A FOXC2 inhibitor, MC-1-F2, as a therapeutic candidate for targeting EMT in castration-resistant prostate cancer

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Abstract: Androgen deprivation therapy (ADT) is the major treatment option for advanced prostate cancer. However, prostate cancer can develop into androgen-independent castration-resistant prostate cancer (CRPC) which is resistant to ADT. Alternative treatments for CRPC have focused on targeting the epithelial-mesenchymal transition (EMT). EMT is governed by a series of transcription factors of which FOXC2 is a central mediator. Our previous research into the inhibition of FOXC2 in breast cancer cells lead to the discovery of MC-1-F2, the first direct inhibitor of FOXC2. During our current study on CRPC, MC-1-F2 has shown a decrease in mesenchymal markers, inhibition of caner stem cell (CSC) properties and decrease in invasive capabilities of CRPC cell lines. We have also demonstrated a synergistic effect between MC-1-F2 and docetaxel treatments, leading to a decrease in docetaxel dosage, suggesting the possible combination therapy of MC-1-F2 and chemotherapeutic drugs for the effective treatment of CRPC.

Prostate Cancer is one of the top five most diagnosed cancers worldwide, with androgen deprivation therapy (ADT) as the most commonly prescribed treatment for advanced prostate cancer.[1, 2] However, ADT leads to the acquisition of an androgen-independent state.[3] This resistance to ADT leads to what is known as castration-resistant prostate cancer (CRPC). 90% of prostate cancer patients progress to this stage.[4, 5] This stage of cancer progression is associated with androgen independence and resistance to chemotherapeutic agents.[6-8] These characteristics lead to high mortality rates, high recurrence rates and high metastatic rates,[4, 5] and create a challenge for treatment, necessitating the development of alternatives to ADT.

Currently, alternatives to ADT focus on the use of chemotherapeutic agents such as docetaxel.[9] Docetaxel is an anti-mitotic compound that increases the stabilization of microtubules in the cell and specifically works by activating apoptosis in CRPC.[10] The use of chemotherapeutic agents after the development of CRPC is standard treatment. However, patients will often face disease progression and chemoresistance after about 7 months of treatment.[11] This proves to be a major challenge in the treatment of CRPC, often leading patients with few treatment options. In order to develop new treatment options for CRPC, we must understand the mechanism behind the development of this chemoresistance.

The acquisition of chemoresistance in CRPC is mediated by the epithelial to mesenchymal transition (EMT).[4, 5, 12] EMT leads to the breakdown of cell-cell contact, acquisition of cancer stem cells (CSC)-like characteristics, and an increase in invasive and chemo-resistant capabilities.[13-15] In addition, the upregulation of ZEB1 and/or FOXC2, transcription factors involved in EMT regulation, are shown to correlate with an increase in CSC subpopulation and ADT resistance.[3, 16] The inhibition of either ZEB1 or FOXC2 has shown to inhibit EMT progression, render cells more susceptible to chemotherapeutic agents and lead to a decrease in CSC characteristics.[3, 17] As a central mediator of EMT progression, FOXC2 is required for the acquisition of CSC characteristics and the initiation and maintenance of a mesenchymal phenotype.[18, 19]

Our previous research into the inhibition of FOXC2 led to the discovery of MC-1-F2 (Figure 1), the first direct small molecule inhibitor of FOXC2.[20] MC-1-F2 was able to show a decrease in EMT markers, inhibition of CSC properties and decrease in invasive capabilities in breast cancer cell lines. In this study, we hypothesize that MC-1-F2 will have the same effects in CRPC. We will focus on the use of MC-1-F2 in the treatment of CRPC and examination of MC-1-F2's potential to increase chemo-sensitivity upon inhibition of FOXC2 and ZEB1 expression.

CRPC cell lines DU145, and PC3 were chosen due to their androgen independence, and relatively high expression of FOXC2 (Figure S1). We first examined the effect of MC-1-F2 on FOXC2 degradation in CRPC cell lines DU145 and PC3 (Figure 2). MC-1-F2 has shown in breast cancer to induce FOXC2 degradation, believed to be caused by an inhibition of the nuclear transport of FOXC2. We saw a similar effect in CRPC. In both cell lines we can see a clear decrease in

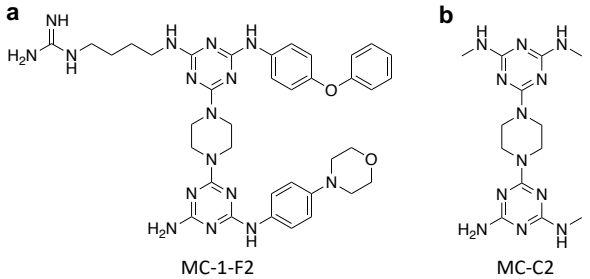

Figure 1. Chemical structures of MC-1-F2 and control compound MC-C2

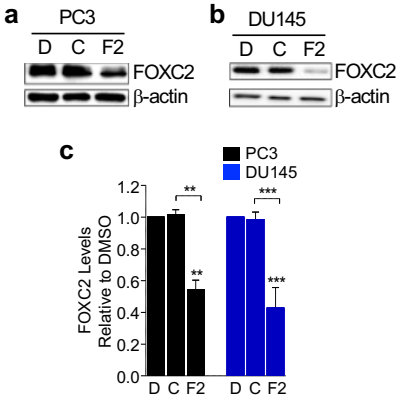

Figure 2. Effect of MC-1-F2 on FOXC2 degradation. (a-c) Western blot analysis of FOXC2 degradation in PC3 or DU145 cells after treatment with DMSO (D), MC-1-F2 (F2, 20 μM) or MC-C2 (C, 20 μM) for 48 hours. Representative image of Western blot analysis of FOXC2 in PC3 (a) and DU145 (b) cells. c) Error bars represent s.d. from triplicate experiments. Statistical comparisons performed by Student's t-test. ***p<0.0005, **p<0.005

FOXC2 protein level upon 48-hour treatment with MC-1-F2. These initial results pushed us to further explore the effects of MC-1-F2 in CRPC.

We next examined the modulation of EMT markers (Figure 3) in both cell lines, exploring both epithelial and mesenchymal markers and their effect upon treatment with MC-1-F2 for 48 hours. As expected from our previous studies, MC-1-F2 was able to downregulate various EMT markers such as ZEB1, Vimentin, *N*-cadherin and Slug in both PC3 and DU145. We also saw a recovery of the epithelial markers ZO-1 and *E*-cadherin. A key change in EMT markers upon MC-1-F2 treatment was a reversal of the cadherin switch involved in EMT progression. During EMT progression, *E*-cadherin expression is downregulated followed by an upregulation in *N*-cadherin expression.[18] A reversal of this switch, evident by Western blot analysis, is a strong indication of a reversal of EMT progression as previously observed in breast cancer.[20] Thus, we next set to explore the effect of MC-1-F2 on CSC properties of CRPC.

CSC properties are acquired with EMT progression and are governed by FOXC2.[1, 18, 19, 21] In CRPC, there exists a higher ratio of CSC to regular prostate cancer cells, making treatment for CRPC more difficult.[22] CSC are linked with higher proliferative, self-renewal and chemo-resistant properties.[23] It is these CSC properties that allow for tumour growth to progress regardless of ADT therapy or chemo-therapeutics administration, ultimately leading to metastasis.[15] Here we examined CSC markers: Nanog, C-myc and KLF4 in CRPC cell lines. All CSC markers showed significant decreases after treatment with 20 μM of MC-1-F2 in both cell lines (Figure 4). A decrease in CSC properties is an indication of a better outcome in the treatment of CRPC, with current research into CRPC treatments focusing on CSC targeting therapies.[24]

Following the determination of both inhibition of EMT and CSC properties, we focused on the inhibition of the invasive capabilities of CRPC cell lines following MC-1-F2 treatment, by a transwell invasion assay. Cell lines PC3 and DU145 both showed remarkable inhibition in invasiveness after treatment with MC-1-F2 for 24 hours as compared to the DMSO condition (Figure 5). In order to confirm this inhibition was only occurring in FOXC2-expressing cell lines, we tested the effect of MC-1-F2 on LNCaP, an androgen-dependent, low FOXC2 expression level cell line (Figure S2). We saw no change in the number of invading cells upon treatment, further supporting the specificity of MC-1-F2's mode of action.

Thus far we have demonstrated MC-1-F2 is capable of inhibiting EMT and CSC properties leading to an inhibition of the invasive capabilities of CRPC. This is an important aspect of MC-1-F2 treatment, as CRPC is marked by an

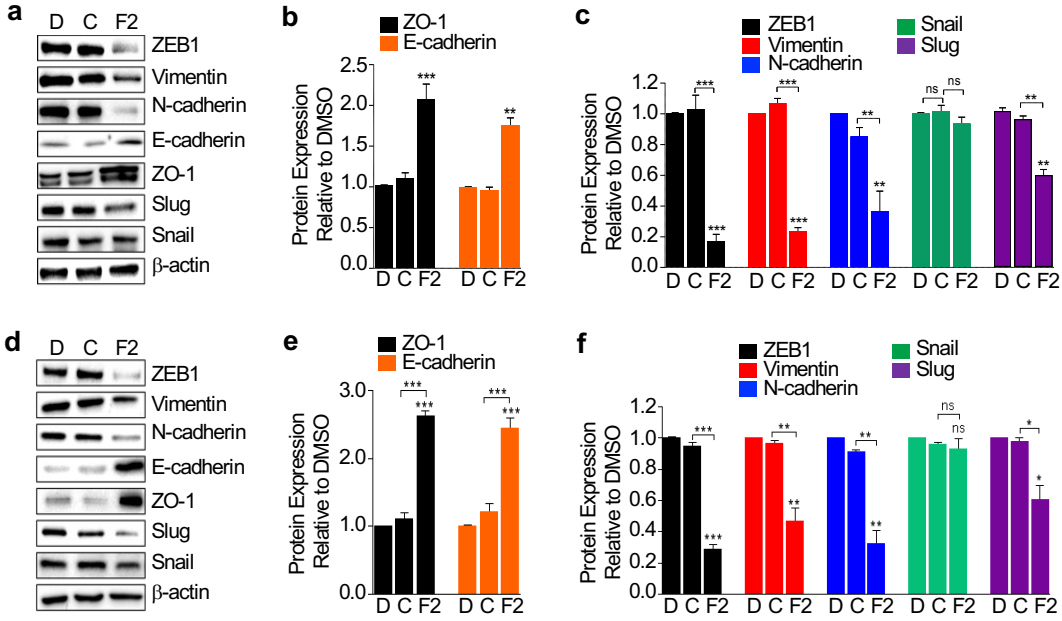

Figure 3. Modulation of EMT by MC-1-F2. Western blot analyses of EMT markers in PC3 (a-c) or DU145 (d-f) cell line upon treatment with 20 μM of MC-1-F2 (F2), 20 μM of MC-C2 (C) or DMSO (D) for 48 hours. Epithelial markers: ZO-1 and E-cadherin / Mesenchymal markers: ZEB1, Vimentin, N-cadherin, Snail, and Slug. Error bars represent s.d. from triplicate experiments. Statistical comparisons performed by Student's t-test. ***p<0.0005, **p<0.005, *p<0.05

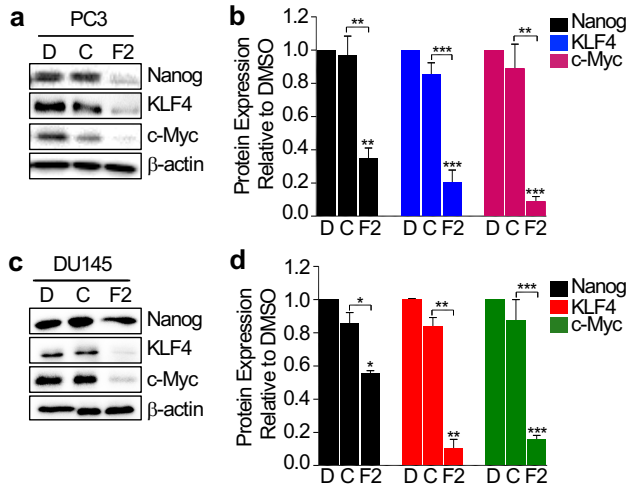

Figure 4. Inhibition of CSC activity by MC-1-F2. Western blot analysis of CSC markers in PC3 (a, b) or DU145 (c, d) cell line upon treatment with 20 μM of MC-1-F2 (F2), 20μM of MC-C2 (C) or DMSO (D) for 48 hours. Error bars represent s.d. from triplicate experiments. Statistical comparisons performed by Student's t-test. ***p<0.0005, **p<0.005, *p<0.05

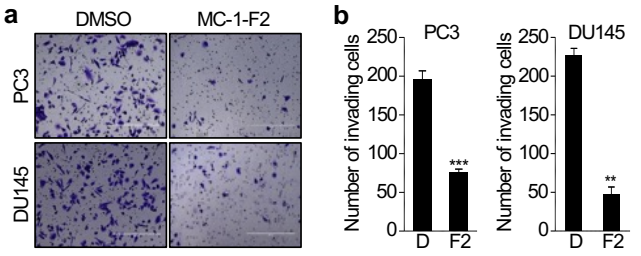

Figure 5. Effect of MC-1-F2 on metastatic capabilities. a) Representative image of transwell invasion assay of PC3 (top) and DU145 (bottom) cell lines upon treatment with 20 μM of MC-1-F2 (F2) or DMSO (D) for 48 hours. b) Quantitation of invasion assay results in PC3 and DU145 cells. Error bars represent s.d. from triplicate experiments. Statistical comparisons performed by Student's t-test. ***p<0.0005, **p<0.005

increase in invasiveness that ultimately leads to metastatic progression.[25] CRPC that has become metastatic has low survival rates, below 30 months.[1, 12, 25] MC-1-F2's ability to inhibit the invasiveness of CRPC cell lines is a good progress in the development of new targeted therapeutics to combat metastatic progression.

However, in order for MC-1-F2 to be considered an effective treatment option for CRPC we needed to determine its efficacy and how well it compares to other chemotherapetic agents. First, we determined the EC_{50} of MC-1-F2 using an MTT cell viability assay in both DU145 and PC3. EC_{50} of MC-1-F2 in DU145 and PC3 respectively are 48.14μM and 53.21μM (Figure S3). These EC_{50} values are higher than those we encountered in MDA-MB-231, a breast cancer cell line. This is most likely due to the overall lower FOXC2 expression levels found in prostate cancer cell lines.

Next, we chose to focus on a comparison between MC-1-F2 and enzalutamide or docetaxel treatment. A main treatment option for prostate cancer is ADT. For ADT, compounds that target androgens (AR) are utilized, aiming to halt the progression and growth of prostate cells.[26] An example of such compounds is enzalutamide. Enzalutamide is a pure antagonist of AR, targeting androgens required for the growth and proliferative capabilities of prostate cell, initially slowing the growth of CRPC.[26] In addition, we chose to examine the effects of MC-1-F2 when in combination with docetaxel. Docetaxel has long been established as the secondary treatment option for CRPC; however, some patients do not respond to therapy and chemoresistance inevitably develops.[6, 26] Docetaxel acts in a similar manner to paclitaxel, another common chemotherapeutic drug, by promoting microtubule assembly and inhibiting microtubule dynamics, leading to mitotic progression impairment and cell cycle arrest.[27]

After having chosen our comparison drugs, we first carried out single drug MTT cell viability studies (Figure S3). Enzalutamide showed no effect on both cell lines, as expected due to the cell lines androgen-independence. This being a major drawback of enzalutamide treatment, the development of resistance following ADT therapy. The second line of treatment are chemotherapeutic agents. In this case docetaxel, a chemotherapeutic agent, had a potent effect on both cell lines with and EC_{50} of 7.60nM in PC3 and 6.05nM in DU145. In comparison, MC-1-F2 alone is not as potent as docetaxel but has a stronger effect than enzalutamide. Since MC-1-F2 is not as potent as other chemotherapeutic drugs, then the possibility of utilizing it as a combinatoric treatment was explored.

We carried out combinatorial experiments between MC-1-F2 and enzalutamide or docetaxel. These combinatorial experiments would give us a better understanding of the role of FOXC2 inhibition in terms to chemo-sensitivity and chemo-resistance. A combinatorial drug treatment assay would help us determine if synergism, additive or antagonistic effects exist between two drugs.[28-31] A combinatorial index of <1 indicates a synergistic effect, =1 is additive effect and >1 an antagonistic effect.[30] We first examined the combinatoric treatment of MC-1-F2 with enzalutamide. We have not examined the effect of MC-1-F2 on the AR signalling in both PC3 and DU145 as they are CRPC cell lines. This led us not to expect any effect by enzalutamide or any synergism with MC-1-F2, confirmed by a combination index greater than 1 (Figure 6a).

However, we know that FOXC2 and ZEB1 inhibition have been shown to decrease chemo-resistance in various cancers with their inhibition linked to an increase in chemo-sensitivity to docetaxel.[3, 17] Thus, we set out to explore the combinatorial treatment of docetaxel and MC-1-F2, hoping to see a change in chemo-sensitivity. This combination treatment demonstrated synergistic effects between MC-1-F2 and docetaxel treatment (Figure 6b). Individually, docetaxel is more potent than MC-1-F2, reaching higher inhibition rates at lower dosage concentrations; however, in combination, MC-1-F2 can lower the required dosage of docetaxel at a given inhibition rate. The new EC_{50} of docetaxel in DU145 and PC3 became 1.75nM and 1.97nM, respectively when in combination with MC-1-F2 treatment.

To ascertain that the synergistic effects of MC-1-F2 are occurring only in FOXC2 expressing cell lines, we examined a combinatoric treatment in LNCaP cells. LNCaP prostate cancer cells express low FOXC2 levels and high AR levels. The combinatoric experiments of MC-1-F2 and docetaxel did not lead to any synergistic effects in LNCaP cells (Figure S4). This further highlights the specificity of MC-1-F2 in only targeting FOXC2 expressing cell lines.

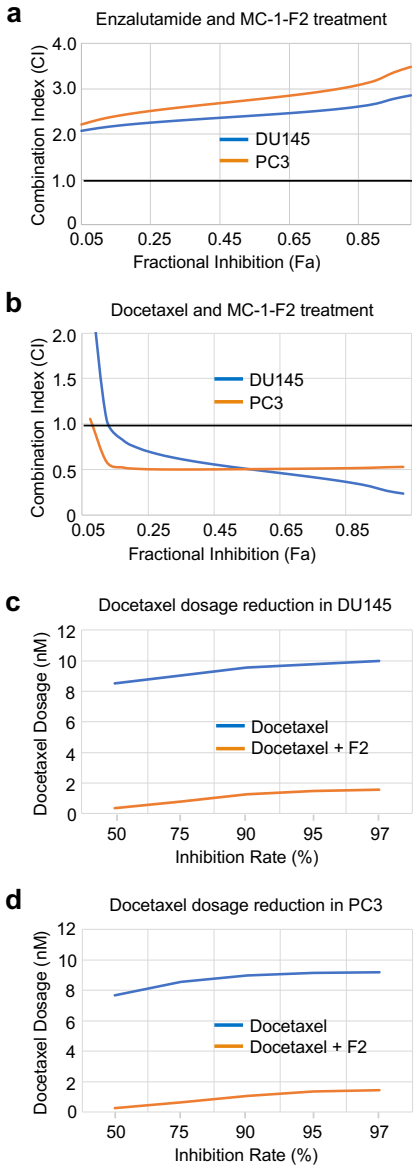

Figure 6. Combinatorial effects of MC-1-F2. (a, b) Combinatorial index of DU145 and PC3 cell lines treated with MC-1-F2 and enzalutamide (a) or docetaxel (b) for 24 hours. (c, d) Dose reduction graph of docetaxel administration in DU145 (c) or PC3 (d) cell upon combinatorial treatment. Analysis carried out using CompuSyn.

The mechanism of the synergistic effect between MC-1-F2 and docetaxel is yet to be understood. Regardless of the mechanism of synergism, the increase in chemo-sensitivity due to MC-1-F2 treatment in CRPC is prominent. The increase in chemo-sensitivity to docetaxel would allow for shorter treatment times at lower dosages, without inducing chemo-resistance. This type of combinatoric treatment can be beneficial to patients who have developed ADT resistance and whose only treatment option is chemotherapy.

In conclusion, MC-1-F2, the first direct small molecule inhibitor of FOXC2 can demonstrate a halt in EMT progression, a decrease in CSC makers and an increase in chemo-sensitivity in CRPC. Chemoresistance in CRPC is linked to EMT progression, where both FOXC2 and ZEB1 play a key role. It has shown that inhibition of FOXC2 and/or ZEB1 lead to a decrease in both CSC properties and chemo-resistance capabilities of various cancer types.[3, 17, 18] Here, we have shown that the inhibition of FOXC2 can lead to an increase in the chemo-sensitivity of CRPC cell lines to docetaxel. MC-1-F2, as the first direct small molecule inhibitor of FOXC2, can aid as an combinatorial treatment for common chemo-therapeutic agents already in use, providing new treatment options for patients. We are currently examning if MC-1-F2 can re-sensitize chemo-resistance prostate cancer to chemotherapy.

SUPPORTING INFORMATION

The Supporting Information is available free of charge.

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Author Contribution

MC and JL contributed to conceptualization. MC, LR, and JO performed experiments. HS made contributions to compound synthesis and Western blot experiments. MC, JL, LR, and JO contributed to writing the manuscript. JL contributed to writing, reviewing, and editing of the manuscript and supervision. All authors have given approval to the final version of the manuscript.

Abbreviations

EMT, Epithelial-Mesenchymal Transition; CSC, Cancer stem cell; FOXC2, Forkhead Box C2; ZEB1, Zinc Finger E-Box Binding Homeobox; AR, Androgen receptor; CRCP, castration-resistant prostate cancer; CI, combination index.

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