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MDSC Trafficking and Function in RCC by CXCR4 in the Presence of a VEGF-R Antagonist is Dependent on HIF-2α Expression

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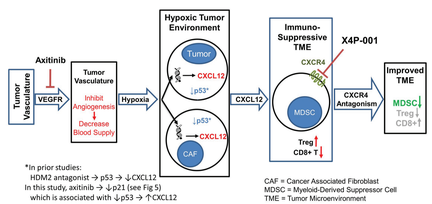
INTRODUCTION

Background: In a murine xenograft model using human RCC 786-0 and A498 cell lines, we have previously demonstrated that acquired resistance to the VEGFR antagonists sunitinib and axitinib was associated with a marked increase in the infiltration of $CD11b^+/Gr\text{-}1^+$ myeloid-derived suppressor cells (MDSC). MDSC express CXCR4 and its ligand, SDF-1/CXCL12, is produced in response to hypoxia induced by VEGFR antagonists. We have recently reported that both the influx of MDSC and resistance to axitinib could be prevented by concurrent administration of X4P-001 (previously AMD11070), a CXCR4 antagonist.

Material and Methods: To investigate the early factors influencing MDSC trafficking with respect to CXCR4 signaling, xenografts from 786-0 cells were established and treated with axitinib, X4P-001, the combination of both agents or saline for 3 or 8 days. At sacrifice tumors were excised and flash frozen in liquid nitrogen for Western analysis, fixed in formalin for IHC and immunofluorescence, or treated with collagenase for the analysis and isolation of MDSC.

Results: As early as Day 3, the combination of X4P-001 and axitinib had additive (synergistic) effects, with 50% suppression of tumor volume compared to controls and to the modest effects of either drug alone. This result paralleled the longer term experiments previously reported. Similarly, by IHC, the tumors from mice receiving axitinib alone had extensive MDSC infiltration by day 3 and continuing to day 8, whereas the tumors from mice receiving either X4P-001 alone or the axitinib/X4P-001 combination had significantly less MDSC infiltration. Mice treated with axitinib alone had an increase in Ki-67 positive tumor cells as early as Day 3, which was not observed in mice that received both X4P-001 plus axitinib, suggesting an anti-proliferative effect of the combination. Of note, mice receiving both X4P-001 and axitinib showed significant suppression of HIF-2α by day 3 as determined by both Western blot analysis and IHC . Furthermore, at Day 8 MDSC were focused near areas of necrosis, suggesting that the hypoxia (and resulting necrosis) induced by axitinib as early as Day 3 of treatment induced SDF-1/CXCL-12 that, in turn, recruits MDSC to the tumor.

Conclusions: The resistance mechanism in RCC xenografts to axitinib occurs by Day 3 after the initiation of treatment, and is dependent on HIF-2α, CXCR4/CXCL12, and the infiltration of MDSC to the tumor. The MDSC then produce proangiogenic factors that mediate VEGFR resistance. Administering X4P-001, a CXCR4 antagonist, concurrently with axitinib, blocks communication between the tumor and the MDSC, suppresses HIF-2α expression, reduces MDSC tumor infiltration, and appreciably improves the anti-tumor treatment effect. X4P-001 in combination with axitinib is currently being evaluated in a phase 1/2 clinical study in relapsed renal cell carcinoma (NCT02667886).



RESULTS

The Combination of Axitinib and X4P-001 Retards Tumor Growth to a Greater Extent than Either Drug Alone

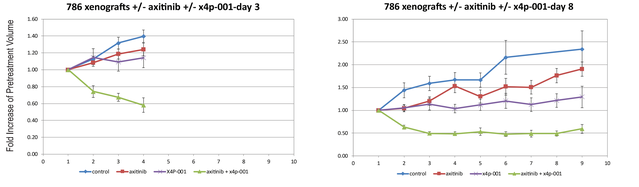


Figure 1. Growth curves of 786 xenografts treated with axitinib +/- X4P-001. Nude beige mice were injected with 10^7 cells sc. When tumors reached 200 mm^3, mice were treated by gavage with axitinib (30 mg/kg), X4P-001 (100 mg/kg), the combination or saline. Data is presented as the fold increase in tumor volume relative to the starting tumor volume.

X4P-001 Prevented Both Ki67 and HIF-2α Increase Caused by Axitinib Treatment

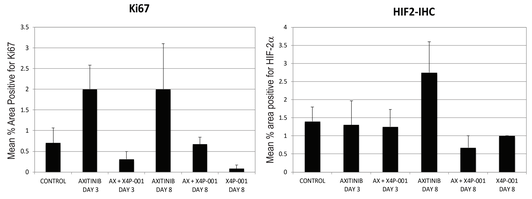


Figure 2. Immunohistochemistry staining of Ki67 (left) and HIF-2α (right) of Day 3 and 8 tumors from 786 xenografts treated with axitinib +/- X4P-001. Data is presented as the percent area positive for Ki67 or HIF-2α.

X4P-001 Reduces MDSC Infiltration Caused by Axitinib as Early as Day 3 Post Treatment

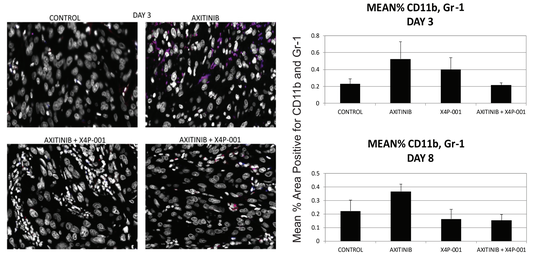


Figure 3. Immunofluorescence for CD11b and Gr-1 positive MDSCs from Day 3 and 8 xenograft tumors treated with axitinib +/- X4P-001. Left. Representative Day 3 tumor tissue from control, axitinib and axitinib + X4P-001 mice. Bar graphs of quantitative analysis of all tumors in each treatment group. Data is presented as the percentage area positive for Cd11b and Gr-1.

X4P-001 Reduces the Increased HIF-2α Caused by Axitinib Treatment

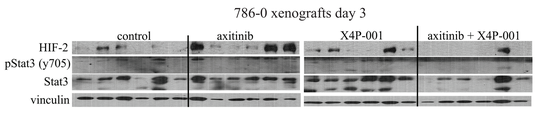


Figure 4. Western blots of 786 xenografts after being treated with axitinib +/- X4P-001.

Axitinib Suppressed the micro-RNAs mir-30a and mir-30c. The Addition of X4P-001 to Axitinib Results in Increased mir-30a and mir-30c at Day 8 Post Treatment in 786-0 Xenograft Tumor

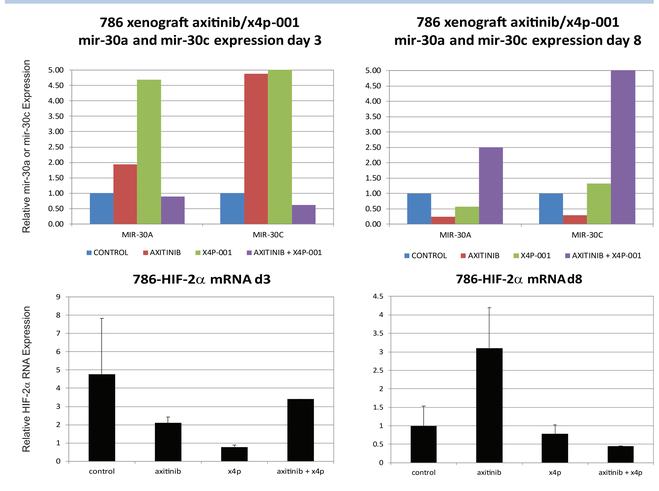


Figure 5. mir-30a and mir-30c microRNA and HIF-2α mRNA expression from tumors of xenografts treated with axitinib +/- X4P-001. Data is presented as mir-30a or mir-30c expression relative to the mean control value (left side) and relative HIF-2α RNA expression.

X4P-001 Results in mir-30a/mir-30c Induction and HIF-2α Reduction in Hypoxic Cells *in vitro*

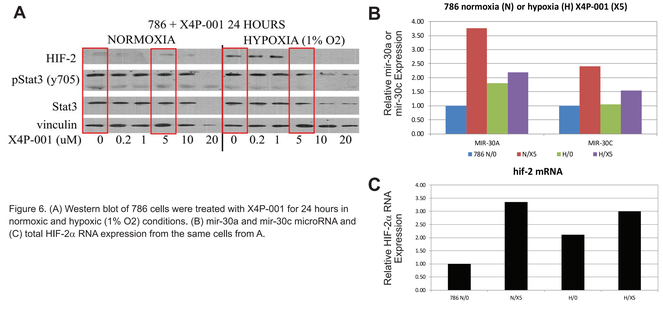


Figure 6. (A) Western blot of 786 cells were treated with X4P-001 for 24 hours in normoxic and hypoxic (1% O2) conditions. (B) mir-30a and mir-30c microRNA and (C) total HIF-2α RNA expression from the same cells from A.

The Suppression of HIF-2α and the Induction of mir-30a and 30c is Dependent on Stat3 Expression

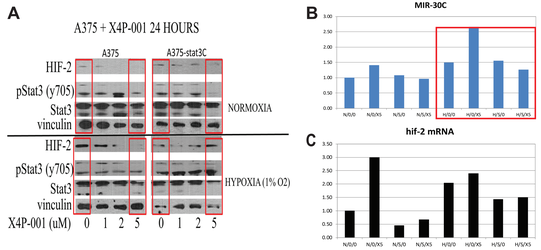
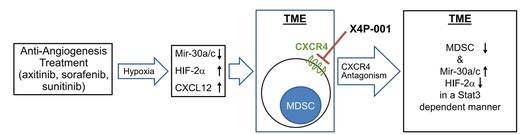


Figure 7. (A) Western blots from lysates of A375 or A375 cells transfected with a constitutively active Stat3 construct. Cells were treated X4P-001 for 24 hours in normoxic or hypoxic conditions. (B) mir-30c microRNA and (C) total RNA expression from the same cells from A.

Schematic of Proposed Mechanism of Action of X4P-001 Under Hypoxic Conditions



SUMMARY

- The combination treatment of X4P-001 and axitinib demonstrated significantly more potent anti-tumor activity than either agent alone in two renal xenograft models.
- X4P-001 suppressed the increased Ki67, increased MDSC and HIF-2α by axitinib treatment as early as Day 3 post treatment.
- Axitinib suppressed the micro-RNAs mir-30a and mir-30c, which have been reported to inhibit HIF-2α translation. The addition of X4P-001 to axitinib *in vivo* and in hypoxic cells *in vitro* results in increased mir-30a and mir-30c.
- Suppression of HIF-2α and induction of mir-30a and mir-30c by X4P-001 could be prevented by constitutive Stat3 expression.

CONCLUSIONS

- The resistance mechanism in RCC xenografts to axitinib occurs by Day 3 after the initiation of treatment, and is dependent on HIF-2α, CXCR4/CXCL12, and the infiltration of MDSC to the tumor. Administering X4P-001, a CXCR4 antagonist, concurrently with axitinib, blocks communication between the tumor and the MDSC, suppresses HIF-2α expression, reduces MDSC tumor infiltration, and appreciably improves the anti-tumor treatment effect. X4P-001 in combination with axitinib is currently being evaluated in a phase 1/2 clinical study in relapsed renal cell carcinoma (NCT02667886).