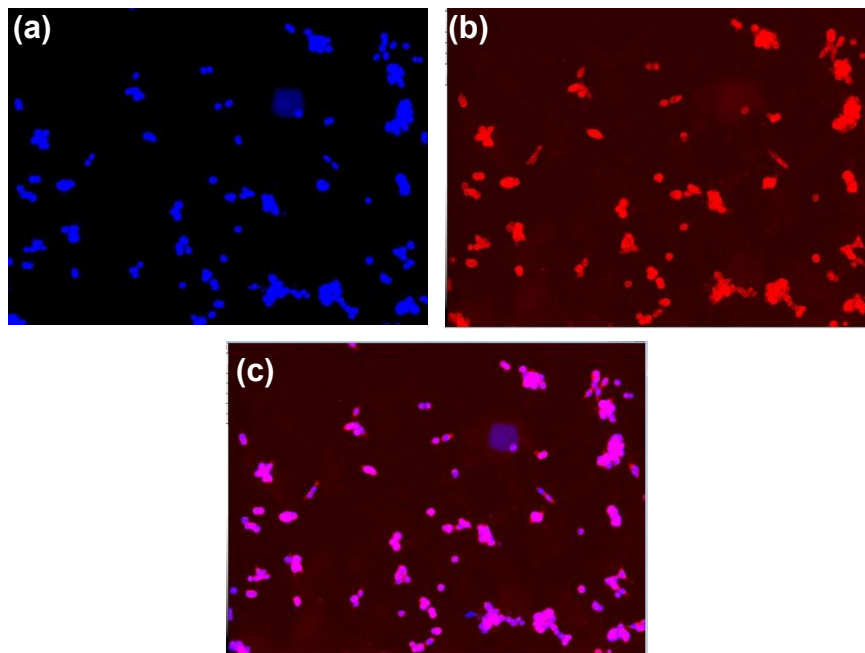


Figure 14: a sample set of images after transfection of a redacted peptide–siGLO complex into HCT116 cells acquired under a 10× objective lens depicting (a) the Hoechst-stained cells in the blue DAPI wavelength, (b) the red CY3 channel showing the peptide–siGLO complexes, and (c) the superimposed image.

REDACTED – Experimental transfection data for Group 1 peptides.

Figure 15a: Percentage of W2-positive HCT116 cells following treatment with redacted peptides, including G3 (blue) and CI-13 (red). Data represent mean $\pm$ SD from three biological replicates, each with four technical replicates ($n = 3$). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test, comparing all peptides within the group. Asterisks indicate statistical significance ($P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). Data from other concentrations were omitted. Non-significant comparisons are not displayed.*

For the HCT116 cell line, only selected concentrations were displayed in Figure 15a. Statistical comparisons were carried out within the group using a two-way ANOVA followed by Tukey's multiple comparisons test. Significant differences between peptides at the displayed concentrations are indicated by the asterisks on the figure.

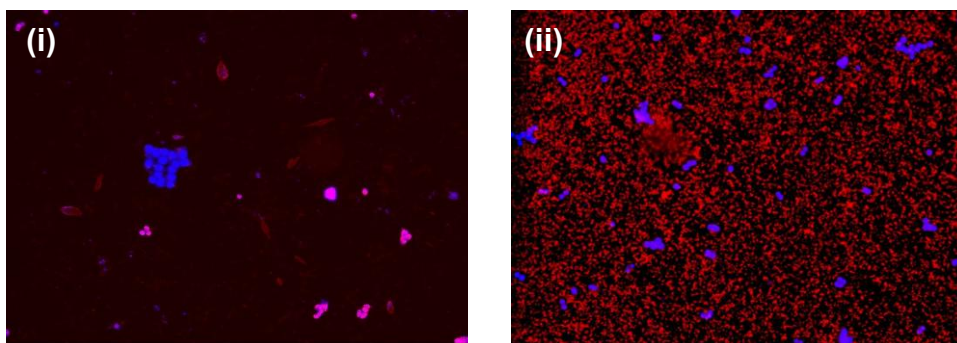


Figure 15b: (i) G3-siGLO complexes as shown under 10x objectivity at a redacted concentration, (ii) CI-13 shown in the same conditions as G3-siGLO.

Figure 15b(ii) displays the presence of CI-13 in the cells, which are coloured purple as a result of the observation of CI-13 and siGLO complexes within them, that are overlapping. Figure 15b(i) reveals redacted trends that confirm the observations from figure 15 (a).

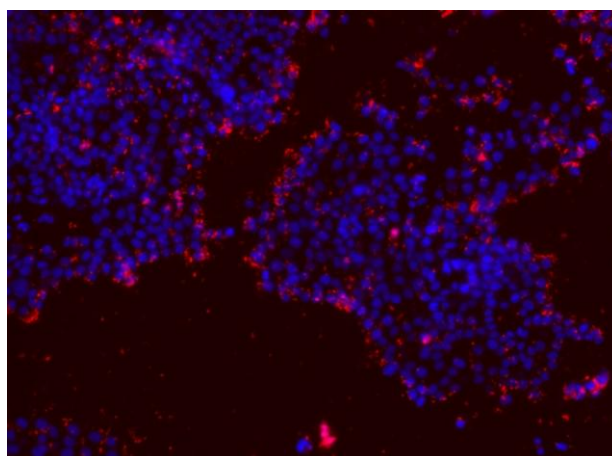


Figure 15c: Redacted peptide-siGLO complex interactions as observed under 10x objectivity.

The image in Figure 15c displays a representative transfection result for one of the redacted peptides. Unlike the published controls shown in Figures 15a–b, detailed interpretation of localisation patterns and cellular uptake for this peptide has been withheld. Observed differences between peptides and across replicates are discussed further in the redacted discussion section.

REDACTED – Experimental transfection data for Group 2 peptides.

Figure 16a: Percentage of W2-positive HCT116 cells following treatment with redacted peptides from Group 3, including G3 (blue) and HHC10 (red). Data represent mean $\pm$ SD from three biological replicates, each with four technical replicates ($n = 3$). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test. Asterisks indicate statistical significance ($P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). Data from other concentrations were omitted. Non-significant comparisons are not shown.*

In Figure 16b, fluorescence differences were observed between the peptides at varying concentrations. Multiple pairwise comparisons revealed statistically significant differences between peptides at the tested conditions ($P < 0.0001$ to $P < 0.01$), as determined by two-way ANOVA with Tukey's multiple comparisons test. Observed fluorescence changes at higher concentrations may be influenced by aggregation or surface binding, and further experiments are needed to clarify the underlying mechanisms (refer to Discussion).

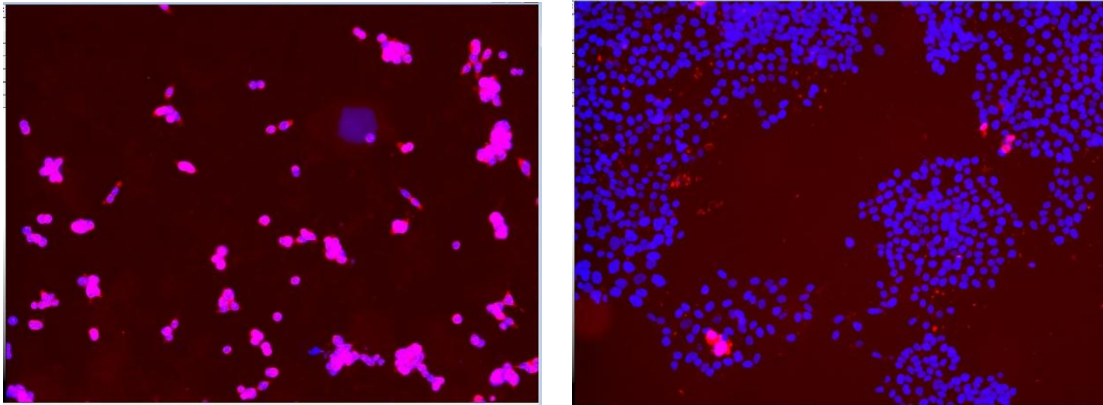


Figure 16b: (i) HHC10-siGLO complexes as shown under 10x objectivity at a redacted concentration, (ii) HHC10-siGLO as shown under the same microscopic settings another redacted concentration.

REDACTED – Experimental transfection data for Group 3 peptides.

Figure 17a: Percentage of W2-positive HCT116 cells following treatment with redacted peptides from Group 2, including G3. Data represent mean $\pm$ SD from three biological replicates, each with four technical replicates ($n = 3$). Two-way ANOVA with Tukey's multiple comparisons test was used to evaluate differences between peptides. Asterisks indicate statistical significance (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). Non-significant comparisons are not shown.

In the Group 2 peptides (Figure 17a), statistically significant differences in W2 positivity were observed between peptides at the tested concentrations, with a subset of peptides displaying markedly higher fluorescence signals. These findings were

consistent with the study's hypothesis that specific amino acid modifications may enhance peptide-mediated siRNA delivery. Further mechanistic insights are discussed in the relevant sections of the discussion.

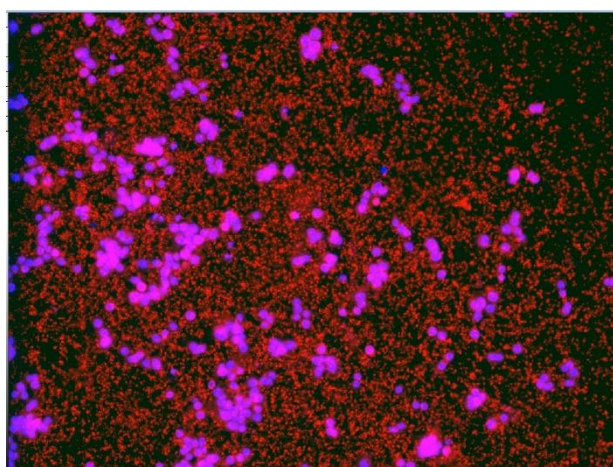


Figure 17b: The detection of W2 (siGLO) within the HCT116 cells at a redacted concentration of a redacted peptide.

Figure 17b displays siGLO-associated fluorescence (W2) in HCT116 cells following treatment with a redacted Group 2 peptide at one of the tested concentrations. The presence of red signal indicates interaction between the fluorescent siRNA and the peptide formulation. At elevated concentrations, the distribution of fluorescence suggested the potential for peptide aggregation or precipitation, which may result from exceeding solubility limits or extracellular accumulation (Zapadka et al., 2017). Further clarification would require additional analyses such as peptide concentration optimisation (Fung et al., 2003) or fluorescence recovery after photobleaching (FRAP) to determine whether the observed fluorescence arises from intracellular uptake or extracellular binding (Braga et al., 2004).

Statistical comparisons were conducted using two-way ANOVA with Tukey's post-hoc test, confirming significant differences between the peptide conditions tested ($P < 0.0001$). These results supported the overall conclusion that peptide concentration influences cellular interaction outcomes, though specific findings for individual peptides remain redacted. Only statistically significant pairwise comparisons are indicated on the figure; non-significant comparisons were omitted for clarity.

REDACTED – Experimental transfection data for Group 1 peptides.

Figure 18a: Percentage of W2-positive Caco-2 cells following treatment with redacted peptides from Group 1, including G3. Data represent mean $\pm$ SD from three biological replicates, each with four technical replicates ($n = 3$). Two-way ANOVA with Tukey's multiple comparisons test was used to evaluate differences between peptides. Asterisks indicate statistical significance ($P \leq 0.05$, ** $P \leq 0.001$). Non-significant comparisons are not shown.*

As seen in Figure 18a, the cysteinated peptide group demonstrated varied uptake patterns across the tested conditions. Details regarding specific concentrations and comparative uptake trends have been redacted. Statistically significant differences were detected between certain peptides within the group ($p < 0.05$ to $p < 0.001$). Representative images are shown for selected peptides in Figure 18b(i) and Figure 18b(ii).

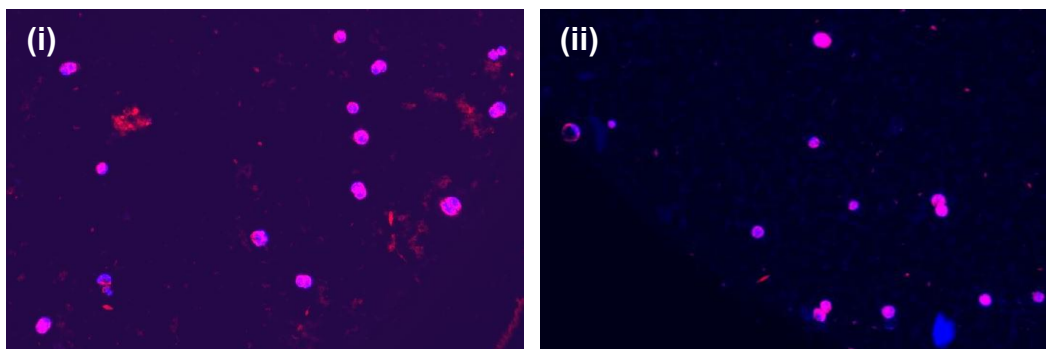


Figure 18b: (i) G3-siGLO as seen inside Caco-2 cells under 10x objectivity at a redacted concentration and (ii) CI-13 as seen under the same conditions.

REDACTED – Experimental transfection data for Group 2 peptides.

Figure 19a: Percentage of Caco-2 cells positive for W2 following treatment with redacted peptides from Group 3. Data represent mean $\pm$ SD from three biological replicates, each with four technical replicates ($n = 3$). Statistical analysis was performed using two-way ANOVA with Tukey's post-hoc test. Asterisks indicate statistical significance (* $P \leq 0.05$, *** $P \leq 0.0001$). Details of specific concentrations and peptide identities have been redacted. Non-significant comparisons are omitted for clarity.

In the second peptide group (data redacted), the comparative analysis originally described differences in siGLO uptake between individual peptides at multiple concentrations, alongside statistical comparisons. These details have been removed to avoid disclosure of sensitive performance trends. Broadly, the findings contributed to the overall evaluation of histidine-containing peptides as siRNA delivery agents, with analysis conducted via two-way ANOVA and Tukey's post-hoc testing.

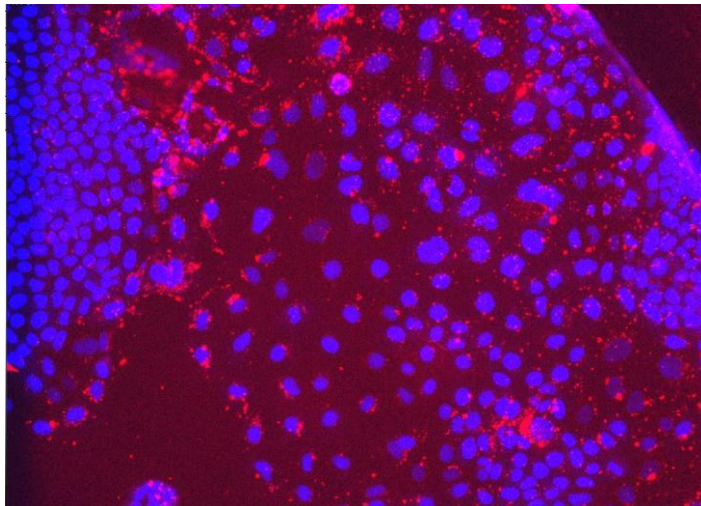


Figure 19b: HHC10-siGLO complexes as seen under 10x objectivity at a redacted concentration against Caco-2 cells.

REDACTED – Experimental transfection data for Group 3 peptides.

Figure 20a: Percentage of W2-positive Caco-2 cells following treatment with redacted peptides from Group 3, including G3. Data represent mean $\pm$ SD from three biological replicates, each with four technical replicates ($n = 3$). Specific concentration values have been redacted. Two-way ANOVA with Tukey's multiple comparisons test was used to assess differences between peptides. Asterisks indicate statistical significance ($P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). Error bars exceeding 100% were noted, but related trends have been redacted. Non-significant comparisons are not shown.*

Among the histidine-modified peptides in Group 2 (Figure 20a), certain peptides demonstrated higher levels of W2-positive Caco-2 cells compared to others. Specific concentration values, uptake levels, and statistical outcomes have been redacted. These results indicate variability in the efficiency of peptide-mediated delivery within the group, with some sequences performing better than others.

These results reinforce the observation that some peptides within Group 2 showed improved transfection efficiency compared to others, with certain sequences performing more favourably at higher concentrations (peptide identities and concentration values redacted) (Figure 20b). Visual inspection suggested differences in peptide behaviour, with some appearing more prone to surface association rather than efficient intracellular delivery.

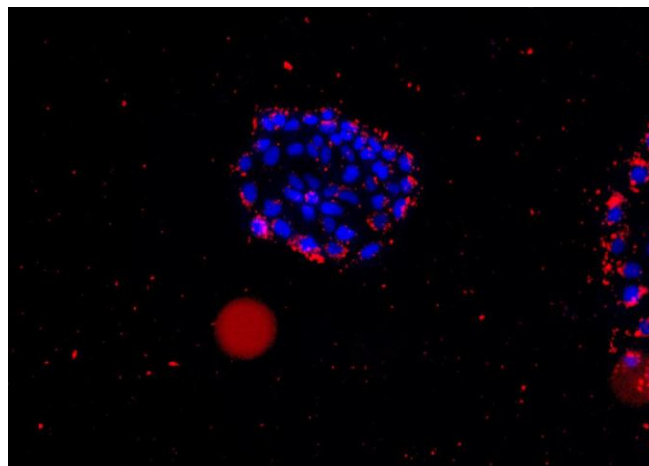


Figure 20b: Example of siGLO-peptide complexes in Caco-2 cells imaged under 10× magnification following treatment with a redacted peptide at a redacted concentration.

A two-way ANOVA confirmed a highly significant effect of both peptide identity and concentration on siGLO uptake in Caco-2 cells ($P < 0.0001$). Tukey's multiple comparisons test identified statistically significant differences between several peptide pairs at higher concentrations (values redacted), supporting the conclusion that histidine-modified sequences can enhance peptide-mediated transfection efficiency.

REDACTED – Experimental transfection data for Group 1 peptides.

Figure 21a: Percentage of HFF-1 cells positive for W2 following treatment with redacted peptides from Group 1, including G3 and CI-13, at redacted concentrations. Data represent mean $\pm$ SD from three independent biological replicates, each with four technical replicates. Statistical analysis was performed using two-way ANOVA with multiple comparisons. Asterisks denote statistical significance ($P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$); non-significant comparisons are not shown.*

In contrast to the two cancer cell lines (HCT116 and Caco-2), the HFF-1 cells exhibited distinct patterns in W2 uptake compared to the other models. Uptake was detected across all tested conditions, but the extent and apparent localisation varied depending on the peptide. (Specific differences between peptides and concentrations have been redacted.)

For the cysteinated group (Figure 21a), specific comparative trends in W2 detection between peptides at different concentrations have been redacted. Despite this, it was

noted that one peptide displayed relatively higher W2 levels than the others, though the associated error bars were notably large, particularly at higher concentrations, suggesting high variability. While the raw data did not show extreme fluctuations, the elevated variability following normalisation likely resulted from subtle inter-replicate shifts in the untreated control, which can become amplified during percentage transformation.

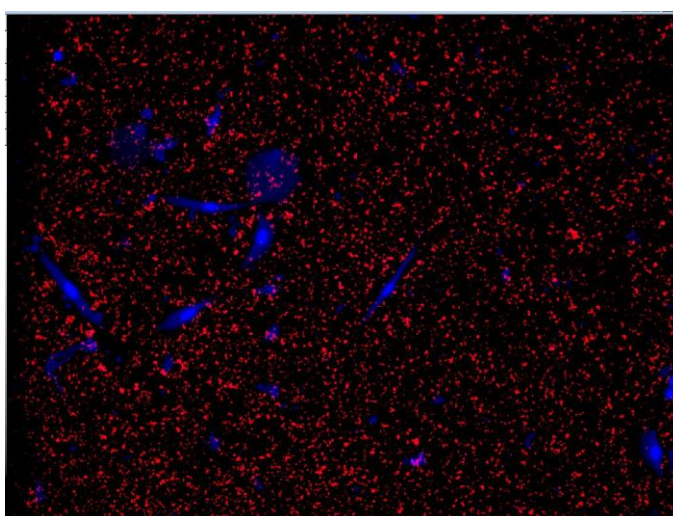


Figure 21b: CI-13-siGLO complexes as seen under 10x objectivity at a redacted concentration against HFF-1 cells.

However, upon analysing the obtained images (Figure 21b), a markedly different narrative emerged. One peptide-siGLO complex appeared to localise around the cells, but actual cellular entry was rare. The algorithm (which observes and denotes wavelength overlaps) may have detected instances where siGLO particles were bound to the cell surface and incorrectly counted them as internalised. This behaviour is consistent with aggregation or precipitation of peptide-siGLO complexes at certain conditions (details redacted), and was not observed in earlier experiments, suggesting that this effect may be influenced by cell-type-specific factors affecting surface interactions.

REDACTED – Experimental transfection data for Group 2 peptides.

*Figure 22a: Percentage of HFF-1 cells positive for W2 following treatment with redacted peptides from Group 3, including G3. Data represent the mean $\pm$ SD from three independent biological repeats, each with four technical replicates. Statistical analysis was performed using two-way ANOVA with multiple comparisons. Significance levels are indicated as follows: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Non-significant comparisons are not shown. Concentration values have been redacted.*

In the HHC peptide group (Figure 22a), W2 uptake patterns varied across peptides, with lower concentrations often showing enhanced detection compared to higher doses. At these lower concentrations, several peptides showed significantly greater uptake than G3 ($P < 0.001$). Interestingly, G3 produced significantly less uptake than nearly all peptides at the next concentration step, except for HHC10 ($P < 0.01$ – 0.001).

These observations suggest that some histidine-modified peptides displayed stronger interactions with HFF-1 cells than the benchmark G3.

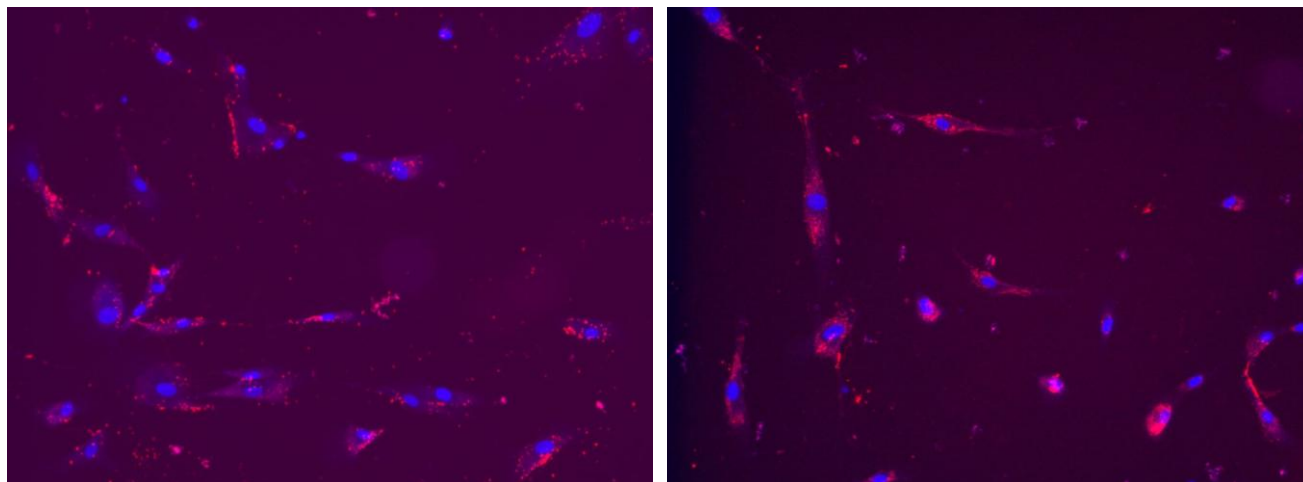


Figure 22b: Representative images of redacted peptides from Group 2 conjugated with siGLO in HFF-1 cells under 10× magnification at the highest tested concentration (identifiers redacted).

However, upon closer examination, it was found that the true circumstances were different from what was initially assumed. As depicted in Figure 22b, at the maximum tested concentration of redacted peptides from Group 2, the peptide–siGLO complexes were observed to accumulate within the cytoplasm of the cells. Importantly, this accumulation did not hinder the growth and proliferation of the HFF-1 cells, unlike what occurred with the malignant HCT116 and Caco-2 cell lines. However, the current analysis is limited by the resolution of the imaging used, which does not allow for detailed subcellular localisation or comparative quantification of peptide accumulation. High-magnification imaging in future studies could provide more insights into these differential interactions, enabling a clearer understanding of peptide behaviour within HFF-1 versus HCT116 and Caco-2 cells.

REDACTED – Experimental transfection data for Group 3 peptides.

*Figure 23a: Percentage of HFF-1 cells positive for W2 staining following treatment with redacted peptides from Group 3 (including G3) across redacted concentrations. Data represent the mean $\pm$ SD from three independent biological repeats, each with four technical replicates. Statistical analysis was performed using two-way ANOVA with multiple comparisons. Significance levels: $p < 0.05$ (), $p < 0.01$ (), $p < 0.001$ (), $p < 0.0001$ (****); non-significant comparisons are not shown. (Peptide identities and concentration values have been redacted.)*

For Group 3 (Figure 23a), redacted peptides at redacted concentrations significantly outperformed G3 in W2 detection ($P < 0.01$ – 0.001), and were also significantly higher than other redacted peptides ($P < 0.001$ – 0.0001). However, this trend reversed at higher concentrations, where W2 levels declined across all peptides, with no statistically significant differences between them. This concentration-dependent drop may reflect peptide aggregation, sequestration at the membrane, or saturation effects, as supported by microscopy findings (Figure 23b), where peptides were observed clustering around cells, suggesting possible false positives from surface-localised siGLO particles (see discussion).

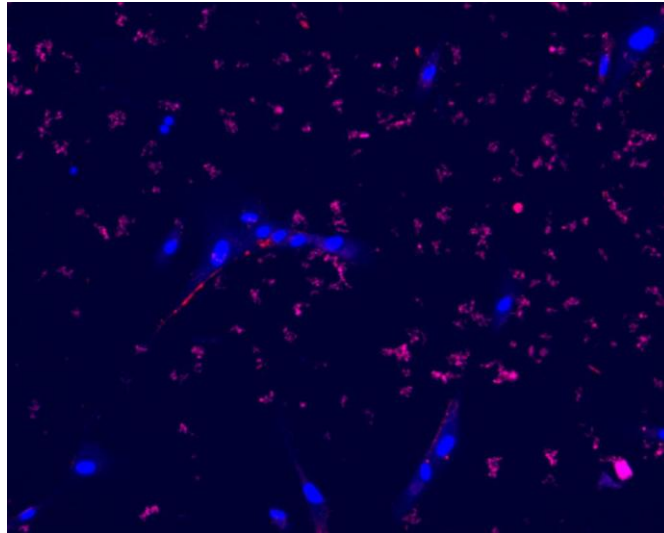


Figure 23b: Peptide-siGLO complexes observed coating the HFF-1 cells at a redacted concentration.

The two-way ANOVA performed on this dataset confirmed a significant effect of concentration on W2 uptake ($P < 0.0001$), while the effect of peptide type also reached statistical significance ($P = \text{redacted}$). Although this justifies rejection of the null hypothesis, the magnitude of this difference was substantially weaker compared to that seen in the cancer cell lines, highlighting the limited and inconsistent peptide uptake in normal HFF-1 cells.

DISCUSSION

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Conclusion/future work

Considering all the findings from this study, one peptide within the series emerged as the most promising candidate for further research, particularly if only a single sequence were to be prioritised. However, the data obtained only partially confirm the initial hypothesis that a histidine-containing peptide, based on the reference sequence, would be the most effective. While one modified peptide demonstrated strong binding affinity and selective cytotoxicity in colorectal cancer cells, another peptide with histidine residues positioned differently showed a distinct efficacy profile. This suggests that both sequence modification and residue positioning play a crucial role in determining peptide performance, warranting further investigation across the wider group.

To build on these findings, future research should explore in vivo studies using tumour-bearing immunocompromised mice to evaluate the therapeutic potential of these peptides, as such models are commonly used to test novel anticancer peptides, particularly in patient-derived xenograft (PDX) models, which offer a more physiologically relevant environment to assess tumour response (Hidalgo et al., 2014). In vivo stability and pharmacokinetics remain critical challenges in peptide-based therapies due to enzymatic degradation and rapid clearance from circulation (Veronese and Pasut, 2005) and strategies such as cyclisation, PEGylation, and the

incorporation of D-amino acids or non-natural amino acids have been shown to enhance peptide stability and half-life (Werner et al., 2016). These approaches could significantly reduce the necessary therapeutic dose while improving bioavailability.

Additionally, 3D tumour organoid models should be employed to evaluate peptide penetration and efficacy in an environment that better mimics tumour architecture compared to traditional 2D cultures (Fatehullah et al., 2016). The impact of these peptides on the tumour microenvironment should also be investigated, as interactions with extracellular matrix components could influence peptide uptake and cytotoxicity (Anderson and Simon, 2020). This could be particularly relevant for understanding the selectivity of certain sequences towards colorectal cancer cells, as opposed to normal fibroblasts.

Another important consideration for future work is the mechanism of action of these peptides. It remains to be determined whether the cytotoxic effects observed in HCT116 and Caco-2 cells result primarily from cell membrane disruption, intracellular targeting, or induction of apoptosis/autophagy (Xie et al., 2020). Future studies should incorporate flow cytometry to assess apoptosis markers such as Annexin V/PI staining, as well as western blot analysis to evaluate caspase activation pathways (Galluzzi et al., 2018). Such investigations will provide critical insights into whether these peptides are suitable for development into targeted anticancer agents.

Furthermore, the potential for combination and hybrid therapies should be explored. Selected sequences from this study may enhance the efficacy of chemotherapeutic agents by increasing membrane permeability, facilitating drug uptake, or inducing

complementary cytotoxic effects. The growing interest in anticancer peptides highlights the need for multimodal treatment strategies that leverage collaborative effects between peptide-based therapies and conventional chemotherapy (Domalaon et al., 2018).

Overall, the results of this study provide valuable insights into the potential of histidine-modified peptides in cancer therapy. Further optimisation through structural modifications and targeted drug delivery approaches will be key to advancing these peptides towards clinical applications.

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