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Foxy-5 – A first-in-class therapy to prevent cancer metastasis

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Table of Contents

Executive summary

Our Goal: The goal of the project is to develop a complementary anti-metastatic treatment that prevents the dissemination of cancer cells and the subsequent establishment of metastasis in patients with breast, colon and prostate cancer.

Rationale: Clinical analysis of breast, colon and prostate cancer revealed that patients with tumours lacking or having a reduced expression of the endogenous protein WNT5A developed metastasis much earlier than patients with tumours expressing this protein and consequently had a shorter cumulative survival than those with tumours that expressed this protein. To reconstitute the effects of WNT5A in patients lacking the expression of this protein, the small WNT5A mimicking Foxy-5 peptide was developed. In animal experiments, Foxy-5 was demonstrated to have a very strong anti-metastatic effect, supporting its development as an anti-metastatic drug candidate in humans.

Scientific Evidence: Analysis of clinical tumour samples from patients and *in vitro* work with tumour cell lines revealed that the major effect of WNT5A was to impair migration and invasion of the tumour cells with little or no effects on tumour cell proliferation and survival. The fact that the WNT5A protein is unsuitable for systemic *in vivo* reconstitution of WNT5A signalling and function in tumours led to the development of the small WNT5A mimicking peptide Foxy-5. The Foxy-5 peptide has been shown *in vitro* to mimic the signalling and functional effects of the WNT5A protein in different tumour cells. These results led to studies testing its anti-metastatic activity *in vivo* in different mouse models. In these studies, in accordance with our previous *in vitro* data from tumour cell lines, we found that Foxy-5 significantly impaired the formation of metastasis from primary tumours as well as from tumour cells directly injected into the blood and that it also reduced the number of cancer stem cells.

Rationale for Clinical Studies: Our *in vitro* and *in vivo* scientific evidence clearly demonstrates that Foxy-5 mimics WNT5A's ability to prevent metastases by playing a pivotal role in the initial as well as the later stages of the metastatic process. Approximately 90% of all cancer deaths are directly related to metastases and this, therefore, meets a critical unmet need. Based on our pre-clinical studies we decided to progress Foxy-5 into clinical development with the aim to realise WntResearch's vision that cancer patients will no longer have to face metastasis.

Overall Status and Significance: WntResearch has completed two phase 1 studies with Foxy-5 and have an active phase 2 study ongoing in Spain and Hungary. The phase 1 first-in-man dose and phase 1b escalation studies with 48 patients with metastatic breast, colon or prostate cancer underlined that Foxy-5 is safe and well tolerated up to the highest dose tested, 2.3 mg/kg. Foxy-5 was detectable in the patient blood for the entire treatment cycle, indicating that the drug has adequate stability and half-life. The finding that Foxy-5 is non-toxic is of prominent importance, as it provides promising possibilities for combining anti-metastatic Foxy-5 treatment with conventional cancer treatments primarily targeting tumour growth. The 1.8 mg/kg dose was selected for further clinical investigation to maximize the potential for Foxy-5. The current phase 2 study is designed to obtain a proof of principle for Foxy-5 to prevent metastasis formation in patients with stage IIc/III colon cancer with a maximum of three affected lymph nodes and no sign of distal metastases. The primary outcomes are safety, tolerability and recurrence of disease. The latter is measured using circulating tumour (ct)DNA as a proxy. The secondary endpoints include recurrence-free survival as determined by traditional imaging techniques and eventually overall survival. Meeting one or several of these endpoints in conjunction with Foxy-5's excellent toxicity profile will provide key clinical evidence for Foxy-5 representing a paradigm shift in cancer treatment, which will provide clinicians with a novel strategic advantage in the treatment of cancer.

Key benefits of Foxy-5

- Foxy-5 is a first-in-class drug candidate selectively targeting the formation and prevention of cancer metastases.
- Foxy-5 significantly decreased the ability of WNT5A low-expressing cancer cells to migrate and invade. Studies with Foxy-5 in WNT5A-low human breast cancer cells showed that Foxy-5 increased adhesion of the cells to collagen in a dose-dependent manner.
- In mouse models of breast cancer metastasis, Foxy-5 treatment by orthotopic injection resulted in the following:
    - a reduction in liver metastasis by approximately 70%,
    - a reduction in lung metastasis of up to 90%.
- Likewise, in a mouse model of prostate cancer, Foxy-5 significantly reduced metastatic spread to regional and distant lymph nodes by 90% and 75%, respectively.
- Foxy-5 not only affects metastatic spread from the primary tumour but also has an anti-metastatic effect on circulating tumour cells:
    - there was a more than 50% reduction in lung metastasis when human breast cancer cells were injected into the tail vein.
- Foxy-5 treatment of mice with inoculated human colon cancer cells induces a reduction in the number of colon cancer stem cells in the cancer tissue. This reduction indicates an additional mechanism whereby Foxy-5 counteracts relapse and the formation of metastasis.
- Foxy-5 treatment has been shown to be non-toxic and safe in the completed toxicology programme in animals and human phase 1 trials. Foxy-5 is a safe and well-tolerated drug with no dose-limiting toxicities observed at any of the doses tested.
- Foxy-5's pharmacokinetic profile in humans displays a half-life of approximately 5-7 hours and no drug accumulation.
- Foxy-5 can be administered together with relevant anti-cancer therapeutics to prevent the metastatic process, complementing therapeutic approaches primarily targeting tumour growth:
    - Foxy-5 treatment can be combined with conventional cancer treatments, including chemotherapy and tamoxifen, as suggested from *in vitro* interaction studies.
    - Foxy-5 has no negative effect on the tumour suppressor activity of checkpoint inhibitors.

WHITE PAPER

Foxy-5 – A first-in-class therapy to prevent cancer metastasis

Cancer is a disease affecting millions of people worldwide with 18.1 million new cases and 9.6 million cancer deaths in 2018[1]. The primary tumour is not the dominant cause of death of patients with cancer. Cancer-associated mortality in the majority of cases is the result of cancer cell dissemination to other organs, that is, the metastatic process[2]. Current adjuvant treatment of patients with cancer includes chemotherapeutic drugs, endocrine treatment, targeted therapies and novel checkpoint inhibitors[3]. These treatment modalities primarily target the increased proliferation and survival of cancer cells and thus the growth of the cancer. None of the current treatment modalities are designed to directly target cancer cell dissemination or cancer stem cells, both of which are essential for metastatic spread and relapse of the disease. Chemotherapy is currently the most commonly used therapy for high-risk patients after removal of their primary tumour. Unfortunately, a large proportion of patients eventually develop relapse of their cancer disease in the form of metastatic spread, indicating a significant deficiency in treatment efficacy[I]. It is envisioned that any treatment specifically targeting cancer dissemination will be an excellent complement to the presently available anti-proliferative and anti-survival treatment options of cancer cells. Thus, neo-adjuvant and peri-operative therapy directed towards the cancer cell dissemination process and/or directed towards reducing the number of cancer stem cells would be expected to result in a significantly improved quality of life and cancer patient survival with a large impact on the lives of millions of people.

This white paper presents Foxy-5, a WNT5A mimic, as a drug candidate to prevent cancer metastasis and thus relapse of the disease and covers the background, the mechanisms of action, pre-clinical development and safety profile as well as prior, on-going and planned clinical studies to obtain human proof of principle for the anti-metastatic property of Foxy-5.

[1] For example, for colon cancer diagnosed at a localized stage (stage I), the 5-year survival rate is 90%. For stages II and III, the 5-year survival rate is 71%. For stage IV (metastasized to distant sites), the 5-year survival rate drops drastically to 14%[Table 6.13 38].

The role of WNT5A in cancer metastasis

WNT5A mechanism of action

WNT5A is a protein expressed in many different normal and non-cancer transformed cells in the body, including both epithelial and blood cells[4,5]. The WNT5A protein is a member of the WNT family of secreted lipoglycoproteins. The secreted WNT5A protein/ligand has been reported to bind to and activate primarily members of the non-conventional G-protein-coupled receptor family of the Frizzled (FZD) family but also ROR and Ryk tyrosine-kinase receptors[5]. Depending on the WNT family ligand, receptor(s) activated, and the cellular context, the downstream signalling events are characterized as either β-catenin dependent (also referred to as canonical) or β-catenin independent (also referred to as non-canonical)[5,6]. WNT β-catenin-dependent signalling is triggered primarily by the WNT1 and WNT3A ligands (see the left and white part of Figure 1). These ligands impair the phosphorylation and degradation of β-catenin, enabling its downstream signalling via the β-catenin-dependent pathway. In cancer cells, essential components of this signalling pathway are frequently mutated, leading to constitutive and elevated activation of this pathway that results in cancer transformation, cancer cell proliferation and expansion of the cancer stem cell niche[7]. In contrast, the WNT5A protein is characterized as a non-transforming ligand that mediates its effects via the β-catenin-independent signalling pathway[5,6]. The above distinction between the two distinct WNT signalling pathways (Figure 1) is crucial since the absolute majority of drug development relates to identifying and characterizing different inhibitors of WNT β-catenin-dependent signalling (see the left and white part of Figure 1).

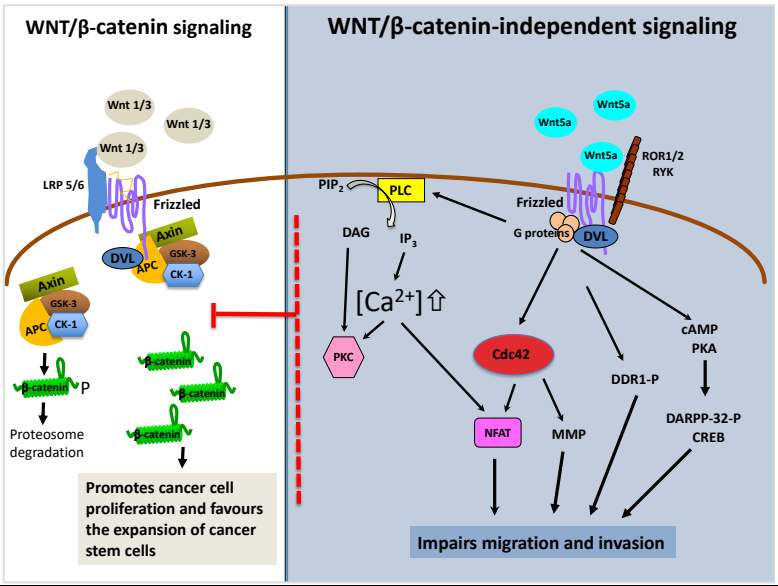


Figure 1. This schematic drawing outlines the two principally different WNT signalling pathways. The white field to the left outlines the β-catenin signalling pathway towards which most antagonistic drug candidates are directed. The blue field to the right outlines the β-catenin-independent signalling pathway towards which our unique agonist is directed. References relating to the blue field[4,5,8,11, 20,25,30,32,39].

This approach is different from the present project, where WntResearch investigates the use of a WNT5A mimicking small peptide to serve as an anti-metastatic and complementary treatment option in breast, colon and prostate cancer, where the endogenous expression of WNT5A is often lost or significantly reduced. In these types of cancer cells, WNT5A does not cause activation of the β-catenin-dependent pathway; if anything, it causes minor inhibition of this pathway, as indicated by the red lines at the interaction between the two pathways[4,8,9,10]. The broken line indicates a mechanism of action (MoA) that is not distinct but appears to involve several different means depending on the cell type and context.

WNT5A and the risk of disease recurrence

In patients with breast cancer, loss of WNT5A expression in breast cancer cells has been correlated with a high risk of disease recurrence emerging as metastasis several years after the initial treatment, resulting in a shortened survival time for the patient[5,11,12]. A similar association between low WNT5A protein expression in tumours and disease outcome has been described for stage II colon cancer[6,8,13]. In one of these studies, the median survival time after diagnosis was approximately 110 months for patients

with WNT5A-positive tumours but less than 60 months for those with WNT5A-negative tumours[13]. In surgically removed prostate cancers, low expression levels of WNT5A protein have been correlated with a significantly worse outcome for patients (shorter recurrence-free survival)[14,15]. Summarized data from these studies are presented in Figure 2. There are also indications that WNT5A may act as a tumour

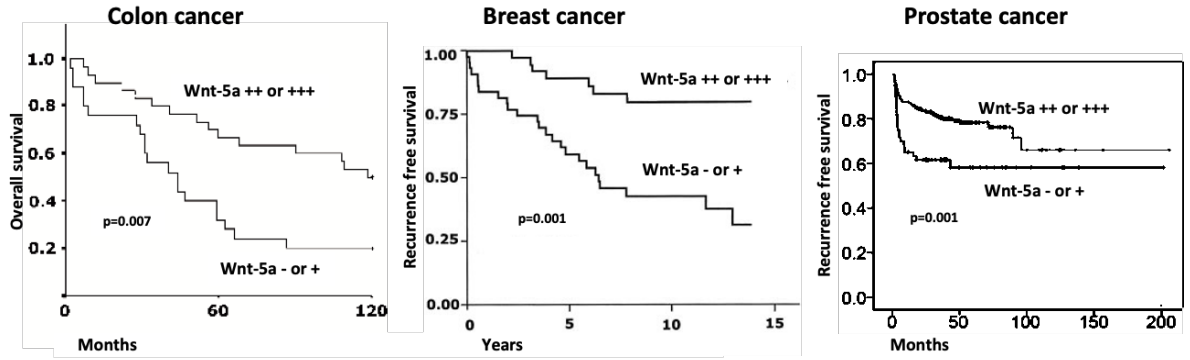


Figure 2. A high expression of the WNT5A protein in Dukes B colon, ductal breast, and prostate cancers correlated with an increased recurrence-free survival (time without metastases) or overall survival of the patient[11,13,14]. Expression level of WNT5A protein is expressed with (-) or (+) for no or low expression levels and (++) or (+++) for near normal or normal expression levels.

and metastasis suppressor in several other cancer types, such as ovarian cancer, thyroid cancer, liver cancer and lymphoid malignancies[16–19]. The evident association between the expression of WNT5A protein and spreading of the tumour to lymph nodes and distant organs leads to the hypothesis that restoring WNT5A signalling in patients with any of the above cancer types and with low WNT5A levels in their cancer cells will decrease the appearance of metastasis and thereby increase their quality of life and prolong their survival.

In line with this hypothesis, in experiments with cancer cells, WNT5A signalling has been shown to inhibit migration and invasion in WNT5A-low breast cancer[20,21,22,23], colon cancer[6,13] and prostate cancer cells[13,22]. As a good example of these effects, we outline the response to recombinant WNT5A of colon cancer cell migration (Figure 3 and Figure 4).

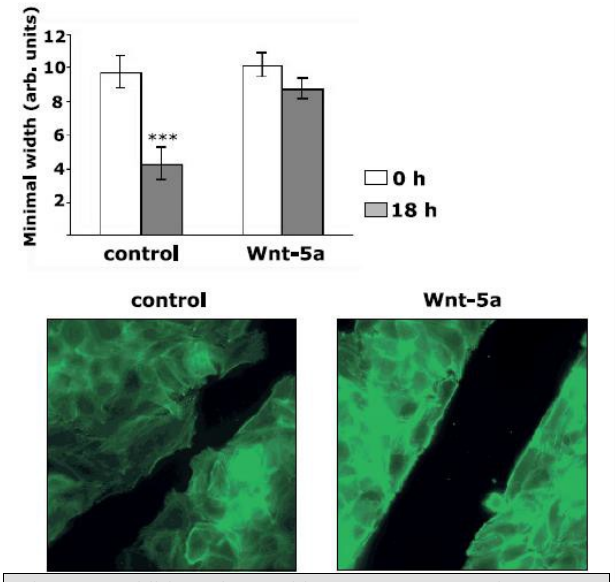


Figure 3. Addition of recombinant WNT5A to colon cancer cells inhibited migration of the cells in a wound-healing assay[13]. Statistical difference, *** p<0.001.

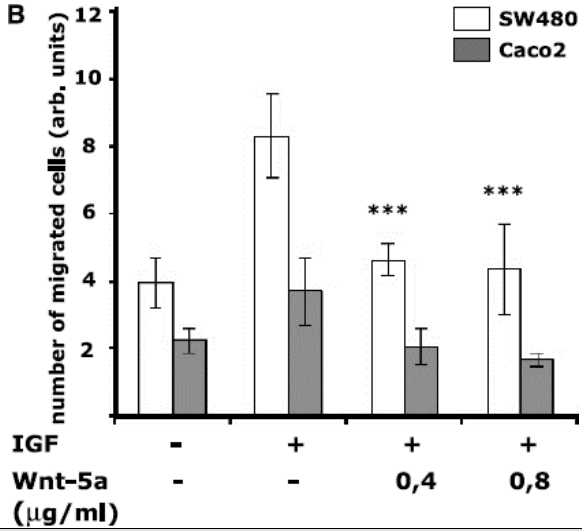


Figure 4. Addition of recombinant WNT5A to two different colon cancer cell lines inhibited migration of the cells[13]. Statistical difference, *** p<0.001.

Mechanisms explaining how WNT5A can reduce disease recurrence

In a study of a clinical breast cancer cohort, we did not find a correlation between the expression of WNT5A and the proliferation marker K_i67[12], indicating that the primary effect of WNT5A on breast cancer recurrence and survival is not caused by its effect on cancer cell proliferation. Instead, it has been documented *in vitro* that WNT5A-induced non-canonical signalling events lead to increased adherence of a cell to its neighbouring cells[24] as well as increased adhesion to the surrounding connective tissue components[25,26] resulting in a decreased ability of the cell to migrate. In breast, colon and prostate cancer cells, the interpretation is that loss of WNT5A *in vivo* increases their ability to detach, migrate and eventually invade lymph and blood vessels. For example, in normal breast epithelial cells as well as in certain tumours, WNT5A is significantly expressed, and due to its auto- and paracrine signalling properties, it has been shown to secure firm adherence between the cells and the basement membrane[24,25]. It has also been shown that recombinant WNT5A impairs the release and activity of matrix metalloprotease 9 (MMP-9) from breast cancer cells[22]. MMPs are enzymes that are capable of degrading all kinds of extracellular matrix proteins, and therefore, their increased presence and activities in tumour tissues are linked to cancer progression and metastatic spreading. Consequently, the ability of WNT5A to impair the release and activity of MMP-9 from breast cancer cells[22] will restrict the migration and invasion of these cancer cells.

Furthermore, overexpression of WNT5A in breast cancer cells has been shown to suppress the number of cancer stem cells in a mouse model, suggesting that this is another important mechanism of the tumour-suppressing function of WNT5A[25]. Although this study did not use a therapeutic approach, it is interesting since the presence of cancer stem cells has been positively correlated with recurrence of a previously surgically removed and treated cancer[26,27].

The discovery of Foxy-5

Recombinant WNT5A is a large protein with a MW of 38,000 Da that is significantly post-translationally modified and possess a high affinity domain for binding to heparin sulphate proteoglycans (linear polysaccharides) that are found on mammalian cell surfaces. This means that recombinant WNT5A will have a limited distribution once it enters the body and thus has a small chance of reaching the tumour cells, making it unsuitable as a drug candidate. Foxy-5 was invented and developed to circumvent the problems associated with administrating WNT5A directly to patients and was developed in order to provide a suitable drug candidate that mimics the signalling and functional effects of WNT5A. When a protein is in its three-dimensional structure, some parts of the amino acid chain are hidden within the core of the protein, and some parts of the amino acid chain are presented on the surface of the protein, exposing the solvent. The amino acid parts exposed on the surface are more likely to be the regions where the protein binds a receptor and mediates its action. With the aim of finding a peptide with the ability to mimic the functional effects of WNT5A, an *in silico* prediction of the human WNT5A protein segments likely to be externally exposed was performed.

The 14 initial peptides originating from this prediction of the WNT5A structure were screened for their ability to induce activation of the collagen-binding receptor called DDR1, increase adhesion and decrease migration of WNT5A-low breast cancer cells, all known effects of the WNT5A protein[5,22,25]. These screenings identified a 12-amino-acid lead candidate (corresponding to amino acids 311-322 of the intact WNT5A protein) with the capacity to mimic the effect of recombinant WNT5A on the adhesion and migration of WNT5A-low breast cancer cells[20]. The lead candidate amino acid sequence is strongly conserved between species, being completely identical in human, dog, mouse, rat and even salamander WNT5A, which further strengthens the belief that this is an important part of the protein[20]. The lead candidate was, thereafter, sequentially shortened by 2 amino acids from the *N*-terminal side to identify the smallest possible peptide with a WNT5A mimicking effect on adhesion. Peptides consisting of 10 and 8 amino acids remained effective, but the six-amino acid peptide was ineffective[20]. This inactive six-amino-acid peptide had a methionine as its N-terminal amino acid. In bacteria, protein synthesis is often initiated with a formylated methionine, and when the protein is about ready, this initial part of the protein is released, thus resulting in different formylated small peptide fragments inside the bacteria[28]. During a bacterial infection, these peptides are released and activate white blood cells at the site of inflammation by binding to seven transmembrane receptors of the same class that WNT5A binds to. These formylated peptides released from bacteria are active at the site of inflammation with high protease activity and low pH, a milieu similar to that of cancer tissue[29]. Furthermore, if the formyl group from these peptides is experimentally removed, the effects of these peptides are reduced by three to four orders of magnitude. We explored the effects of such a modification of the N-terminal methionine of our non-effective six-amino-acid peptide and found that this formylation of the N-terminal methionine (Figure 5) completely restored and increased the ability of the six-amino-acid peptide to induce adhesion of breast cancer cells to collagen[20]. The peptide was named Foxy-5, which is a truncation of FOrmylated heXapeptide derived from the WNT5A sequence.

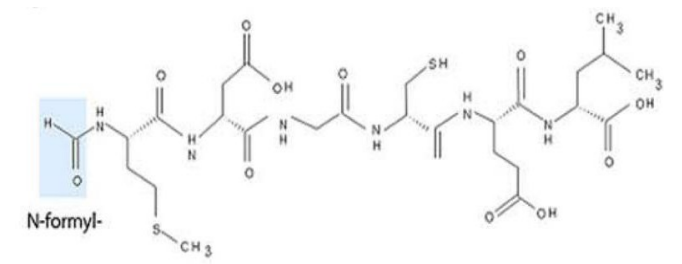


Figure 5. The Foxy-5 hexapeptide sequence (Met-Asp-Gly-Cys-Glu-Leu) with the formyl group at the N-terminal (indicated by the blue area at the left) has a Mw of 695f Da, which is 1.8% of the recombinant WNT5A molecule.

Foxy-5 mimics WNT5A's effects *in vitro*

Inducing WNT5A signalling

Receptor engagement

The WNT5A protein is known to activate different types of receptors, including members of the Frizzled family of seven-transmembrane receptors as well as the tyrosine kinase receptors ROR and Ryk[5]. To investigate whether the Foxy-5 peptide and the WNT5A protein used the same receptor for their effects on migration, we employed a WNT5A-low human breast cancer cell line.

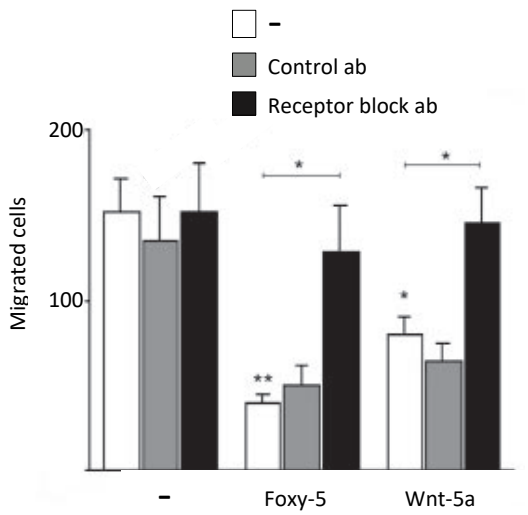


Figure 6. The effect of Foxy-5 and that of recombinant WNT5A on breast cancer cell migration were not affected by a control anti-Frizzled 2 antibody but were affected by an anti-Frizzled 5 antibody[20]. Statistical significance, *p<0.05, **p<0.01.

With the intention of further verifying that the functional effect of Foxy-5 is mediated through a specific Frizzled receptor, migration studies in the presence of an antibody specifically blocking the Frizzled 5 receptor were performed. In the presence of a Frizzled 5 receptor blocking antibody, the effect of both WNT5A and Foxy-5 on the migration of WNT5A-low breast cancer cells was abolished (Figure 6). A control antibody against the ectodomain of the Frizzled 2 receptor did not influence the effect of either WNT5A or Foxy-5 on migration[20]. These experiments not only support the conclusion that Foxy-5 mimics the effect of WNT5A on intracellular signalling but also that Foxy-5 mediates its effect via a receptor that is also utilized by WNT5A.

Intracellular signalling

As previously outlined on page 5, the binding of WNT5A to its receptor(s) triggers the activation of several intracellular signalling pathways. One of the first intracellular signals is a short-lived increase in the intracellular free calcium concentration, a so-called calcium signal. This calcium signal then participates in triggering downstream signalling events that will further promote other downstream signalling events, including activation of protein kinase C (PKC) and NFAT[30]. These signals are then responsible for the changes in cellular activities. As a means to investigate whether Foxy-5 can mimic the effects of WNT5A concerning signalling events in the cell, the ability of Foxy-5 to generate a calcium signal was investigated. As shown in Figure 7, both recombinant WNT5A and

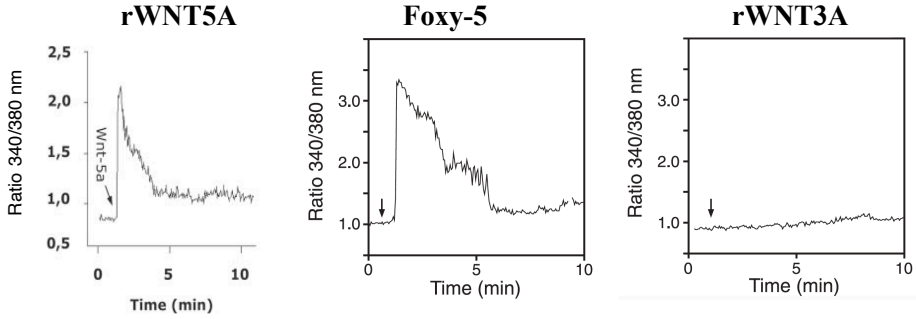


Figure 7. Addition of recombinant WNT5A or Foxy-5 induced a prompt intracellular calcium signal in breast cancer cells that is in contrast to the addition of recombinant WNT3A, an activator of the β-catenin signalling pathway[20,30].

Foxy-5 generated a prompt calcium signal in breast cancer cells (as well as in colon cancer cells[8]), suggesting that Foxy-5 indeed activates the Frizzled 5 receptor and therefore mimics the intracellular signalling of WNT5A.

In addition to our previous finding that Foxy-5 did not cause activation of β-catenin-dependent WNT signalling in breast cancer cells[20], we also demonstrated that both recombinant WNT5A and Foxy-5 can cause minor inhibition of β-catenin signalling in colon cancer cells[8] (Figure 8; indicated by the red lines in Figure 1, p.5). Accordingly, these findings provide evidence that the pharmacological effect of Foxy-5 is related to the same principle of action as that of WNT5A.

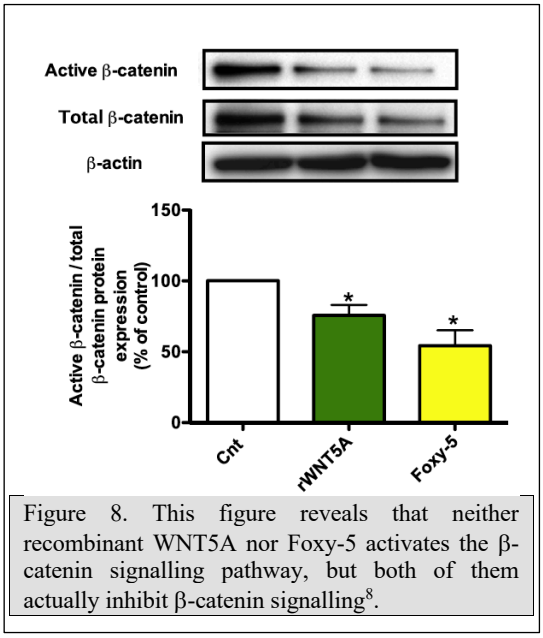


Figure 8. This figure reveals that neither recombinant WNT5A nor Foxy-5 activates the β-catenin signalling pathway, but both of them actually inhibit β-catenin signalling[8].

Decreased migration and invasion

Having found that Foxy-5 mimicked the receptor engagement and signalling properties of WNT5A, we next investigated the ability of Foxy-5 to decrease the migration and invasion of cancer cells. To analyse the effect on the migratory and invasive capacities, WNT5A-low human cancer cells were allowed to migrate through either uncoated (migration; Figure 6, p.9)[20,21,23] or Matrigel/collagen pre-coated (invasion)[14,21,31] cell culture inserts with 8-µm pore size membranes. In the latter experiments, not only the migratory capacity but also the capacity to secrete enzymes that breakdown extracellular matrix components is tested. The analysis showed that Foxy-5 significantly decreased the ability of WNT5A low-expressing cancer cells to migrate and invade[14,20,21,23,31] (Figure 6, p.9). These results were the first reported experiments revealing that Foxy-5 was able to mimic the ability of WNT5A to inhibit the migration and invasion of cancer cells in an *in vitro* setting. These findings are essential since migration is considered an essential step in the metastatic process[5]. These clear findings generated the important question as to what are the mechanisms behind the observed inhibitory effect of WNT5A/Foxy-5 signalling on cancer cell migration and invasion?

Increased adhesion

To form metastases in lymph nodes and distant organs, an essential and initial step is a decrease in the adhesive property of the cancer cells to neighbouring cells and proteins, in particular collagen, present in the basement membrane and in the extracellular matrix. WNT5A is known to increase the adhesion of breast cancer cells to surrounding cells[24] and to collagen structures present[25,26], which readily explains how WNT5A reduces migration and invasion of these cancer cells, which can then explain the more limited formation of metastases in patients with high WNT5A expression in their tumour cells[11,12,32] as well as in colon[8,13] and prostate[14,15] cancer. Therefore, the ability of Foxy-5 to affect the adhesion of cancer cells was an important aspect to investigate. Studies with Foxy-5 in WNT5A-low human breast cancer cells showed that Foxy-5 increased adhesion of the cells to collagen in a dose-dependent manner (Figure 9). Thus, Foxy-5 can restore firm adhesion to collagen in breast cancer cells lacking WNT5A, making it quite plausible that this effect contributes to the ability of Foxy-5 to impair the migration and invasion of these cells (Figure 6, p.9)[20].

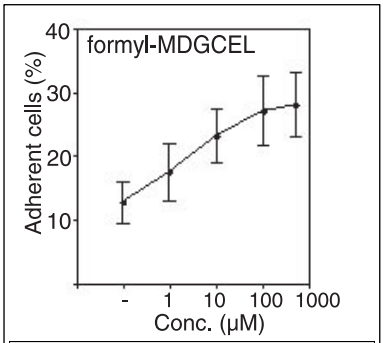


Figure 9. Increasing concentrations of Foxy-5 resulted in the increased adhesion of WNT5A-low human breast cancer cells[20].

Reduced release of matrix metalloproteases

Apart from increasing the adhesion of breast cancer cells to one another and to collagen structures in the basement membrane and in the extracellular matrix, it has been shown that WNT5A signalling can reduce the expression and activity of MMP-9 in human breast cancer cells[22]. This finding adds an important mechanism whereby WNT5A signalling can hinder tumour metastatic dissemination of cancer cells since MMPs are enzymes that are capable of degrading all kinds of extracellular matrix proteins, and, therefore, their increased presence and activities in tumour tissues are linked to cancer progression and metastatic spreading. It can be proposed that the WNT5A mimicking peptide Foxy-5 most likely impairs the production and release of MMPs responsible for the degradation of basement membranes as well as structural components of the extracellular milieu that would otherwise hinder the invasion of the cancer cells[22].

Normalization of cancer cell metabolism

It is generally known that tumour cells often develop an altered cell metabolism that includes an increased production and release of lactate. From a tumour cell invasive and metastatic perspective, this is interesting since the presence of lactate drives tumour cell migration and invasion[23]. It has been found that both recombinant WNT5A and Foxy-5 normalize the altered metabolism in breast cancer cells and thus reduce the production and release of lactate. This was explained by their abilities to downregulate the expression of the glycolytic enzyme phosphofructokinase platelet-type and possibly by their abilities to reduce the expression of the membrane bound lactate transporter mono-carboxylate transporter-1[23]. Another important finding in this report was that the drug candidate Foxy-5 could impair not only the basal migration of breast cancer cells but also the migration induced by the addition of exogenous lactate (Figure 10). This means that our drug candidate is still active in a tumour environment with a reduced pH. Thus, we have identified an additional mechanism whereby Foxy-5 can contribute to the reduction in cancer cell migration and invasion and that Foxy-5 also functions at a low pH, such as that often encountered in and around a tumour[23].

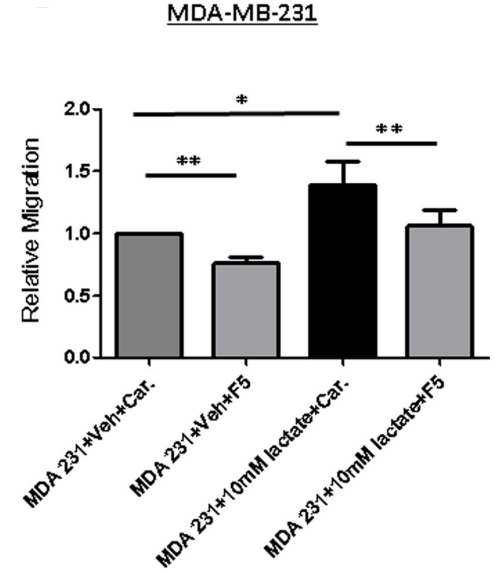


Figure 10. Foxy-5 impairs the migration of MDA-MB-231 breast cancer cells even in the presence of exogenous lactate[23].

Reduction in cancer stem cell promoting signals

The presence of cancer stem cells is positively correlated with the recurrence of a previously surgically removed and treated cancer[26,27]. This means that any substance that has the ability to reduce the number of cancer stem cells, known to be resistant to chemotherapy, would delay and hinder recurrence that presents itself as metastatic spread of the original cancer disease. We had a suspicion that Foxy-5 might have such an anti-cancer stem cell property since we had previously shown that Foxy-5 could have a limited effect on β-catenin signalling and the generation of prostaglandin E_2 (PGE_2)[8], both known promoters of the cancer stem cell niche in colon cancer. Interestingly, a reduction in PGE_2 signalling was predicted by the ability of WNT5A and Foxy-5 to increase the expression of 15-PGDH, which is often downregulated in colon cancer tissue and is responsible for the degradation of PGE_2, as well as by the ability of WNT5A and Foxy-5 to reduce the expression of COX2, which is often upregulated in colon cancer tissue and is responsible for the generation of PGE_2.

Anti-metastatic effects of Foxy-5 in animal models

Up to this point in its development, Foxy-5 has shown promising potential to become an anti-metastatic treatment based on the results from experiments performed *in vitro* on cancer cells. However, demonstrating proof of concept of an anti-metastatic effect can only be done in an animal model. The limited distribution of the full-length native WNT5A protein hinders it from effectively reaching tumour cells *in vivo*. Therefore, WNT5A is not suitable as a drug or for use in comparative *in vivo* animal studies with Foxy-5. Thus, the following *in vivo* experiments were performed with Foxy-5 alone.

Breast cancer models

The ability of Foxy-5 to inhibit the metastatic spread of cancer cells was primarily tested in a syngeneic mouse model of breast cancer metastasis[21]. As the WNT5A sequence from which Foxy-5 originates is completely identical in mouse and human WNT5A[20], the effect of Foxy-5 can also be studied in a mouse model.

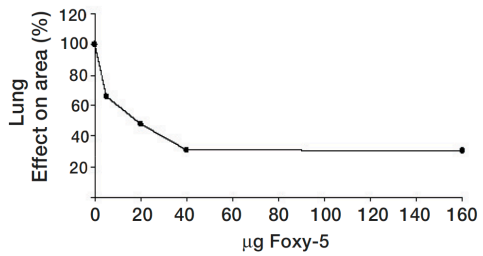


Figure 11. Effects of different doses of Foxy-5 on lung metastases in the syngeneic mouse breast cancer model. Forty micrograms equals approximately 2 mg/kg[21].

In this model, murine WNT5A-low breast cancer 4T1 cells were inoculated into the mammary glands of normal mice, resulting in the formation of primary breast tumours and subsequent metastasis to the liver and lungs. Each mouse was treated with vehicle alone (PBS; control), a formylated control hexapeptide or Foxy-5 administered intraperitoneally every fourth day. For these initial studies, we had no scientific data to guide the determination of 1) when the treatment would be started, 2) how often it should be given and 3) for how long. Based on other related studies and our understanding of the MoA, we decided in this first study to initiate the treatment directly after the inoculation of the tumour cells and then repeat it every fourth day for approximately three weeks[21]. Before the actual experiments, we performed an experiment to decide a suitable dose level (Figure 11). Based on these dose experiments, we decided to employ 40 µg of Foxy-5 for each treatment since it was the lowest dose with maximal effect (Figure 11). This approach resulted in a reduction in liver metastasis by approximately 70% and lung metastasis by up to 90% compared to the control treated animals (Figure 12). In this study, we also investigated the effect of Foxy-5 on the metastatic spread of the murine cell line 4T1 breast cancer cells when injected into nude mice. The results we obtained here were similar to those found in the above described syngeneic set-up[21]. As the main mechanism of Foxy-5 found in *in vitro* studies is inhibiting the migration and invasion of breast cancer cells, no effects were anticipated on tumour growth or tumour cell survival. As expected, Foxy-5 did not show any or only minute effects on the growth of the primary tumour, the weight of the animals, or the number of breast cancer cells undergoing programmed cell death (apoptosis) or proliferation[21]. Based on these results, we concluded that the main mechanism whereby Foxy-5 inhibits the formation of metastasis is via reduced tumour cell migration and invasion.

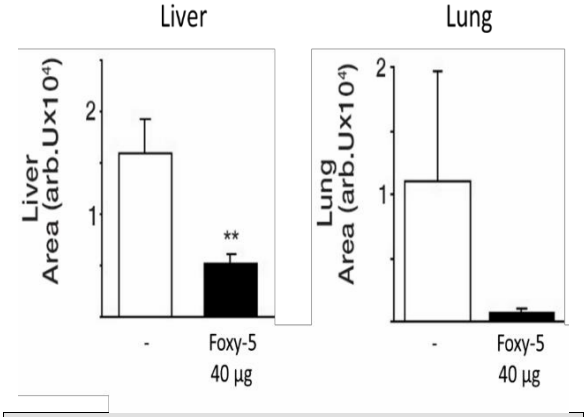


Figure 12. Effect of 40 µg of Foxy-5 on liver and lung metastases in the syngeneic mouse breast cancer model[21].

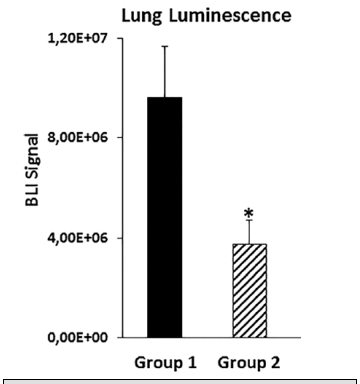


Figure 13. Effect of 40 µg of Foxy-5 on the lung metastases of human breast cancer cells injected into the tail vein[5].

In addition to the above metastatic data that were based on orthotopic injection of the cancer cells into a mammary fat pad of the mouse, we also tested the effect of Foxy-5 on breast cancer cells injected directly into the tail vein. If Foxy-5 would lack effect in such a model, it would then indicate that Foxy-5 only targets the primary tumour to accomplish its anti-metastatic effect. However, to our surprise, we also observed a more than 50% reduction in lung metastasis when human breast cancer cells were injected into the tail vein (Figure 13). In these experiments, we started treatment with 40 µg of Foxy-5 directly after the tumour cells were injected into the tail vein and then continued this treatment every second day for 13 days, after which the experiments were terminated. We conclude from these experiments that Foxy-5 not only affects metastatic spread from the primary tumour but also has an anti-metastatic effect on circulating tumour cells.

Taken together, these results clearly suggest that Foxy-5 is an attractive drug candidate for anti-metastatic cancer treatment, providing that the results can be repeated in a human setting.

Prostate cancer model

Prostate cancer is the most common cancer type affecting men. As previously described on page 5, a high expression level of WNT5A in prostate cancer correlated with a significantly better outcome in patients who had undergone surgical removal of their prostate cancer. Studies of different prostate cancer cells with low expression of endogenous WNT5A revealed that Foxy-5 impaired their invasive capacities[14], as also previously demonstrated for breast cancer cells[5]. To directly test whether the anti-invasive effects of Foxy-5 on prostate cancer cells might correlate to an anti-metastatic spread and thus constitute a promising therapeutic drug candidate for inhibiting metastasis in prostate cancer, we performed a study in a mouse prostate cancer model.

In this model, human prostate cancer cells expressing a low level of WNT5A were inoculated into the prostate of nude mice and allowed to form tumours prior to the start of Foxy-5 (40 µg) treatment. We believe that this approach better mimics the clinical situation where anti-cancer treatment is only initiated after a tumour has been detected. To compensate for the fact that the primary tumours were already established when the treatment was initiated, we administered Foxy-5 every second day in these experiments. The data revealed that Foxy-5 treatment had no effect on cell viability or on the growth of the prostate tumour but significantly reduced metastatic spread to proximal and distant lymph nodes in this model (Figure 14). This *in vivo* proof of concept obtained in a prostate cancer model further emphasizes that Foxy-5 is a promising anti-metastatic drug candidate as a complementary treatment for patients with WNT5A-low prostate cancers.

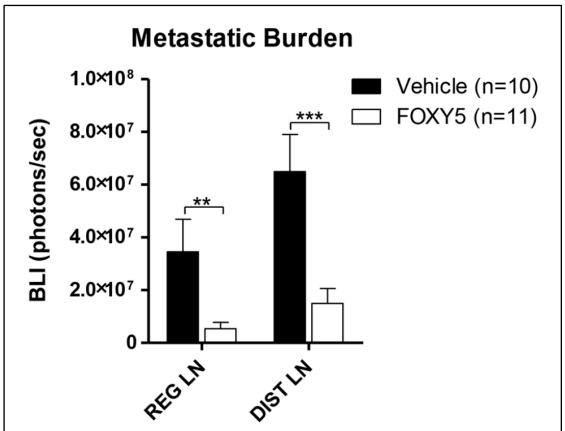


Figure 14. Effect of 40 µg Foxy-5 on metastasis to regional and distal lymph nodes in an orthotopic prostate cancer mouse model[31].

Colon cancer model

Unfortunately, no appropriate model with a reasonable take and an absence of ascites formation was available, meaning that the ability of Foxy-5 to promote colon cancer metastasis could not be properly tested. To study the effect of Foxy-5 on the metastatic process from the initiation of a primary tumour

to the formation of distal metastasis in mice, one has to perform orthotopic injections of the cancer cells into the location where the primary tumour is initiated and grown. These findings need to be done in accordance with our studies of the anti-metastatic effect of Foxy-5 in breast and prostate cancer[21,31].

Although an orthotopic colon cancer mouse model has been described where human colon cancer cells are injected into the caecum, this model is quite impractical to use. The main reason for this is the fact that only 50% of the control animals developed liver metastases. Furthermore, for our experimental approach where we administered Foxy-5 intraperitoneally, there is a problem with this model since 75% of the mice that have had colon cancer cells injected into their caecum develop ascites[33]. In a more recent study where the authors used the mouse colon cancer cell line MC38 in a syngeneic model, the tumour take was only 25%, and only 10% of the injected animals developed liver metastases[34].

Despite the lack of metastatic mouse data, we still believe that the clinical data available for the shorter recurrence-free survival of patients with colon cancer lacking or with low expression of WNT5A[8,13] and the data from experimental work with human colon cancer cells[8,13] make it very likely that Foxy-5 would show an anti-metastatic effect if such animal experiments could have been performed. This conclusion is further supported by the finding outlined below that Foxy-5 affects the number of colon cancer stem cells.

Reduction in colon cancer stem cells

Based on our previous *in vitro* findings that Foxy-5 has the capacity to affect both β-catenin and PGE_2 signalling[8], both of which are known to support the cancer stem cell niche, we next tested whether Foxy-5 could actually reduce the number of colon cancer stem cells in a xenograft mouse model. We found that Foxy-5 treatment for 2 weeks reduced the number of colon cancer stem cells by approximately 30%, as indicated by a reduction in the specific colon cancer stem cell marker DCLK-1 and the general stem cell marker ALDH (Figure 15)[10]. These findings suggest that the observed reduction in colon cancer stem cells might contribute to the tumour suppressive effect of Foxy-5. This finding is in agreement with the finding that overexpression of WNT5A in breast cancer cells also causes a reduction in cancer stem cells[27]. In addition, these data suggest that Foxy-5 can complement the traditional adjuvant chemotherapeutic treatment of colon cancer to which colon cancer stem cells are resistant.

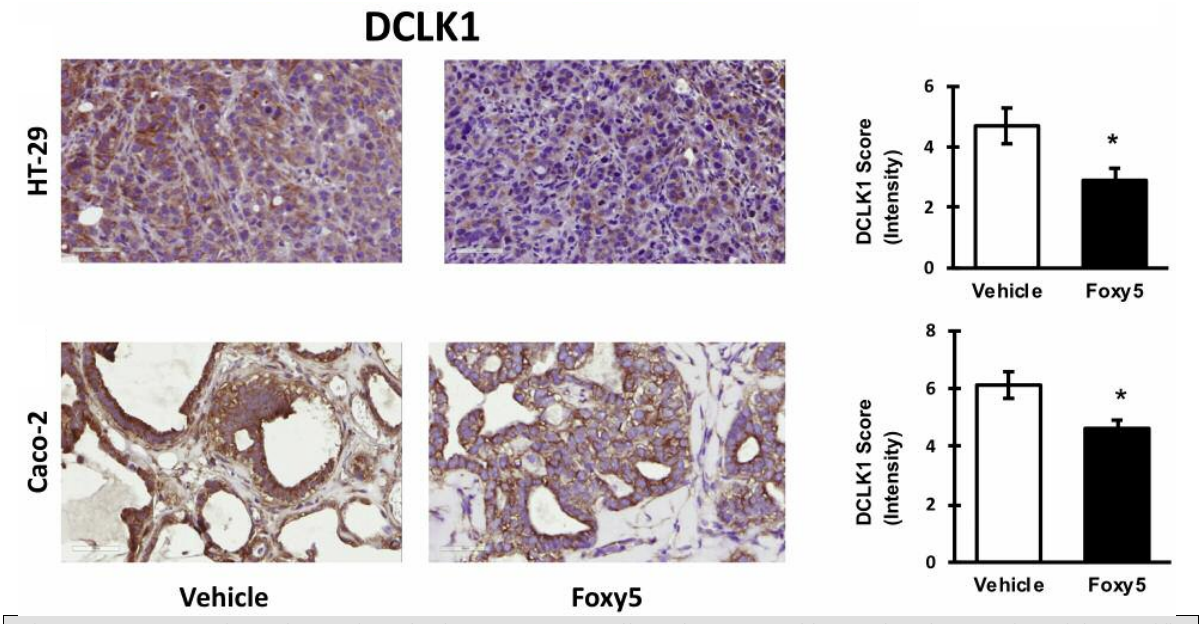


Figure 15. Foxy-5 reduces the number of colon cancer stem cells as demonstrated by a reduced expression of the specific colon cancer stem cell marker DCLK-1 in two different human colon cancer cell lines[10].

Foxy-5 safety profile in pre-clinical toxicology models

The potential toxic effects of Foxy-5 were investigated in a series of studies in rats (dose range finding, 4-week, 6-month, and 4-week supplementation performed prior to phase 1b clinical study) and in dogs (maximum tolerated dose, 4-week, and 9-month). In addition to the traditional elements of toxicology, the four-week study in rats was designed to include elements of safety pharmacology. A determination of the effects on respiration and on the central nervous system, and further a micronucleus test on circulating erythrocytes, was included. It is also important to certify that the drug does not induce genetic changes, since genetic changes may in the long run induce cancer. The mutagenic potential of Foxy-5 was assessed in an Ames test. Notably, the WNT5A sequence from which Foxy-5 originates is completely identical in human, mouse, rat and dog WNT5A[20].

The results from the completed toxicology programme showed that Foxy-5 was very well tolerated with only a few minor observations.

The dose levels selected in initial animal studies (dose range finding (DRF) study in rats and maximal tolerated dose (MTD) study in dogs) were based on the 40 µg/25 g dose shown to be most effective in the pharmacodynamic study in mice and were selected as 1/10, 1 and 10 times this dose level equivalent to 0.08, 0.8 and 8 mg/kg in rats and 0.025, 0.25 and 2.5 mg/kg in dogs (according to the Human Equivalent Dose (HED) Principles).

As the dose levels used in the DRF study in rats and the MTD study in dogs appeared appropriate, the same dose levels were used in the four-week studies and later in the chronic studies (6-month rat and 9-month dog). However, following human clinical exposure, it has been observed that higher clinical doses than those previously qualified in the toxicological programme could be applicable to achieve the desired clinical effect. A 2-week DRF study and a 4-week repeated dose study in rats, with daily intravenous administration of Foxy-5 at dose levels of 8, 24 and 72 mg/kg, were therefore performed to qualify higher doses for human clinical use.

The toxicokinetic properties of Foxy-5 were evaluated following daily administration in all toxicology studies performed. Foxy-5 showed linear kinetics in both species. The initial half-life was found to be rather short (approximately 0.24 hours to 0.40 hours in both species) and non-sex specific, and there were no clear differences between day 1 and at the end of the study in the two species; hence, no accumulation over time was observed.

The potential to cause local irritation at the site of injection was examined in all studies in rats and dogs. No signs of irritation were detected at any occasion; therefore, Foxy-5 intravenous injections are considered devoid of local irritation properties.

In the initial rat study (DRF study), the groups treated with Foxy-5 alone did not exhibit any changes compared to controls.

The initial dog study (MTD study) provided results that could support a suspicion of an effect on the immune system caused by administration of Foxy-5, but similar clinical signs were not observed in any of the following studies and are hence regarded as chance findings not related to treatment with Foxy-5.

Analysis of plasma samples revealed a clear dose response-related exposure of the animals to Foxy-5. Due to the higher doses, a considerably higher exposure than in the earlier studies was reached. No accumulation with time occurred. The plasma concentrations declined post dosing in a bi-phased manner with an elimination half-life comparable to what has been observed in earlier studies.

Interactions with other therapies (*in vitro* and *in vivo*)

Treatment paradigms in cancer are often complex and multidimensional. Foxy-5 is envisioned to prevent the metastatic process and administered together with other anti-cancer therapeutics, complementing their therapeutic approach primarily targeting tumour growth.

Pharmacokinetic interactions

Foxy-5 is a peptide and is expected to follow normal metabolic pathways for proteins, resulting in degradation to individual amino acids. Therefore, the metabolic pathways are generally understood, and classical biotransformation studies performed for pharmaceuticals are not needed for peptides such as Foxy-5. Similarly, as the degradation of Foxy-5 is not expected to interact with processes associated with absorption, distribution, metabolism and elimination of small peptide drugs, pharmacokinetic interactions with other drugs are not expected.

Pharmacodynamic interactions

FOLFOX

Currently, Foxy-5 is administered as neo-adjuvant treatment in patients until the start of FOLFOX (fixed combination of folinic acid, 5-fluorouracil and oxaliplatin). To investigate the potential for an adverse interaction with the treatment mechanism of FOLFOX, the effect of Foxy-5 in combination with FOLFOX on cell viability has been tested *in vitro* using the human colorectal cell line HT29. The combination results revealed that Foxy-5 did not enhance or reduce the inhibitory effect of FOLFOX on cell viability. The IC50 value for FOLFOX in combination with the Foxy-5 peptide was 1.5 μM, which was the same as FOLFOX treatment alone.

Checkpoint inhibitors

The use of checkpoint inhibitors is increasing for a variety of cancer indications. Foxy-5 is currently not used in combination with checkpoint inhibitors. To explore potential adverse interactions, a combination of checkpoint antibody inhibitors of the CTLA-4 and PD-1/PD-L1 classes was tested together with Foxy-5 both *in vitro* and *in vivo*. In both series of experiments, it was shown that there was no negative effect on the tumour suppressor activity of checkpoint inhibitor treatment by simultaneous administration of Foxy-5. Further an *in vitro* study has suggested a potential synergistic effect of Foxy-5 when given in combination with checkpoint antibody inhibitors, an effect that is further explored for different cancer cell types, including tests in functional assays in collaboration with an external research group at Dublin City University.

Tamoxifen

The safety of combining high-dose Foxy-5 therapy with the breast cancer drug tamoxifen was investigated in rats and dogs. The study showed a slight decrease in body weight in rats and a few changes in biochemical and haematological parameters compared to the control and the rats treated with only Foxy-5. However, these effects are considered to be irrelevant for the toxicological assessment of Foxy-5 at the current stage of development in colon cancer. In dogs, the combination therapy with Foxy-5 and tamoxifen showed an increase in the number of leucocytes as the only treatment-related finding differing from treatment with Foxy-5 alone. The increase in leucocyte count is presently considered irrelevant, as treatment with tamoxifen is not included in the clinical trial.

Pre-clinical data summary and rationale for clinical studies

Foxy-5 pre-clinical profile

Our pre-clinical studies showed that Foxy-5 increases adhesion and impairs the migration and invasion of breast, colon and prostate cancer cells. In *in vivo* models of cancer metastasis, Foxy-5 has been shown to reduce the metastatic burden by 70 – 90% in mouse models of breast cancer, as well as to decrease metastasis to a similar extent in a mouse model of prostate cancer. Foxy-5 not only affects metastatic spread from the primary tumour but also has an anti-metastatic effect on circulating tumour cells. Additionally, in mice inoculated with human colon cancer cells, Foxy-5 treatment induces a reduction in the number of colon cancer stem cells in the cancer tissue. Foxy-5 has been shown to be non-toxic in pre-clinical toxicology, which is of prominent importance as it provides promising possibilities for combining anti-metastatic Foxy-5 treatment with conventional cancer treatments primarily targeting tumour growth. The possibility of such combination therapies was investigated, and it was revealed that Foxy-5 did not enhance or reduce the inhibitory effect of FOLFOX on cell viability. Furthermore, there was no negative effect on the tumour suppressor activity of checkpoint inhibitor therapy.

Due to the documented ability of Foxy-5 and WNT5A to engage an identical receptor, it is believed that Foxy-5 will be effective in cancer types where a low or devoid WNT5A expression correlates with the increased appearance of metastases and where WNT5A can inhibit migration and invasion of WNT5A-low cancer cells, implicating that Foxy-5 may be an anti-metastatic treatment suitable for breast, colon and prostate cancer and possibly also ovarian, thyroid and liver cancer as well as haematological malignancies.

Based on our pre-clinical studies, we decided to enter Foxy-5 into clinical development with the aim of realising our vision that patients with cancer will no longer have to face metastasis.

Predicted doses for clinical studies

Experiments were performed to identify a suitable dose level before the first *in vivo* study was started. The lowest dose with maximal effect was 40 µg of Foxy-5 for each treatment (Figure 11, p.12) and consequently, this dose was chosen for the subsequent *in vivo* experiments. In the mouse models, 40 µg equals approximately 2 mg/kg.

The No Observed Adverse Effect Level (NOAEL) in the rat studies was considered to be the high dose level in the supplementary 28-day rat study, 72 mg/kg, and the NOAEL in the dog studies was 2.5 mg/kg. Compared to plasma values for the 1.8 mg/kg dose level, which is the employed phase 2 dose, the dose level in rats represented more than 45 times the anticipated human therapeutic dose level and in dogs approximately 2 to 4 times the anticipated human dose level based on estimated C_{max} values and as such showed a favourable safety margin.

The first-in-man dose was selected as 1/10 of the intermediate (NOAEL) dose in rats (0.8 mg/kg) and converted to a human equivalent dose using the principles outlined in the FDA guideline for estimation of first-in-man human therapeutic dose levels. The dose is based on observations from toxicology studies, where the intermediate dose level was selected as the NOAEL in female rats and is selected according to guidelines.

Rationale for the dosing interval

The Foxy-5 peptide is administered via a 15-minute intravenous infusion (IV administration). The Foxy-5 peptide mediates its action via engagement of several different receptors and co-receptors, including Frizzled receptors and the tyrosine-kinase receptors Ryk and ROR. The subsequent downstream activation of the non-canonical pathway has been substantially described (Figure 1, p.5)[5], although a complete understanding is still lacking. The half-life of small peptides in the circulation of rodents is in the range of 30-50 minutes. However, the knowledge of a very short half-life of peptides

in mice must be balanced by the observation that Foxy-5 stimulation of tumour cells has the characteristics of a "hit-and-run" effect, as revealed by the fact that the anti-invasive effect of a 2-hour pre-incubation with Foxy-5 was still present during a subsequent 22-hour invasion assay in the absence of Foxy-5[31]. Consequently, the half-life of Foxy-5 is not correlated to its anti-invasive effect, which is also in agreement with the *in vivo* results from different anti-metastatic studies in pre-clinical mouse models. Foxy-5 dosing every second to fourth day in different mouse models resulted in very significant anti-metastatic effects (see Breast cancer models, p.12 and Prostate cancer model, p.13), thus supporting a dosing interval using two, three or four administrations per week.

Implications for clinical studies

Foxy-5 is considered to be a safe drug, with very few and inconsistent adverse effects in toxicological and safety studies. The lack of clear dose-related side effects at C_{max} and AUC concentrations 9 times over the effective concentrations in the pre-clinical studies caused no concern for starting a clinical phase 1 study. MTD was not established in the first phase 1 study (0.013 to 1.3 mg/kg), which necessitated a new toxicological 4-week study with doses up to 72 mg/kg and a further dose escalation phase 1 study to determine MTD (0.8 to 2.3 mg/kg). The completed toxicological studies supported a dose escalation range in phase 1 from 0.013 to 2.3 mg/kg.

The reported significant effects seen in the pre-clinical setting using administration every second to fourth day support studies in phase 1 being conducted using a three-weekly administration with increasing dose. A more frequent dosing increases the complexity of the logistics of administration in larger clinical trials and was considered not to be feasible for a future phase 2 study, meaning no other dosing intervals have been tested.

Clinical data for Foxy-5 (phase 1 and phase 1b studies)

Two phase 1 studies were performed with Foxy-5. A total of 48 patients with metastatic breast, colon or prostate cancer were included in these two studies (see Table 1). A phase 2 study in patients with colon cancer is ongoing.

Table 1 Overview of phase 1 clinical studies

Study population	Study design	Phase/ Countries	Study Identification	Status	No. of Patients
WNT5A-negative/low Patients with Metastatic Breast, Colon or Prostate Cancer	MTD study open-label, standard 3+3 dose escalation study (3 dosings per week)	Phase 1 /DK	SMR-2562 (EudraCT no. 2012-004200-35)	Finalised	31
WNT5A-negative/low Patients with Metastatic Breast, Colon or Prostate Cancer	MTD study open-label, standard 3+3 dose escalation study (3 dosings per week)	Phase 1b/DK, UK	SMR-3164 (EudraCT no. 2015-004767-36)	Finalised	17

As the MTD could not be established in the first-in-man study, a phase 1b study was initiated to continue dose escalation and establish the MTD. In addition, measurements of circulating tumour cells, buffy coat and gene expression analysis from tumour biopsies were introduced to explore the potential for establishing a target engagement marker. The phase 1b study included the two top dose levels tested in the phase 1 studies (0.832 mg/kg and 1.3 mg/kg). Only the 0.832 mg/kg dose was approved by the Medicines and Healthcare Products Regulatory Agency based on their assessment that this dose level was covered by available toxicology and toxicokinetic data. As a mitigation action, a supplementary 4-week toxicology study in rats with higher doses was performed to support the use of higher dose levels (see Foxy-5 safety profile in pre-clinical toxicology models).

The two phase 1 studies showed that Foxy-5 was very well tolerated in humans, with no dose-limiting toxicities (DLTs) observed.

Foxy-5 clinical safety profile

The safety of Foxy-5 treatment has been tested in a clinical phase 1 study including 31 patients with low WNT5A expression in the primary tumour (27 evaluable) with metastatic breast, colon or prostate cancer where Foxy-5 was administered intravenously at eight dose levels three days a week for up to 17 weeks. The results showed Foxy-5 to be safe and well tolerated up to the highest dose tested (1.3 mg/kg). Foxy-5 was detectable in the patient blood for the entire treatment cycle, indicating that the drug has adequate stability and half-life. The finding that Foxy-5 is non-toxic is of noticeable importance, as it provides promising possibilities for combining anti-metastatic Foxy-5 treatment with conventional cancer treatments targeting tumour growth. The pharmacokinetic data showed that there was a close to linear dose-proportionality relationship, a half-life of 5-7 hours, and no drug accumulation. It is normal to anticipate a longer half-life in humans compared to the half-life prediction in rodents.

Phase 1 study - SMR-2562 (first-in-man study)

The study was designed as an open-label, standard 3+3 dose escalation study, where 3 to 6 patients were treated at each dose. Patients received one intravenous slow infusion (15 minutes) of IMP administration three times weekly for three weeks, constituting one cycle. The next cycle followed immediately, with no interruption between cycles. Patients continued to receive Foxy-5 treatment at the investigator's discretion until disease progression or unacceptable toxicity.

A total of 31 patients in 8 cohorts were treated in a dose range from 0.013 mg/kg to 1.3 mg/kg. Nine patients were included in the highest dose cohort.

The majority of the adverse events were of grade 1 or 2 severity and unrelated to IMP. There were no adverse events confirmed as definitively related to Foxy-5 treatment. The adverse events reported by most patients were fatigue (n=25, 81%), decreased appetite (n=17, 55%), nausea (n=16, 52%) and malaise (n=16, 52%). A total of 17 serious adverse events (SAEs) were reported, and all were deemed unrelated or unlikely to be related to Foxy-5.

The results from this phase 1 study demonstrate that from a safety perspective, Foxy-5 is a safe and well-tolerated drug with no dose-limiting toxicities observed at any of the doses (0.013 mg/kg to 1.3 mg/kg).

Phase 1b study - SMR-3164

The study design was similar to the first-in-man study: an open-label, standard 3+3 dose escalation study. A total of 17 patients were treated in a dose range from 0.8 mg/kg to 2.3 mg/kg. The majority of the adverse events were of grade 1 or 2 severity and unrelated to IMP. There were no adverse events confirmed as definitively related to Foxy-5 treatment. The adverse event types occurring in more than 50% of patients were nausea (n=11, 65%), fatigue (n=10, 59%), decreased appetite (n=10, 59%), vomiting (n=10, 59%), constipation (n=9, 53%) and malaise (n=9, 53%). Nine SAEs were reported in the study, and 1 serious adverse reaction (SAR) was reported as possibly related to Foxy-5 and was reported as a suspected unexpected serious adverse reaction (SUSAR); however, it did not qualify as a DLT.

The results from this phase 1b study demonstrate that from a safety perspective, Foxy-5 is a safe and well-tolerated drug with no DLTs observed at any of the doses (0.8 mg/kg to 2.3 mg/kg).

The MTD was not determined from this study. The protocol allowed escalation up to a dose of 7.0 mg/kg (cohort 8); however, analysis of exploratory efficacy biomarkers suggested that a biological response, displayed by changes in mRNA expression in tumour biopsies, was observed at all doses of Foxy-5 tested (0.8 mg/kg, 1.3 mg/kg, 1.8 mg/kg and 2.3 mg/kg), and the study was therefore stopped in accordance with the protocol. Cohort 3 was selected as the expansion cohort, as preliminary results suggested that the biological response was highest for this dose level. There were no consistent or significant changes in the number of circulating tumour cells in response to treatment.

Overview of the serious adverse events observed in phase 1 studies

A total of 26 SAEs were reported in 17 of 48 subjects (35%) in the safety population of the two phase 1 studies. The majority of the SAEs were of grade 2 to 3 severity and considered to be unrelated or unlikely related to Foxy-5. One SAE (oppression in the chest) was deemed possibly related to treatment by the investigator and was recorded as a SUSAR.

Few clinically significant abnormalities were discovered for the clinical safety laboratory parameters. None of the SAEs observed in the two phase 1 studies were considered dose limiting by the investigator or the Safety and Data Monitoring Committee; therefore, no MTD was defined.

Foxy-5 human pharmacokinetic properties.

The pharmacokinetics of Foxy-5 in humans are similar to those in animals with a close to linear dose exposure relation and the absence of time-related accumulation. There was a rapid initial elimination covering approximately 80% of the plasma level within the first hour followed by a slower bi- or triphasic elimination of the remaining 20%. The reported half-life is longer than that observed in animals, but this represents the terminal portion of the curve and thus a minor portion of the total AUC.

Foxy-5 preliminary efficacy

Phase 1 study - SMR-2562 (first-in-man study)

At the end of treatment, 24 patients had progressive disease, 3 patients had stable disease, and 4 patients were not evaluably defined according to CT scan evaluation using response evaluation criteria in solid tumours (RECIST) criteria version 1.1. Two of the patients with stable disease were in the 1.3 mg/kg cohort (received 28 and 52 drug administrations), and one patient was in the 0.208 mg/kg cohort (received 51 drug administrations). The data are, however, far too limited to make any conclusions regarding the treatment effect.

Results from the mRNA analysis of tumour biopsies showed results indicative of a regulation of certain genes being expressed by the tumour cells from 4 biopsies; however, with the limited results, it was not possible to draw any firm conclusions. None of the other exploratory biomarkers showed any systematic changes.

Phase 1b study - SMR-3164

Of the 17 patients dosed with Foxy-5 in the study, 14 had progressive disease at 8 weeks. One patient (1.3 mg/kg) had stable disease at 8 weeks but progressive disease at 12 weeks. One patient (2.3 mg/kg) had stable disease at 8 and 12 weeks but progressive disease at 20 weeks. One (2.3 mg/kg) had stable disease at 8, 12 and 20 weeks but progressive disease at 28 weeks. All 17 patients had progressive disease at the end of treatment, defined as the end of all treatments. In most dose cohorts, there were only 3 patients; therefore, the data are far too limited to make any firm conclusions on the relationship between the dose and Foxy-5 effect.

Blood sample (buffy coat) analysis investigating the changes in genome-wide mRNA expression was included in this study as an exploratory biomarker, as non-clinical data obtained from the nine-month toxicology study in dogs indicated that a response to Foxy-5 treatment could be detected using this method. No changes in gene expression could be observed during Foxy-5 treatment; hence, the results obtained in the nine-month dog study are considered a chance finding. The results indicate that there is no untoward effect observed by increasing WNT5A signalling.

Analysis of genome-wide mRNA expression in pre- versus post-treatment tumour biopsies from patients showed that a biological response was observed at all dose levels of Foxy-5 (0.8 mg/kg, 1.3 mg/kg, 1.8 mg/kg and 2.3 mg/kg) indicated by gene expression changes. A total of 18 patients (15 patients from the phase 1b study and 3 patients from the phase 1 study) with colon, breast and prostate cancer were evaluated.

Due to the heterogeneous nature of the test samples, a descriptive statistics approach was used to evaluate the dose-response results for Foxy-5. A set of dose-response results was made for all the greatest/top 5-, 10- and 20-fold changes (both up- and downregulated genes) in mRNA from the blood samples and tumour biopsy samples, and an approximate 2-fold change was considered a positive biological response.

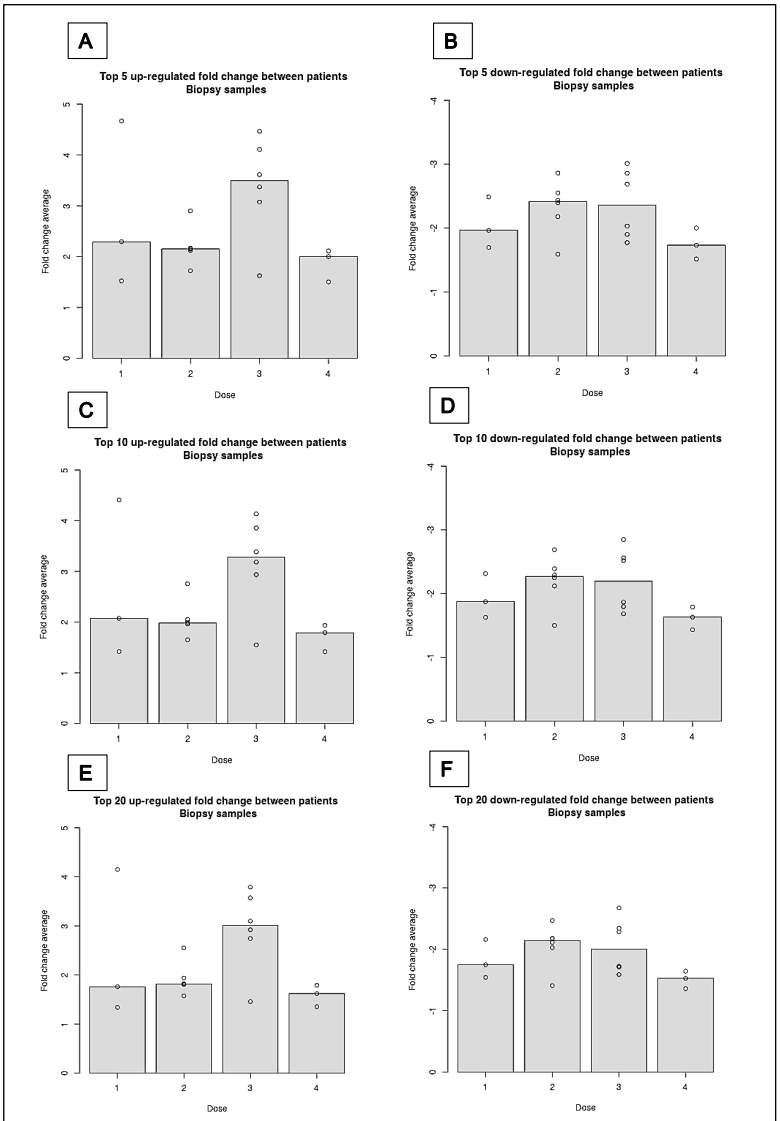


Figure 16. Genome wide mRNA analysis of tumour biopsies. Dose 1 = 0.8 mg/kg, Dose 2 = 1.3 mg/kg, Dose 3 = 1.8 mg/kg and Dose 4 = 2.3 mg/kg

The results are shown in Figure 16 and are presented as the dose-response between the patient's top gene fold changes (genes that showed the largest change) for each given dose. Each point represents the mean of the top 5, 10 or 20 genes with the greatest fold changes, while the bar-plots represent the median of the points (median of the fold change means).

The greatest fold change in gene expression of upregulated genes was observed at the 1.8 mg/kg dose level (cohort 3). The greatest fold change in gene expression of downregulated genes was observed at the 1.3 mg/kg dose level (cohort 2), and this level of change was maintained at the 1.8 mg/kg dose level (cohort 3).

Predicted doses

It has not been possible to establish an MTD for Foxy-5, and there is presently a lack of clear target engagement markers that are not dependent on clinical outcome. Thus, the trends in the changes in up- and down-regulation of gene expression from tumour biopsies obtained in the phase 1 study have been used for dose selection. Based on the limitations given by the study design, it was predicted that a dose of 1.3 to 1.8 mg/kg would be efficient, and so to maximize the potential for Foxy-5 in light of the benign safety profile, the higher dose was chosen for phase 2.

Clinical phase 1 data summary

Initiation of the clinical phase 1 studies was based on observed pre-clinical efficacy and a favourable safety profile. In addition to confirming the safety and tolerability at predicted efficacy levels, an additional objective was to establish an MTD.

Two phase 1 studies were performed with Foxy-5, including a total of 48 patients with metastatic breast, colon or prostate cancer with three administrations of Foxy-5 per week for up to 17 weeks. The MTD could not be established in the first-in-man study; therefore, the phase 1b study was initiated to continue dose escalation and establish the MTD. In addition, an objective was to explore the potential to establish a target-related effect. For this purpose, changes in genetic markers in the buffy coat and tumour biopsies as well as circulating tumour cells were explored.

The accumulated evidence from the two phase 1 studies showed that Foxy-5 is a safe and well-tolerated drug with no dose-limiting toxicities observed at any of the doses tested (0.013 – 2.3 mg/kg). Foxy-5 was detectable in the patient blood for the entire treatment cycle, indicating that the drug has adequate stability, a half-life of 5-7 hours and no drug accumulation. The finding that Foxy-5 is non-toxic is of noticeable importance, as it provides promising possibilities for combining anti-metastatic Foxy-5 treatment with conventional cancer treatments targeting tumour growth.

No effects on either circulating tumour cells or gene expression in the buffy coat were observed. The origin and number of circulating tumour cells were not clear due to study limitations. The lack of effect on gene expression in the buffy coat at any dose level was regarded as a sign of no effect on non-cancerous cells. Based on the heterogeneity and limited size of the patient cohort, changes in unspecific gene expression were not statistically significant. The benign safety profile combined with trends in the changes in up- and downregulation of gene expression was the basis for the selection of the 1.8 mg/kg dose for further clinical investigation to maximize the potential for Foxy-5.

Obtaining human proof of principle for Foxy-5

It is important to emphasize that the aim of the study design is to obtain a human proof of principle (POP) for Foxy-5 to prevent metastasis formation and should not be considered as a preamble and limitation for the use of Foxy-5 as a commercial product. The prerequisite is to optimize the ability to demonstrate an effect on disease recurrence, with limited patient material, by starting treatment as early as possible in a patient population with a high risk of recurrence and within the scope of the Foxy-5 mode of action with the aim of preventing the metastatic process.

Rationale for the phase 2 study

Indication selection

Most pre-clinical work with Foxy-5 has been performed in breast cancer; however, these results translate well to both prostate and colon cancer (see page 5). The decision to perform the phase 2 study in colon cancer was a combination of availability and competition for patients, as well as severity of the various cancer forms. Colon cancer is one of the three major cancer forms and affects both men and women. Fewer patients with non-stage IV colon cancer are enrolled in ongoing clinical studies[II], and treatment paradigms in breast and prostate cancer are complex with effective therapies in the non-metastatic setting. Disease progression to metastatic disease after surgical removal of the primary tumour is more rapid in colon cancer, and the reported incidence is higher compared to breast and prostate cancer, as shown in Figure 2, p.6. The limitation of the study cohort to colon cancer rather than colorectal cancer was based on the different treatment paradigms for colon and rectal cancer. While surgery followed by chemotherapy, if required, is the main treatment scheme for colon cancer, treatment of rectal cancer can be initiated by chemotherapy, radiation or surgery. From a study design perspective, multiple treatment schemes introduce a high degree of complexity in rectal cancer indications.

Selection of colon cancer population

The clinical outcome/prognosis for patients diagnosed with colon cancer is critically dependent on the stage once detected. Stage I and most stage II patients are considered to be cured by surgery, and a rare minority of these patients will recur with future metastasis. Stage IV patients have a very poor outcome and are signified by visible metastasis and are not within scope of the mode of action of Foxy-5. The late stage II, i.e., stage IIc, together with stage III patients have a progressively worse disease with a recurrence rate of 27-69% within 5 years[35]. Recurrence occurs relatively rapidly, and most recurrences appear within 24 months post-surgery[36]. A further consideration for the study population was the decision to allow up to a maximum of three adjacent lymph nodes presumed to be cancerous, as more nodes and more distant nodes present a higher risk to also have occult, non-visible metastases.

To further enrich the patient material with respect to risk for recurrence, the primary evaluation will be performed on the basis of the expression of WNT5A in the resected primary tumour, as retrospective analyses of patients presenting with tumours demonstrate a clear difference in recurrence on the basis of WNT5A levels. Patients with a low expression of WNT5A have a higher risk than those with high levels, which has also been demonstrated in other research studies[27]. Thus, although it is not evidence of the cause of the severity, it is a noteworthy correlation for consideration. The expression level of WNT5A is heterogenic in the primary tumour, so the discrimination is based on a combination of the area and intensity of staining. As the "cut-off" between low and high expression of WNT5A is arbitrarily set, there is also an interest in comparing the full cohort of patients treated with Foxy-5 vs the control group in order to establish whether patients with higher levels of WNT5A in the primary tumour may also benefit from the treatment.

[II] ClinicalTrials.gov; Cancer Today, GLOBOCAN 2018 accessed 18 May 2020; Non-stage IV patients in interventional studies, incidence in brackets: Breast cancer 834,000 (676,000); Prostate cancer 232,000 (864,000); Colon cancer 247,000 (449,000)

Treatment initiation and duration

The treatment paradigm is based on three aspects of Foxy-5's MoA: metastatic spread from the primary tumour by decreasing cancer cells' ability to migrate and invade, anti-metastatic effect on circulating tumour cells to penetrate distant organs/tissue and the indicated effect on the number of cancer stem cells. Therefore, treatment should start as early as possible after diagnosis, prior to surgery, and continue through surgery until the start of chemotherapy. Thus, from a study perspective, it is important that early diagnosis by radiological methods can correctly identify the risk population with therapy starting as soon as possible. It is also important to treat patients in the post-surgery setting in the event that there are metastatic cells that have already escaped from the primary tumour. The duration of treatment is almost certainly the most important factor to address cancer stem cells. These are most likely present at diagnosis in this patient population and are only partially removed by surgery.

Dose selection and dosing interval

Phase 1 studies were performed in patients with metastatic disease (stage IV) of breast, prostate and colorectal cancer origin. The objective to establish an MTD was not met, while both the safety and tolerability were shown to be excellent. In lieu of an MTD, the selection of dose for the clinical phase 2 study was based on signs of engagement by exploring the up- and downregulation of unspecified genes from biopsy material. A significant change was not found, but a clear trend for an effect was observed in the range of 1.3-2.3 mg/kg body weight, with the most pronounced change at 1.8 mg/kg. Based on the combination of no toxicity or tolerability issues together with the observed trend, the 1.8 mg/kg dose was chosen for the phase 2 study.

A three times per week administration was chosen for the phase 1 studies based on the significant effects seen in the pre-clinical setting using administration every second to fourth day. From a feasibility point of view, the decided dosing interval was continued in the phase 2 study.

The phase 2 study

Today's guidelines for cancer care are to resect the primary tumour within 2 weeks after diagnosis. These guidelines are incorporated in most national directives. It is evident that there is a large variation in the ability to follow these guidelines at the national, regional and local level in a number of European countries. Thus, the choice of countries to start the study was based on a feasibility study, where one of the criteria was the actual time from diagnosis to surgery according to current statistics. Spain, the Netherlands and Hungary were chosen for the study based on the fact that the ongoing screening activities for colon cancer have timelines well beyond the recommended two weeks.

Number of patients for inclusion

The patient population is patients with stage IIc/III colon cancer with a maximum of three affected lymph nodes and no sign of distal metastases. The statistical calculation for the number of patients to include to obtain a meaningful clinical difference in a sufficient number of patients included both the skill by radiologists to accurately predict stage from a clinical picture versus the true stage obtained by pathological examination post-surgery and the prediction of the number of patients who will have low expression of WNT5A in the primary tumour. A 20% relative difference in recurrence was considered to be a clinically meaningful benefit. To demonstrate this, there is a need to evaluate 60 patients in a 1:1 design. To obtain this number of evaluable patients, the estimates suggest the need to randomize between 100 and 130 patients.

Exclusion criteria

The standard exclusion criteria are implemented, with a very important exclusion of patients with a prior history of cancer and/or other deleterious conditions from the trial.

Dosing intervals

Foxy-5 is administered three times weekly for a maximum of 39 administrations but stops once the patient starts chemotherapy. The reason for stopping treatment at chemotherapy is a combination of logistics and to avoid an overreporting of adverse events, which may be caused by chemotherapy. There is no limitation due to safety concerns, as Foxy-5 has no negative effect on chemotherapy efficacy based on pre-clinical data. Foxy-5 should be administered for at least three weeks prior to surgery and given in at least 9 administrations. The administration of Foxy-5 is allowed to be given to patients directly after surgery and, if stopped, has to be restarted within 7-14 days post-surgery. Administration of Foxy-5 requires handling by a trained nurse in a hospital setting.

Open study with a control arm not receiving any vehicle

Due to the logistical challenges associated with three intravenous administrations of Foxy-5 per week, the decision was to conduct an open study with a control arm not receiving vehicle administration. One important aspect of the decision is the binary outcome of the study, i.e., either there is a recurrence or there is not. A clear drawback is the skewed reporting of AEs and SAEs, although historical data are of great use for the review.

Endpoints and outcomes

The primary outcomes are safety, tolerability and recurrence of disease. The latter is measured using a surrogate marker, i.e., circulating tumour (ct)DNA. ctDNA has been detected up to 12 months prior to the appearance of visible metastases and in some tumour forms even earlier. The method for ctDNA detection is the SAGAsign® technology (formerly known as KROMA) developed by SAGA Diagnostics and is based on using the patient's own primary tumour to produce "probes" that can be detected in plasma. The precision is on the order of 90-95%, with exceptionally few false positives, if any. The secondary endpoints include disease-free survival, overall survival and the recurrence-free interval as required by regulatory authorities. The patients will be followed up for 2 years post-surgery, and plasma samples for analysis of ctDNA will be withdrawn every three months.

There is also an exploratory endpoint for the correlations among plasma levels of thymidine kinase (TK), WNT5A expression and primary outcome. TK has been shown in previous studies to be correlated with outcome, and the purpose is to explore whether there is a potential to use TK as a surrogate marker for patients who would benefit from Foxy-5 treatment.

Next steps for Foxy-5

Partnerships

WntResearch's focus is on asset creation in the pre-clinical and clinical setting aiming at demonstrating phase 2 POP for assets. For assets reaching maturity, WntResearch is pursuing an out-licensing/acquisition model to leverage partners' late-stage clinical development, regulatory and commercialisation expertise and capability. WntResearch seeks partnerships with larger, preferentially oncology-focused, pharmaceutical companies that have the capability and financial strength to run large clinical programmes and are able to successfully launch globally or at a regional level. The outcome of the current phase 2 POP trial is the basis for how to proceed with Foxy-5 development, either in house or in collaboration. WntResearch is looking for either licensing or acquisition after availability of POP data or an option deal before POP data are available.

Manufacturing

A number of non-clinical activities have been initiated to further develop Foxy-5, with a special focus on the manufacturing of active pharmaceutical ingredients (APIs). Although not as complex as the native WNT5A protein, the current method of manufacturing Foxy-5 is sub-optimal with limited potential for scaling up to commercial batches. Reduction of the cost of goods (COGS) will reduce commercialisation risk. WntResearch has investigated a number of manufacturing improvements with partners, leading to the invention of a novel manufacturing method that is suggested to be more robust, scalable and less costly than the current product. New IP has been filed, which brings down COGS considerably but also reduces the risk for commercial manufacturing. Any further clinical work with Foxy-5 will be performed using the new manufacturing method, which may or may not require a bridging study.

New formulation

The current use of Foxy-5 is limited to intravenous administration due to the fairly low solubility of the current formulation. Although not considered to be a major hurdle for the acceptance of Foxy-5 as a viable treatment option, increasing the solubility opens up several other options for administration and, ultimately, self-administration by the patients. A programme to achieve better solubility has been initiated with the potential for additional patent protection of Foxy-5 and in time for use in pivotal phase 2b and phase 3 trials.

Opportunities beyond colon cancer

Although colon cancer is a leading indication and is used to obtain human POP for Foxy-5, there is a correlation between the low expression of WNT5A in primary tumours and poor prognosis in several different types of cancers, including breast, prostate, rectal, hepatocellular, thyroid-oesophageal, lymphoma and ovarian cancer. It is envisaged that all these different cancer types can constitute possible new areas of application for Foxy-5 and demonstrate the huge scalability potential of Foxy-5. These cancers together represent more than 6 times the number of new colon cancer cases and represent 50% of all cancer cases worldwide[37].

There are other potential routes forward, including combination therapy with chemotherapy, checkpoint inhibitors and other drugs. Other treatment paradigms can also be explored. Preventing metastasis in a chemotherapy treatment exclusion period could be the key to improving patient prognosis and overall survival when timely cancer eradicating surgery or chemotherapy is not offered. Examples are patients not fit for chemotherapy or patients with complications that prohibit timely surgery.

Summary

The main cause of cancer-related death is the formation of metastases. Today, there are no specific treatment methods preventing tumour cells from invading and disseminating to distant unaffected tissues and forming metastases. Low expression of WNT5A in breast, colon and prostate cancer tumours has been correlated with an increased number of disease recurrences due to the formation of metastases and a shortened survival of the patient.

WNT5A is known to inhibit the migration and invasion of cells of the above cancer types, and the addition of recombinant WNT5A has been shown to impair the migration and invasion of these tumour cells and supress the number of cancer stem cells. Consequently, an attractive approach would be to administer WNT5A to patients lacking the expression of this protein in their tumour cells as treatment to prevent metastasis. The WNT5A protein has major disadvantages as a therapeutic drug candidate, being that it is a large protein with several post-translational modifications and a heparan sulphate-binding domain that limits its distribution in the body. Therefore, a peptide that mimics the effect of WNT5A was discovered and developed. Based on sequence analysis of WNT5A, a short list of peptides was analysed for their WNT5A mimicking effects. The lead candidate, having the desired effects, was a formylated hexapeptide named Foxy-5.

Foxy-5 was subsequently shown *in vitro* to impair migration and invasion by increased adhesion, reduced release of matrix metalloproteases and normalization of cancer cell metabolism of cancer cells. In *in vivo* models of cancer metastasis, Foxy-5 has been shown to reduce the metastatic burden by 70% in the liver and 90% in the lungs in mouse models of breast cancer, as well as to decrease metastasis in a similar range in a prostate cancer mouse model. Foxy-5 not only affects metastatic spread from the primary tumour but also has an anti-metastatic effect on circulating tumour cells. In a mouse model of colon cancer, Foxy-5 reduced the number of cancer stem cells.

Foxy-5 has been shown to be non-toxic with an excellent safety profile in pre-clinical toxicology studies as well as in human phase 1 studies. Foxy-5 can be used with standard adjuvant chemotherapy or checkpoint inhibitors with no interactions. Foxy-5 has significant potential to prevent the metastatic process and complement today's therapeutic approach, primarily targeting tumour growth.

While the maximum tolerated dose was not established in phase 1 studies, signs of engagement by exploring the up- and downregulation of unspecified genes from biopsy material showed a clear trend for an effect in the range of 1.3-2.3 mg/kg body weight. Combining Foxy-5's favourable toxicity and tolerability profile together with the trend, 1.8 mg/kg was chosen for further clinical investigation. Currently, Foxy-5 is being studied for human POP in a phase 2 study with patients with colon cancer. Stage IIc and III patients with colon cancer are included in the study to enrich the study with respect to risk of recurrence. The primary tumours will be evaluated for their expression of WNT5A to further define the cohort with a high risk of recurrence. In the open study, the recurrence of disease is evaluated every three months by ctDNA, and patients are followed up for 2 years post-surgery.

Foxy-5 mimics WNT5A, and Foxy-5 may be effective in other cancer types where loss of WNT5A promotes progression of metastases, implicating that Foxy-5 may prevent the metastatic process in breast, colon and prostate cancer and possibly also ovarian, thyroid and liver cancer as well as haematological malignancies.

With Foxy-5, WntResearch is developing a novel approach to challenge and prevent the metastatic process. We aim to prevent the recurrence of cancer and ensure that millions of patients do not end up in the devastating situation of being diagnosed with metastatic cancer. WntResearch is focusing on solving what truly matters for patients with cancer and ultimately reaching our vision in which patients with cancer will no longer have to face metastasis.

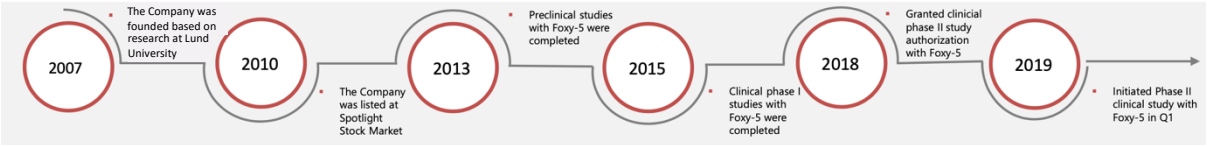
About WntResearch AB

WntResearch is an oncology-focused discovery and clinical development company that is developing therapies to prevent metastases. Our emphasis is on WNT5A, a protein involved in the metastatic process, as a target, and WntResearch's vision is that patients with cancer will no longer have to face metastases.

One in three persons will be diagnosed with cancer during their lifetime. Although modern cancer treatment has become more successful, effective ways to prevent metastases are absent. Approximately 90% of all cancer-related deaths are directly associated with metastases. WntResearch's mission is to prevent the metastatic process by providing novel cancer therapies. We are relentless in pursuing our mission, and our strategy has five key priorities:

1. Focusing on revealing the functional properties of the WNT5A agonist Foxy-5
2. Pursuing our clinical phase 2 POP study
3. Optimizing the manufacturing and formulation of Foxy-5, including associated IP
4. Establishing partnership(s)
5. Exploring new WNT5A opportunities

Fulfilling our mission has become a real opportunity through the discovery and development of our lead drug candidate Foxy-5, which is in a phase 2 study in patients with colon cancer where it is tested as a WNT5A mimic for its ability to prevent cancer metastases. Our business model is designed to create assets and value in the pre-clinical and clinical setting, and we intend to enter commercial cooperation agreements with one or several pharmaceutical companies when the time has arrived to further develop and commercialize the company's drug projects for the benefit of patients with cancer. WntResearch's product portfolio also comprises Box-5, a drug candidate with the potential to counteract metastasis in other cancer types, such as melanoma and gastric cancer. WntResearch AB is a public company listed at Spotlight Stock Market. WntResearch was founded in 2007 based on research at Lund University. For more information, please visit our website at www.wntresearch.com.



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