

Grant proposal (F Demichelis, UniTrento) – February 2023

Title: Exploring pharmaceutical sensitivities exposed by 9p21 loss in bladder cancer

Background and Study Hypothesis

Bladder cancer is the fifth most common cancer in the western world. Most patients (around 80%) present with non-muscle invasive bladder cancer (NMIBC) at diagnosis, which has a relatively favorable prognosis but is associated with considerable morbidity and high cost for managing treatment¹. Unfortunately, 70% of NMIBC cases will recur after surgery, and 10-20% will eventually progress to muscle-invasive bladder cancer (MIBC). Patients with MIBC have a relatively poor prognosis with a 5-year survival rate of 10-50% depending on the tumor stage at the time of diagnosis^{2,3}. While immunotherapy offers a potentially valuable option to a selected number of patients, bladder cancer remains a major cause of cancer-related death, underlying the need for new therapeutic strategies for this tumor type.

Synthetic lethality-based strategies - two genes are defined as synthetic lethal when their simultaneous inactivation causes cell death^{4,5}, whereas a deficiency of only one of these genes does not – can be exploited to nominate potential targets in specific genomic backgrounds. This concept has been extended to the interactions between pharmacological agents and deletion or mutation in tumor suppressor genes, where drug treatment kills mutated cancer cells while having little effect on normal (wild-type) cells. Deletion of the 9p21 locus is a very frequent event in bladder cancer, identified in 27.2% of the TCGA muscle-invasive bladder cancer cohort, making it a perfect candidate for the search of synthetic lethal interactions⁶. The locus includes the tumor suppressors *CDKN2A/2B* and the metabolic gene *MTAP*, involved in the salvage pathway of methionine and adenine. Large-scale short-hairpin RNAs (shRNAs) depletion screens identified a selective dependence of *MTAP*-deleted cancer cells on protein arginine methyltransferase 5 (PRMT5), methionine adenosyltransferase 2A (MAT2A) and PRMT5 binding partners WDR77, pICln and RIOK1⁷⁻⁹. Indeed, *MTAP*-deleted cells have increased levels of methylthioadenosine (MTA) that partially inhibits PRMT5 activity and makes them more sensitive to a further PRMT5 inhibition. Literature findings suggest that PRMT5 and MAT2A inhibition causes splicing defects in *MTAP*-deleted cells that favor the formation of RNA/DNA structures called R-loops¹⁰. The accumulation of R-loops is responsible for replication fork stalling, ultimately leading to DNA damage. These exciting findings stimulated the design and development of highly specific PRMT5 and MAT2A inhibitors that are now in clinical trials for a broad range of tumors harboring *MTAP*-loss (NCT03435250, NCT04794699 and NCT05245500).

To identify bladder cancer specific vulnerabilities associated with 9p21 loss, the Demichelis laboratory generated *CDKN2A/2B/MTAP* isogenic bladder cancer cell lines employing CRISPR/Cas9 technology. We selected three cell lines with different genomic backgrounds: HT1197 (RB1^{+/+}, TP53^{+/+}), T24 (RB1^{+/+}, TP53^{mut/mut}) and TCCSUP (RB1^{-/-}, TP53^{mut/mut}) and first evaluated the functional impact of the deletion in a competitive proliferation assay. Our results show that HT1197 and TCCSUP 3KO clones have higher proliferation rates compared to WT clones. On the other hand, T24 clone proliferation is not altered by 9p21 loss. We next used the HT1197 isogenic pair (WT/3KO) to perform a multi-parameter drug screening aimed at uncovering pharmaceutical sensitivities exposed by the deletion. Methotrexate (MTX), raltitrexed (RTX) (two antifolate agents) and cytarabine (a nucleoside analog) were found and confirmed to be more effective in 3KO clones.

Study Aims

Based on the current results (see next section), we here propose the following specific aims:

Aim 1: To combine multiple pharmacological approaches to maximally exploit the synthetic lethal vulnerabilities of 9p21-deleted cancer cells.

Aim 1.1: To test additional drugs that may have a genotype-specific response based on evidence from the literature.

Aim 2: To validate the selective effect of methotrexate, raltitrexed, and cytarabine on other 9p21-deleted cellular models.

Supporting preliminary results

Multi-parameter drug screening

We designed a High Content drug Screening (HCS) based on multi-parameter phenotypic profiling. Two HT1197 clones, one 9p21 WT, and one 3KO, were treated with a library of 2,351 compounds at a single concentration for 48 hours (**Fig. 1A**). The experiment was performed in duplicate, with or without pre-treatment with the CDK4/6 inhibitor palbociclib at the dose of 2.5 μ M for 24 hours. The screened compounds include two collections: a) 349 anticancer compounds (Selleck): anti-cancer small molecules in clinical use or in clinical trial; and b) MicroSource Spectrum Collection (2100) which includes FDA approved drugs, known drugs, natural products, and bioactive compounds. Cells were fixed and stained with Hoechst and Alexa 594-conjugated phalloidin. Fluorescence images were acquired with Operetta® and analyzed for differential compound effects using 13 informative features. The analysis nominated 75 compounds of therapeutic potential as single agents and 76 active only when combined with palbociclib. The nominated candidates were re-tested for technical validation on the same platform, using the same protocols and more stringent statistics. 45.8% of the compounds were validated supporting the accuracy of the compound selection and offering a set of drugs to be further investigated for dose- and time-response. Of note, the effect on the morphological features was confirmed for all the compounds tested but did not translate in a differential effect on cell viability (or a different IC₅₀).

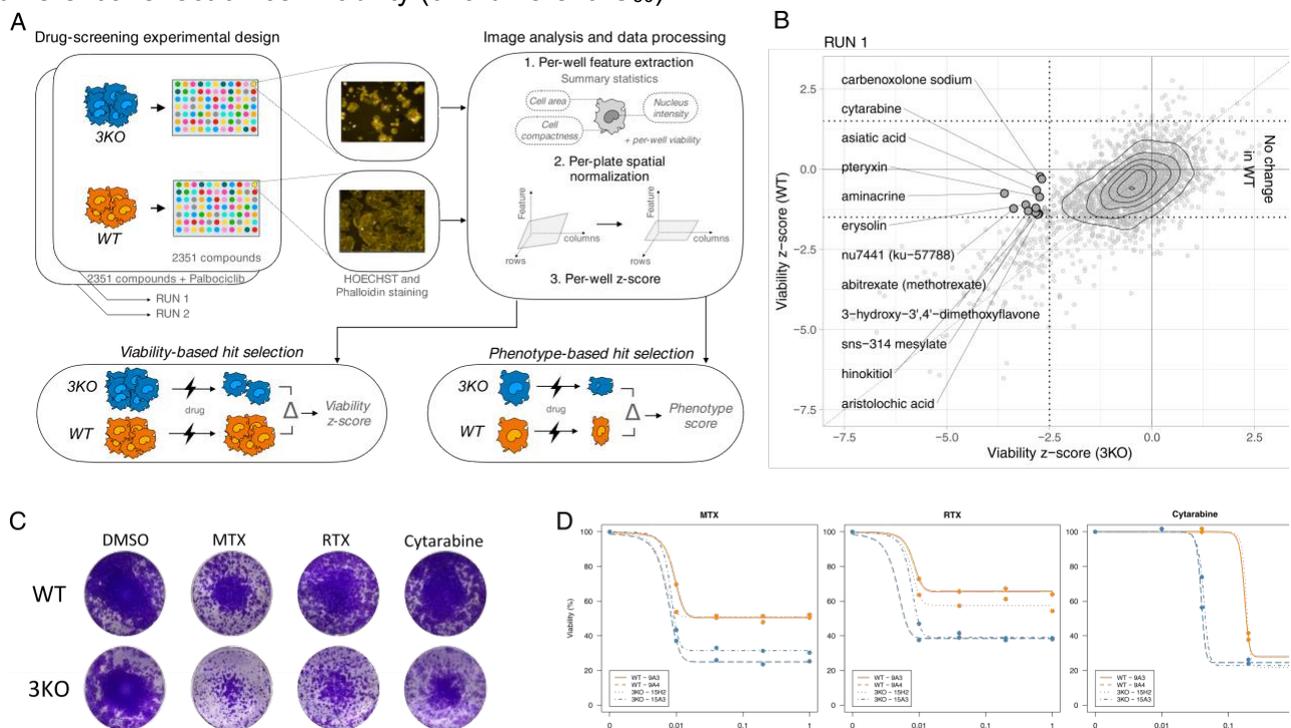


Figure 1: CDKN2A/2B/MTAP deficient bladder cancer cells are selectively sensitive to MTX, RTX and cytarabine. (A) Schematic illustration of the multi-parameter drug screening workflow in the HT1197 9p21-isogenic pair. **(B)** Viability Z-scores of the tested drugs in WT and 3KO clones. The compounds that specifically affected 3KO clone viability are listed. **(C)** Crystal violet assay of HT1197 9p21-isogenic pair treated with DMSO, MTX (40nM), RTX (40nM) and cytarabine (40nM) for 7 days.

(D) Cell viability after treatment with increasing concentrations of MTX, RTX and cytarabine (0.01-1 μ M) for 7 days.

Therefore, we concentrated our efforts on a selection of 18 compounds that specifically affected 3KO clone cell number (Fig. 1B). These compounds interfere with DNA synthesis, steroid biosynthesis and the metabolism of folate, serotonin, and choline. A genotype-specific response was confirmed for two drugs that affect folate metabolism (MTX and RTX) and for a drug that affects DNA synthesis (cytarabine) (Fig. 1C). Dose-response proliferation assays (crystal violet) and metabolic assays (CCK8 assay) were performed after 3, 7 and 14 days of treatment and demonstrated a higher sensitivity of HT1197 3KO clones to the tested drugs compared to HT1197 WT clones (Fig. 1D). Our findings are corroborated by the recent findings of Alhalabi et al. which reported that the antifolate agent pemetrexed is selectively effective in *MTAP*-deficient bladder cancer patients and preclinical models¹¹.

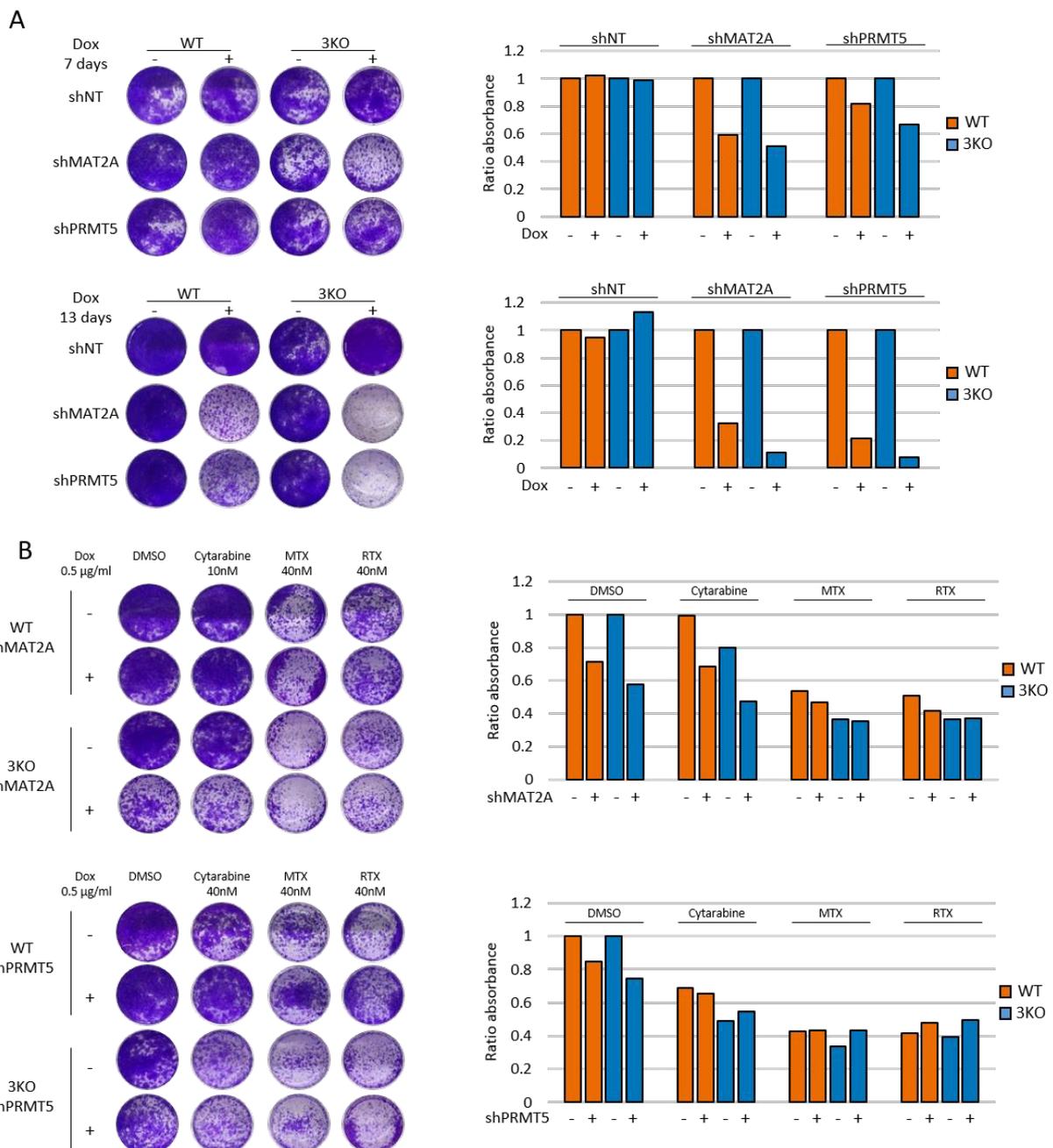


Figure 2: Combination of 9p21-selective drugs with MAT2A and PRMT5 depletion. (A) Crystal violet assay of HT1197 9p21-isogenic pair expressing inducible shNT, shMAT2A or shPRMT5. shRNAs were induced with doxycycline (Dox 0.5 μ g/ml) for 7 and 13 days. **(B)** Response of HT1197

9p21-isogenic pair to MTX, RTX and cytarabine treatment upon depletion of MAT2A/PRMT5. Cells were treated for 7 days.

Combination of therapeutic strategies

We hypothesized that PRMT5 and MAT2A inhibition could synergize with MTX, RTX and cytarabine. In order to verify whether our 3KO clones are selectively sensitive to PRMT5 and MAT2A inhibition, we genetically depleted PRMT5 and MAT2A using doxycycline-inducible shRNAs. As expected, 3KO clones are more sensitive to PRMT5 and MAT2A knockdown (particularly evident at 13 days of induction), confirming the synthetic lethal interactions (**Fig. 2A**). We then combined the two treatments by inducing shRNAs expression and adding the three drugs to the isogenic pair for 7 days (**Fig. 2B**). Our preliminary results suggest that MAT2A depletion and cytarabine treatment have additive effects, while the treatment with MTX and RTX does not (even if MTX and RTX alone have stronger effects in 3KO cells). On the other hand, we did not detect any combinatorial effect with PRMT5 depletion with this time frame, possibly because 7 days of silencing are not enough to observe a sufficient genotype-specific effect on cellular viability. We expect that a schedule optimization of silencing and treatments may improve the outcome.

Methods

Combination of pharmacological inhibitors to assess synergistic effects. Given the promising results obtained with the combination of cytarabine and the genetic depletion of MAT2A, we will evaluate the synergy with pharmacological inhibitors of MAT2A. Indeed, the potent *MTAP*-null selective inhibitor of MAT2A AG-270 (currently tested in clinical trials) has been recently made available to the market¹⁰. Several PRMT5 inhibitors have been designed and tested in clinical trials. However, because of their mechanism of action, these agents do not selectively inhibit PRMT5 activity in *MTAP*-deleted tumors. More recently a new drug was proposed that targets and stabilizes the PRMT5-MTA complex, causing selective PRMT5 inhibition in *MTAP*-deleted cells has been developed¹². We plan to test these highly specific MAT2a and PRMT5 inhibitors in our isogenic model to verify if they recapitulate the effect of genetic depletion. While the silencing of PRMT5 and MAT2A is an effective proof of concept, the efficacy of this approach has to be proven with the pharmacological treatment. Furthermore, the specific PRMT5 and MAT2A inhibitors will be tested in combination with the drugs identified by the drug screening, to explore combinatorial effects. Additionally, we plan to test the CDK4/6 inhibitor abemaciclib. Although *CDKN2A* status might be a reasonable genetic determinant of sensitivity to CDK4/6 inhibitors, *CDKN2A* has both been reported to be and not to be a promising biomarker of response to abemaciclib in clinical trials^{13,14}. However, CDK4/6 inhibitors can be combined with other agents to increase their efficacy. Prolonged treatment with CDK4/6 inhibitors has been shown to induce replicative stress in cancer cells, causing DNA damage and increasing sensitivity to chemotherapeutic agents¹⁵. We will test different treatment settings: pre-treatment with abemaciclib followed by MTX, RTX and cytarabine treatment or a combined and simultaneous treatment. Furthermore, we plan to test abemaciclib with the genetic interference of PRMT5 and MAT2A. Indeed, it has been reported that CDK4/6 inhibitors reduce PRMT5 activity and when combined with PRMT5 inhibitors strongly suppress proliferation of melanoma cells¹⁶.

Assessment of the selective effect of methotrexate, raltitrexed and cytarabine on other 9p21-deleted cellular models. We will take advantage of our established T24 and TCCSUP 9p21-isogenic cellular models in order to confirm the genotype-selective effect of MTX, RTX and cytarabine. The impact on cellular viability will be studied by dose-response proliferation assays at different time points to best delineate the therapeutic window of the treatments. In addition to the

9p21-isogenic pairs, we will perform the same experiments using a small panel of *MTAP*^{+/+} (HT1197, T24, TCCSUP and J82) and *MTAP*^{-/-} (UMUC-3, RT4, RT112 and 253J) bladder cancer cell lines. Finally, we will characterize the effects of the three drugs using apoptosis and cell cycle assays.

Expected pitfalls No significant pitfalls are expected for the current study as most assays and techniques are already in the laboratory. It is possible that some of the drugs identified are effective on a specific cell line, that our findings may not be generalized on other urothelial cell models or that the drug combinations we are proposing have no combinatorial effect.

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Budget and Timeline

Budget

	Amount (Euros)
Drugs	2,000
Antibodies	2,000
Consumables	4,000
Facilities	3,000
Personnel	14,000
Total	25,000

Timeline	Months				
	1-4	5-8	9-12	13-16	17-18
Aim 1: To combine multiple pharmacological approaches to maximally exploit the synthetic lethal vulnerabilities of 9p21-deleted cancer cells.					
Aim 1.1: To test additional drugs that may have a genotype-specific response based on evidence from the literature.					
Aim 2: To validate the selective effect of methotrexate, raltitrexed, and cytarabine on other 9p21-deleted cellular models.					
Submission and revision of the manuscript					