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A Chemical Genomics Approach to Drug Reprofiling in Oncology: Antipsychotic Drug Risperidone as a Potential Adenocarcinoma Treatment

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Abstract. Drug reprofiling is emerging as an effective paradigm for discovery of cancer treatments. Herein, an antipsychotic drug is immobilised using the Magic Tag® chemical genomics tool and screened against a T7 bacteriophage displayed library of polypeptides from *Drosophila melanogaster*, as a whole genome model, to uncover an interaction with a section of 17-β-HSD10, a proposed prostate cancer target. A computational study and enzyme inhibition assay with full length human 17-β-HSD10 identifies risperidone as a drug reprofiling candidate. When formulated with rumenic acid, risperidone slows proliferation of PC3 prostate cancer cells *in vitro* and retards PC3 prostate cancer tumour growth *in vivo* in xenografts in mice, presenting an opportunity to reprofile risperidone as a cancer treatment.

Highlights. The highly novel aspects of our program include:
- demonstration that our recently developed Magic Tag® chemical genomics platform can be used to "reprofile" drugs into cancer indications;
- the use of fruit fly *Drosophila melanogaster* as an unusual model organism for cancer drug discovery;
- the further exploration of 17-β-hydroxysteroid dehydrogenase 10 as an emerging cancer target;
- the potential of VAL401 as a candidate for clinical trials in adenocarcinomas.

Keywords.
- chemical genomics
- drug reprofiling
- *Drosophila melanogaster*
- 17-β-hydroxysteroid dehydrogenase 10
- adenocarcinoma

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1. Introduction

In the search for new cancer treatments, the potential of existing drugs that have been or are being used for non-cancer indications remains under-investigated [1]. Drug reprofiling, (or repurposing or repositioning), is a commercially attractive route to new therapies, since it reduces risks in the development process by exploiting the well-known safety and pharmacokinetic profiles of the drugs [2]. In oncology, reprofiling successes include thalidomide (anti-emetic to multiple myeloma) and zoledronic acid (anti-bone resorption to multiple myeloma, prostate cancer and breast cancer) [3].

Very recently, systematic experimental [4] and *in silico* [5] approaches to reprofiling for oncology have emerged. Our Magic Tag® chemical genomics tool was designed to link the known biological activity of an organic chemical with the genome as expressed through a phage displayed library of polypeptides [6,7]. Herein we demonstrate that Magic Tag® has the potential to be a new and complementary method for systematically screening known drugs to identify reprofiling opportunities. Specifically, we present the widely used antipsychotic therapy risperidone as a possible treatment for prostate cancer and other adenocarcinomas.

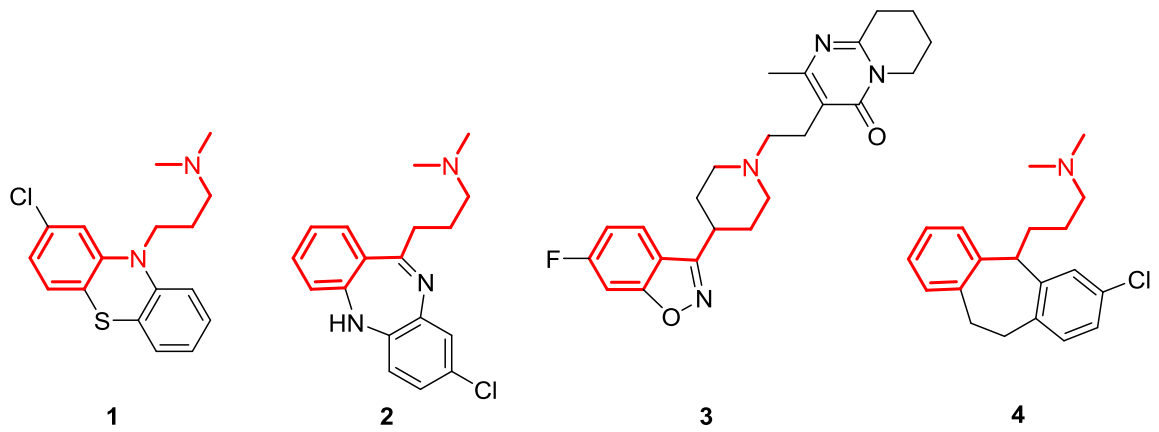


Figure 1. Chemical structures of chlorpromazine **1**, clozapine **2**, risperidone **3** and clomipramine **4**, with the common pharmacophore highlighted in red.

Antipsychotic drugs such as chlorpromazine **1**, clozapine **2** and risperidone **3** have long been known to display polypharmacology [8]. Known to have multiple protein targets, such drugs are interesting candidates for drug reprofiling. We decided to photoimmobilise one drug from this class, chlorpromazine **1**, using Magic Tag® and screen it against a library of polypeptides displayed on T7 bacteriophage, as previously described [7].

For drug reprofiling, a whole genome model is needed; we elected, as in our previous study of flecainide acetate, to use a display library originating from *Drosophila melanogaster* [9]. Despite its central importance to developments in both genetics and genomics, the common fruit fly, *D. melanogaster*, is underexploited in the field of drug discovery [10, 11]. Around 60% of *D. melanogaster* genes have human orthologs [12]. Significantly, around three quarters of genes related to human disease states

have orthologs in *D. melanogaster* [7] and all major human signaling pathways are present in *D. melanogaster* [10].

2. Materials and Methods

The Magic Tag® screen and enzyme kinetics studies used published methods [7, 13, 14]. For other experiments chemicals, drugs and cell line media were acquired from Sigma Aldrich, Cell lines were acquired from ECACC or ATCC. The CytoTox 96 Non-radioactive cytotoxicity assay (LDH Assay) was acquired from Promega (G1780).

Preparation of drug treatments for cell line experiments. Stocks of rumenic acid were prepared at 100mM and risperidone at 10 mM in ethanol, aliquotted for storage. For use, stocks were thawed at room temperature for 15 mins then dissolved 1:10 into F12K media containing 125μM fatty acid-free BSA, mixed by gentle vortexing and observations made. Where necessary preparations were placed in water bath at 37 °C to aid solubility.

For preparation of VAL401 for testing *in vitro*, rumenic acid stocks were first prepared in fatty acid free BSA-F12K media, with serial dilution to a final "working" concentration of 100μM, vortexed gently to mix and incubated at 37 °C for 1 hour. Risperidone was added to the fatty acid BSA-media to the appropriate concentrations before addition to the cells. Negative control wells were generated by the treatment of cells with media containing an equivalent concentration of ethanol; and positive control wells were generated by the use of wells containing media and treatment, but no cell monolayer.

PC3 lysed cell LDH Assay. PC3 cells were seeded at a density of 4000 cells per well in a 96 well plate, and incubated at 37 °C, 5% CO_2 for 48 hours in standard growth media (F12K nutrient mix + 7% fetal calf serum + 2 mM L-glutamine + 45 mg/L ascorbic acid). The media was then removed by pipette and replaced with the appropriate drug treatment in treatment media (F12K nutrient mix + 125 μM fatty acid free BSA + 2 mM L-glutamine + 45 mg/L ascorbic acid), and incubated for a further 96 hours at 37 °C, 5% CO_2.

The supernatant was removed from all wells of cells, and cells washed with 200 μl PBS. After removal of the PBS, the cells were lysed with 0.9% Triton-X in PBS for 2 hours at 37 °C, 5% CO_2. 50 μl of this cell lysate was transferred to a fresh 96 well plate, 50 μl of CytoTox 96 assay reagent was added and the plate incubated at room temperature in the dark for 20 minutes. 50 μl stop reagent was then added and the optical absorbance at 492 nm was recorded.

The percentage cell count was counted by normalising the untreated wells of cells as 100% growth, and wells containing no cells normalised to 0%. The mean and standard error of the replicate wells was calculated.

Mouse xenograft experiments. Male MF-1 nude mice were obtained from the commercial suppliers (Harlan, UK) and were at least 7 weeks of age before being recruited to the study. Mice were maintained in sterile isolators within a barriered unit illuminated by fluorescent lights set to give a 12 hour light-dark cycle (on 07.00, off 19.00), as recommended in the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. The room was air-conditioned by a system designed to maintain an air temperature range of 21 ± 22 °C. Mice were from the same litter and

housed in social groups of 4 during the procedure in plastic cages (Techniplast UK) with irradiated bedding and provided with both nesting materials and environmental enrichment. Sterile irradiated 2019 rodent diet (Harlan Teckland UK, product code Q219DJ1R2) and autoclaved water were available *ad libitum*. Each animal was allocated a unique identification number by implantation of a transponder (Microchip Identification Devices - Fingerprint UK).

The PC·3M·luc cells were maintained *in vitro* in RPMI culture medium (Sigma, UK) containing 10% (v/v) heat inactivated foetal bovine serum (Sigma, Poole, UK) & 2 mM L·glutamine (Sigma, UK) at 37 °C in 5% CO_2 and humidified conditions and selected once a week with Zeocin (0.2mg/ml). Cells from sub-confluent monolayers were harvested with 0.025% EDTA, washed in culture medium and counted. On day of initiation cells were harvested from semi-confluent monolayers with 0.025% EDTA, washed twice in the culture medium and counted. Cells with viability of >90% were re-suspended, for *in vivo* administration, in sterile phosphate buffered saline, pH 7.4 (PBS) at $5x10^7$ cells/ml.

Tumours for the orthotopic xenograft efficacy study were surgically initiated in the prostate of 80 male MF-1 nude mice in 3 batches over 3 days (1 batch per day) with the middle day being designated as day 0. Mice were imaged on either day 6 or day 7 prior to dosing in order to obtain bioluminescent (BLI) photon counts for randomisation and assignment to treatment groups. In order to maintain social groups, mice were randomised on a per cage basis. Mice that had very low or no BLI signal were excluded from the randomisation cohort as were mice with complications arising from surgery or those with extensive fighting injuries.

Mice were dosed orally, twice daily from day 7, with the exception of imaging days when the morning dose was not given to allow for the imaging anaesthetic and a period of unconsciousness. The positive control, docetaxel was dosed twice weekly intravenously. VAL401 and risperidone dosing was initiated under an escalation schedule such that intended doses were reached by Day 14 according to the groups in the schedule below:

Vehicle Group	Days 7 – 42	Vehicle
Risperidone Group	Days 7 – 10	Risperidone 0.25 mg/kg
	Days 11 – 14	Risperidone 0.5 mg/kg
	Days 12 – 42	Risperidone 1 mg/kg
Rumenic Acid Group	Days 7 – 10	Rumenic Acid 0.17 mg/kg
	Days 11 – 14	Rumenic Acid 0.35 mg/kg
	Days 12 – 42	Rumenic Acid 0.68 mg/kg
VAL401 low dose Group	Days 7 – 10	VAL401 0.25 mg/kg
	Days 11 – 42	VAL401 0.5 mg/kg
VAL401 high dose Group	Days 7 – 10	VAL401 0.25 mg/kg
	Days 11 – 14	VAL401 0.5 mg/kg
	Days 12 – 42	VAL401 1 mg/kg
Positive control Group	Twice weekly Days 7 - 42	5 mg/kg i.v. Docetaxel

VAL401 concentrations are quoted with respect to the risperidone content, with the rumenic acid being equimolar. Risperidone was made to a stock of 10 mM and rumenic acid to a stock of 100 mM in ethanol. These ethanol solutions were diluted into water to achieve concentrations such that for 0.25 mg/kg dosing the mice received 5ml/kg, for 0.5 mg/kg 10 ml/kg and for 1 mg/kg 10 ml/kg based on risperidone concentrations. The vehicle group was dosed with a comparable volume of diluted ethanol solution.

Mouse body weights were monitored for the duration of the study. Generally, body weight was well maintained and increased over the duration of the study. Non-surviving mice were terminated according to home office regulations dictated by size of tumour.

When humane endpoints, associated termination days and BLI values ($1x10^{10}$ photons/s) were used to conduct a surrogate endpoint survival analysis, median survival times ($t_{1/2}$) were calculated to be 34 days for the vehicle treated group, and 40 days for both the single agent risperidone and rumenic acid groups. Due to the high proportion of surviving mice in the VAL401 group and the docetaxel positive control group, median survival times could not be calculated. No premature terminations were carried out in the docetaxel treated group demonstrating success of the experimental conditions.

3. Results

Using the method we described previously [9] we immobilized chlorpromazine **1** in the wells of a Magic Tag® plate. Our method ensures that non-specific binding of proteins from the library is minimized, by using "protein resistant" oligo(ethylene glycol) groups and also that the bioactive molecule is presented to the library in numerous different orientations, thus maximizing the number of specific interactions with the library.

The immobilized chlorpromazine **1** was exposed to the *D. melanogaster* phage-displayed library of polypeptides in three rounds of biopanning. After all three rounds, phage that did not bind were removed by washing and the remaining, bound

phage were amplified by re-infection of *E. coli*. After the final amplification step, 46 significant plaque forming units were selected and the oligonucleotide sequences were obtained. Elimination of 11 clones that also occurred in the negative control wells (no drug added) left 35 clones of interest. These were submitted to a selective elution experiment, as we have described previously [6, 7] with a solution of free chlorpromazine. Significant elution in this experiment should indicate that a clone binds to unmodified drug as well as to a surface-modified analogue. Eight clones were judged to merit further study on this basis.

Broadly in accordance with the extent of homology between humans and our chosen model *D. melanogaster*, discussed above, six of the eight clones had DNA inserts that corresponded to sections of orthologous human genes, of which one had previously been proposed as a therapeutic "target". 17-β-Hydroxysteroid dehydrogenase 10 (17HSD10, also known as ABAD, ERAB, Type II HADH, SCHAD *etc.*) is an intracellular binding partner for Amyloid β-Peptide (Aβ) and Kissinger *et al.* suggested inhibiting 17HSD10 as a new approach to treating of Alzheimer's Disease [13]. Additionally, 17HSD10's function in dihydrotestosterone synthesis led some authors to propose it as a prostate cancer target [15]. The enzyme is highly expressed in prostate cancer bone metastases, as compared with non-malignant and primary tumor tissues [16]. Recently, it has become apparent that 17HSD10 is overexpressed in many cancerous cells, particularly those of adenocarcinoma types [17], with the enzyme's multifunctional nature contributing more broadly to the stabilisation of cancer cells [18].

To test whether antipsychotic drugs such as chlorpromazine would interact with normally expressed human 17HSD10 *in vitro*, we studied their effects on the recombinant 17HSD10 catalyzed oxidation reaction of estradiol and corresponding reduction of (acetylaceto) coenzyme A, using literature procedures [13, 14]. As well as chlorpromazine **1**, we investigated two further antipsychotic drugs, clozapine **2** and risperidone **3** that share a pharmacophore (Fig. 1). All three drugs **1**, **2** and **3** might be expected to exhibit similar polypharmacology, potentially including an association with 17HSD10. Clomipramine **4** was also included as a pharmacologically distinct antidepressant that, nevertheless, contains the same pharmacophoric element as chlorpromazine, clozapine and risperidone. The antihistamine diphenhydramine has neither the polycyclic structure of drugs **1** to **3** nor their pharmacological profile and was not expected to inhibit 17HSD10.

	Drug type	Oxidation rate / $\mu M\ min^{-1}$	Reduction rate / $\mu M\ min^{-1}$
No drug		8.4	8.8
Chlorpromazine	Antipsychotic	7.7	7.2
Clozapine	Antipsychotic	4.8	7.5
Risperidone	Antipsychotic	1.0	3.3
Clomipramine	Antidepressant	2.3	7.5
Diphenhydrazine	Antihistamine	8.0	8.3

Figure 2. Inhibition by chlorpromazine and related drugs of reactions catalyzed by 17HSD10. Oxidation and reduction were studied using estradiol and (acetoacetyl) Coenzyme A substrates, respectively [13, 14]. Reaction progress was monitored by following the NAD absorbance at 340 nm.

After initiation of the oxidation reaction by addition of estradiol, chlorpromazine, like the sedative diphenhydramine, was shown to have virtually no effect on the 17HSD10-catalyzed dehydrogenation of estradiol. The other drugs tested showed higher levels of inhibition (Fig. 2). In the reduction reaction, chlorpromazine,

clomipramine and diphenhydramine were shown to have little inhibitory effect on the 17HSD10-catalyzed hydrogenation of acetoacetyl coenzyme A. Clozapine and risperidone showed some inhibition (Fig. 2). To better understand why risperidone **3** was the best inhibitor of 17HSD10 in both the oxidation and reduction reactions, we undertook a computational study. The crystal structure of human 17HSD10 in the presence of a potent inhibitor **5** has been disclosed [13], allowing us to attempt *in silico* docking studies. Docking of risperidone **3**, AG18051 **5**, known inhibitor RM-532-46 **6** [15], and the NAD co-factor in 17HSD10, both in the presence and absence of water and the NAD co-factor respectively, was performed using AutoDock 4.2 [19, 20] and Glide docking software [21, 22]. After analysis of the resulting substrate-enzyme models (see the Supporting Information), the different docking poses, calculated using Glide and AutoDock 4.2 respectively, were scored and compared (Table 1, Supporting Information).

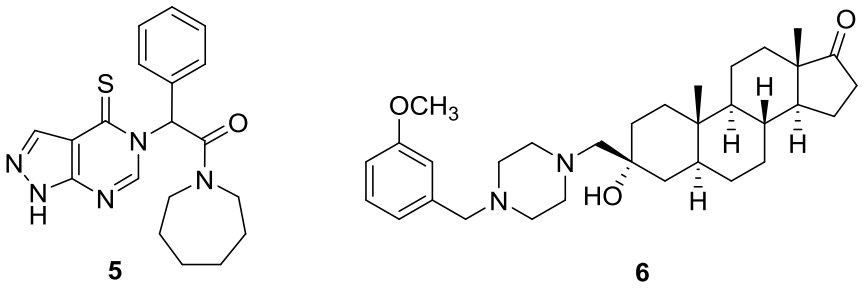


Figure 3. Known inhibitors of 17HSD10 ASG18051 **5** [13] and RM-532-46 **6** [15].

Risperidone was predicted to bind best when there was no NAD co-factor present, which implies a competitive binding mode. This is a similar mode of binding to that predicted and observed for RM-532-46 **6** [15] (Fig. 4). Unlike the case of the AG18051 inhibitor, risperidone is unable to form a covalent interaction with the NAD co-factor which may therefore favour the predicted competitive mode of binding [13].

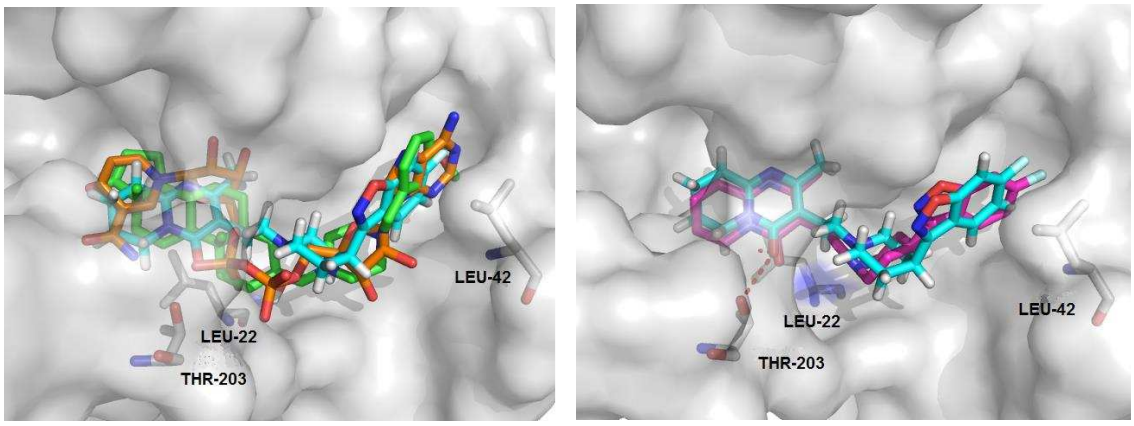
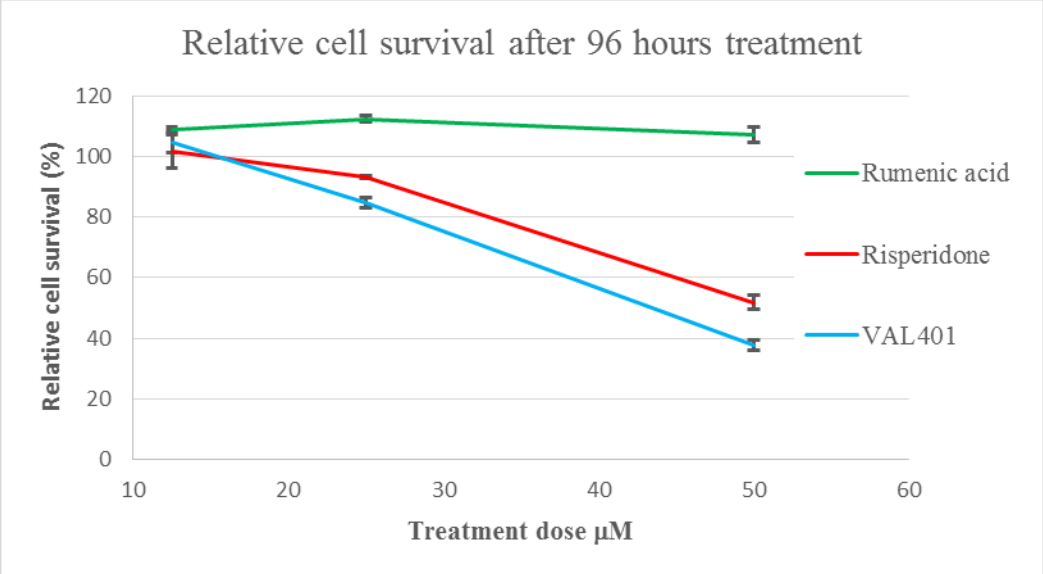


Figure 4. Predicted binding of risperidone to 17-HSD10: (a) overlay of risperidone (cyan), RM-532-46 (green) and NAD co-factor (orange), modelled in the active site of 17-HSD10 (PDB-ID: 1U7T); and (b) overlay of risperidone docked with Glide (cyan) and AutoDock (pink).

To further investigate the binding of risperidone, chlorpromazine, clozapine and clomipramine to 17HSD10, we performed additional *in silico* docking studies. Docking of each substrate into the crystal structure of 17HSD10 (PDB-ID: 1U7T) in the absence of the NAD co-factor was performed using AutoDock 4.2 and Glide docking software respectively. Following analysis, each substrate-enzyme model (see the Supporting Information) was scored using Glide and AutoDock 4.2 (Table 2, Supporting Information). The highest-ranking substrate in terms of both the Glide and AutoDock scores is risperidone.

The sequence of our isolated clone includes most of the amino acids in close contact with the bound inhibitor **5** in the crystal [13]. The key tyrosine 168 and glutamine 165 residues are both present in our analogue, as are the flanking alanines 95 and 97. Leucines 205, 206 and 209, which have been shown by Kissinger *et al.* to be in the active site [13], lie beyond the phage-displayed polypeptide sequence in our experiment.

Given the literature observations on the importance of 17HSD10 expression in both Alzheimer's disease and in prostate cancer, the finding that risperidone may be a competent inhibitor of the enzyme presents two drug reprofiling opportunities. Given the interest in reprofiling for oncology discussed above, we proceeded with the formulation of a risperidone-based drug for prostate cancer. We were aware of a significant literature summarized recently by Polavarapu *et al.* that suggests that polyunsaturated fatty acids, in particular in combination with certain other drugs, could potentially have anti-cancer activity [23]. Following both scientific (data not shown) and commercial evaluation, we designed VAL401 [24], a formulation of risperidone in rumenic acid. Rumenic acid, or 9-*cis* 11-*trans* linoleic acid is an important naturally occurring conjugated linoleic acid (CLA) found in particular in cow's milk [25].



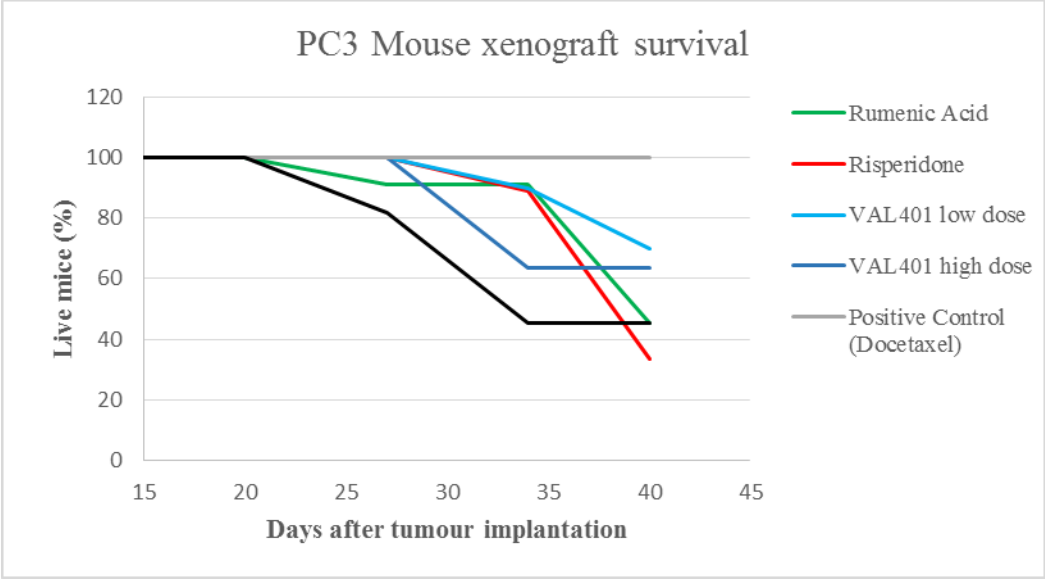


Figure 5. Effects of risperidone, rumenic acid and the VAL 401 formulation on prostate cancer cells and xenografts: (a) survival of PC3 prostate cancer cells at varying dose after 96 hours continuous treatment; and (b) survival of mice with PC3 orthotopic prostate cancer xenografts after daily oral treatment.

VAL401 was tested *in vitro* against the PC3 prostate cancer cell line, which originates from bone cancer metastases. The experiment measured the number of cells still viable after 96 hours treatment using a total cell count assay which lyses the remaining cells and measures the level of lactate dehydrogenase (LDH) produced (Promega Cytox96 non-radioactive cytotoxicity assay). The percentage cell count was calculated by normalizing untreated cells as 100% growth and wells containing as 0%. The mean of the four replicate wells was calculated, and the standard error used to produce the error bars. While risperidone alone was seen to have some activity against PC3 cells, rumenic acid alone had no effect at this level of dosing (Fig. 5a). The formulation of risperidone in rumenic acid (VAL401) was most effective in slowing PC3 cell growth in this experiment.

Finally, VAL401 was administered *in vivo* to mice implanted with PC3 prostate cancer xenografts. The survival of mice during this experiment is displayed according to group (Fig. 5b). The VAL401 low dose group shows 70% survival, whereas risperidone and rumenic acid on their own offered no survival benefit after 40 days, and the docetaxel positive control (administered intravenously) demonstrates the overall success of the experiment.

4. Discussion

Herein we have demonstrated the power of the Magic Tag® chemical genomics tool in a drug reprofiling context. Magic Tag® was successful in immobilizing an antipsychotic drug in a manner that allowed effective screening against a T7 phage displayed library. The use of *D. melanogaster* as a whole genome model uncovered a range of interesting interactions of potential relevance in humans, including apparent binding of the drug to a portion of *D. melanogaster* scully, an ortholog of human 17HSD10. Enzyme inhibition studies coupled with computational modelling indicated that, of the antipsychotic and related drugs in our study, risperidone was the best inhibitor of full length human 17HSD10 *in vitro*. Formulation of risperidone with rumenic acid created a new drug VAL401. VAL401 reduced activity of PC3 prostate cancer cells *in vitro* and prolonged the lifetime of mice with PC3 prostate cancer xenografts *in vivo*. VAL401 is expected to proceed to clinical trials as a reprofiled drug for prostate cancer and other adenocarcinomas.

The relationship between risperidone and NAD^+ proposed here may have broader significance. Increasingly, the role of NAD^+ in controlling both energy and signalling

is being studied in relation to cancer cell proliferation [26]. For example, determination of [NAD^+]/[NADH] ratios showed marked differences between prostate and breast cancer cells [27]. Effects on NAD^+ levels may also explain further aspects of the broad pharmacological profile of risperidone [27].

5. Acknowledgements

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