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Preliminary communication

Antiproliferative activity of phenylbutyrate ester of haloperidol metabolite II [(±)-MRJF4] in prostate cancer cells

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ABSTRACT

Complex mechanisms of prostate cancer progression prompt to novel therapeutic strategies concerning a combination of drugs or of single molecules able to interact with more crucial targets. Histone deacetylase inhibitors and sigma ligands with mixed σ_1 antagonist and σ_2 agonist properties were proposed as new potential tools for treatment of prostate cancer. (±)-MRJF4 was synthesized as phenylbutyrate ester of haloperidol metabolite II, which is a molecule consisting of a histone deacetilase inhibitor (4-phenylbutyric acid) and a sigma ligand (haloperidol metabolite II). Antiproliferatives activities of 4-phenylbutyric acid, haloperidol metabolite II, equimolar mixture of both compounds and (±)-MRJF4 were evaluated *in vitro* on LNCaP and PC3 prostate cancer cells. Preliminary binding studies of (±)-MRJF4 for σ_1, σ_2, D_2 and D_3 receptors and inhibition HDAC activity were reported. MTT cell viability assays highlighted a notable increase of antiproliferative activity of (±)-MRJF4 (IC_{50} = 11 and 13 μM for LNCaP and PC3, respectively) compared to 4-phenylbutyric acid, haloperidol metabolite II and the respective equimolar pharmacological association. (±)-MRJF4 was also used in combination with σ_1 agonist (+)-pentazocine and σ_2 antagonist AC927 in order to evaluate the role of σ receptor subtypes in prostate cancer cell death.

1. Introduction

Prostate cancer is one of the most common causes of death in western countries and is the second leading cause of cancer-related death in men [1]. Prostate cancer is a heterogeneous tumor composed of multiple types of cells that have a variable response to endocrine therapy [2]. The conversion of an androgen-sensitive cancer into an androgen-refractory cancer consequently increases the risk of metastasis and remains a relevant clinical issue [3]. Several pharmacological agents, alone or in combination, have been proposed for the treatment of hormone-resistant prostate cancer. However, complex mechanisms underlie prostate cancer progression, and novel therapeutic strategies use a combination of drugs that interact with crucial targets involved in prostate cancer [4,5]. The development of several human cancers, including prostate cancer, is related to an alteration of histone deacetylase (HDAC) activity, which results in aberrant transcription of key genes that regulate important cellular functions such as cell proliferation, cell-cycle regulation and apoptosis [6]. Histone acetylation is a dynamic reversible process regulated by histone acetyltransferase (HAT) and HDAC enzymes that are very essential to the epigenetic regulation of gene transcription [7]. An imbalance in the equilibrium of acetylation and deacetylation has been associated with carcinogenesis and cancer progression [8]. HDAC overexpression causes a decrease in histone acetylation. This effect is associated with an alteration of oncogene and oncosuppressor activity as well as tumor development [9]. Therefore, histone deacetylase inhibitors (HDACi), alone or in combination with other drugs, are emerging as a new class of anticancer agents and have been demonstrated to exert antitumor effects such as growth arrest, differentiation and apoptosis [10].

Butyrate was the first HDACi discovered [11]. A related compound, 4-phenylbutyric acid, has reached phase I/II clinical trials for the treatment of refractory solid tumor malignancies. This compound has good compliance and tolerability but has lesser potency compared to current HDAC inhibitors [12,13].

Several studies have demonstrated that prostate cancer cell lines overexpress sigma (σ) receptors, a class of membrane receptor

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proteins composed of σ_1 and σ_2 subtypes [14]. σ receptors are found in different areas of the central nervous system and of healthy tissues, such as liver, lungs, gonads, endocrine glands and kidneys. They are located at subcellular level in plasma membranes, mitochondria and endoplasmic reticulum. The two subtypes can be distinguished pharmacologically, functionally, and by their molecular size. The σ_1 receptors have been cloned and have shown to be distinct from any other known receptor class [15]. The σ_1 receptor is a 25 kDa, single polypeptide with two putative trans-membrane regions. The σ_2 receptor is a 18–21 kDa protein which has not yet been cloned [16]. Both receptor subtypes have been detected in multiple types of tissue and are highly expressed in various tumor cell lines. The high density of σ receptors, particularly the σ_2 receptors in cells and tumor tissue compared to normal tissue, suggested their use in diagnostic imaging of tumors such as PET (Positron Emission Tomography) [17] and SPECT (Single Photon Emission Computed Tomography) [18]. In particular, some σ ligands were used as markers for prostate cancer because of the high expression of these receptors in hormone sensitive prostate cell lines (LNCaP) and hormone refractory (DU-145 and PC3) [19,20]. Moreover, recent studies using σ ligands that are conjugated to drug delivery systems, such as liposomes and nanoparticles, have demonstrated the selective delivery of chemotherapeutic substances, antisense oligodeoxynucletides or small interfering RNAs to tumor cells that overexpress σ receptors [21–23].

Among all σ ligands, we focused our studies on haloperidol metabolite II, which inhibits the proliferation of various cancer cells, by depleting Ca^{2+} from the endoplasmatic and mitochondrial stores, thereby inducing apoptosis [24,25]. In contrast to the conventional antipsychotic haloperidol, haloperidol metabolite II displays a preferential activity at σ receptors compared to dopamine receptors [26,27]. In addition, σ compounds decrease the expression of P-glycoprotein (P-gp), a multidrug resistance transporter involved in the efflux transport of several cytotoxic drugs and increase the efficacy of conventional chemotherapy and P-gp-mediated chemosensitivity in some drug-resistant tumors [28,29]. Recent study showed that exposure of cancer cells to HDACi promotes drug resistance and reduces treatment efficacy [30], the inhibition of P-gp expression by σ ligands might counteract the HDACi effect and provide an effective combinational therapy.

The role of different σ receptor subtypes on tumor cells has been recently evaluated with several σ ligands. These studies led to the conclusion that σ ligands that have σ_2 agonist and σ_1 antagonist effects may be potential antineoplastic agents [28,31].

In this report we evaluate antiproliferative effects of a novel compound, named (±)-**MRJF4**, an ester of 4-phenylbutyric acid (HDACi) and haloperidol metabolite II (σ_1 antagonist and σ_2 agonist) (Fig. 1). The antiproliferative activity of (±)-**MRJF4** in prostate cancer cell lines LNCaP and PC3 was compared to the effects of 4-phenylbutyric acid, haloperidol metabolite II and an equimolar mixture of both compounds. Preliminary, binding affinity of this new compound to sigma (σ_1, σ_2), dopamine (D_2, D_3) receptors as well as HDAC inhibition were reported. Moreover, with the use of putative σ_1 agonist (+)-Pentazocine and σ_2 antagonist AC927 the contribution of each σ subtypes on antiproliferative activity was established.

2. Chemistry

(±)-**MRJF4** was obtained by the reduction of 4-chloro-1-(4-fluorophenyl)-1-butanone **1** with $NaBH_4$ in EtOH (Scheme 1). The reaction of alcohol **2** and 4-phenylbutanoyl chloride gave ester **3**, which was treated with 4-(4-chlorophenyl)piperidin-4-ol in DMF to achieve the final compound as racemic mixture.

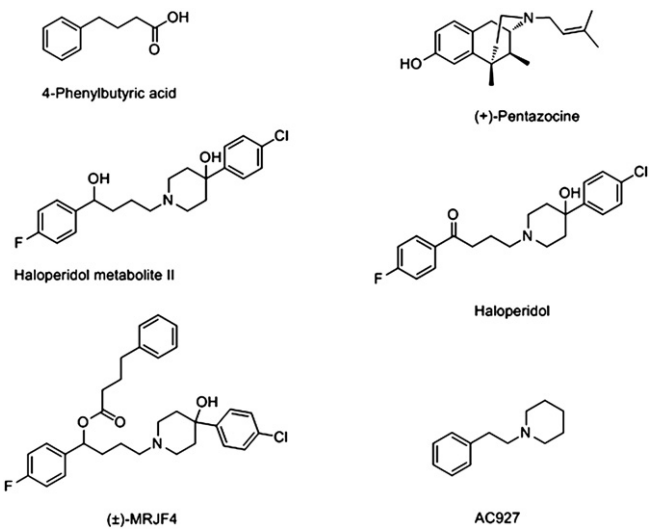


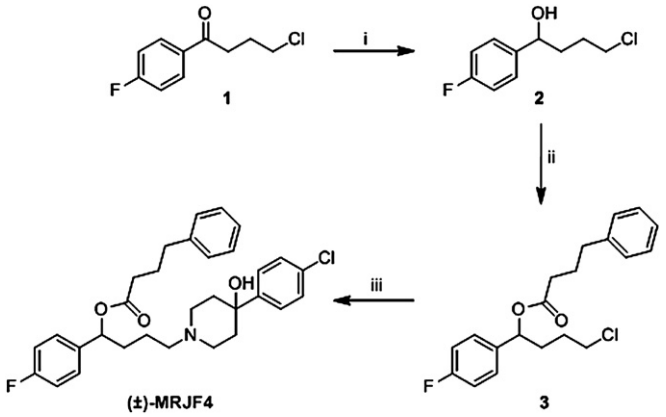
Fig. 1. Chemical structures of 4-phenylbutyric acid, haloperidol metabolite II, (±)-MRJF4, (+)-pentazocine, haloperidol and AC927.

3. Result and discussion

Evaluation of the σ_1 and σ_2 affinity of (±)-**MRJF4** determined that the esterification of the hydroxyl group on the haloperidol metabolite II with 4-phenylbutyric acid decreased the affinity for σ_1 and σ_2 receptors. However, the compound maintained a low micromolar affinity and the ability to interact with σ receptors (Table 1). In regard to dopaminergic D_2 and D_3 receptors, (±)-**MRJF4** had a low or negligible binding affinity in comparison to haloperidol metabolite II and haloperidol keeping a good selectivity for σ receptors. As expected, 4-phenylbutyric acid did not have affinity for the σ receptor subtypes.

The effects on the proliferation of LNCaP (androgen-sensitive) and PC3 (androgen-refractory) prostate cancer cells were measured using an MTT assay. Table 2 shows the IC_{50} values of (±)-**MRJF4** compared to 4-phenylbutyric acid, haloperidol metabolite II and an equimolar mixture of both compounds.

(±)-**MRJF4** was by far the most potent compound to inhibit cell growth in the two prostate cancer cell lines with an IC_{50} value of approximately 11 μM for LNCaP cells and 13 μM for PC3 cells at 24 h. Moreover, in comparison with the equimolar mixture, (±)-**MRJF4** was approximately 15-fold more potent. Superimposable results



Scheme 1. Synthesis of (±)-MRJF4. Reagents and conditions: (i) EtOH, $NaBH_4$, r.t.; (ii) $Ph(CH_2)_3COCl$, THF, 4-DMP, r.t.; (iii) 4-(4-chlorophenyl)-piperidin-4-ol, DMF, 75 °C.

Table 1
Sigma-1, sigma-2, D_2 and D_3 binding assays of (±)-MRJF4, haloperidol metabolite II, haloperidol and 4-phenylbutyric acid.

Compounds	K_i (nM) ± S.E.M.			
	σ_1	σ_2	D_2	D_3
(±)-MRJF4	162 ± 20	105 ± 12	>5000	>5000
Haloperidol metabolite II	2.3 ± 0.7	0.98 ± 0.3	232 ± 46	1095 ± 245
haloperidol	2.2 ± 0.5	16 ± 1.7	2.1 ± 0.3	5.8 ± 3.7
4-phenylbutyric acid	> 10000	>10000	n.d.[a]	n.d.[a]

[a] Not determined.

were obtained from the 48, 72 and 96 h treatments (data not shown).

LNCaP and PC3 cells were treated with various concentrations of (±)-**MRJF4** (1–25 μM) and vehicle (0.1% DMSO) for 24, 48, 72 and 96 h. As shown in Fig. 2, cell growth was inhibited to a variable degree in the presence of (±)-MRJF4 having half-maximum effects at approximately 10 μM.

(±)-**MRJF4** exerted potent antiproliferative effects in a time- and dose-dependent manner in LNCaP cells (IC_{50} = 10.87 μmol/l at 24 h; IC_{50} = 6.65 μmol/l at 96 h). A similar result was obtained in PC3 cells, except that these cells required a longer incubation time. Higher IC_{50} values were obtained in comparison to the LNCaP cells (IC_{50} = 12.86 μmol/l at 24 h; IC_{50} 6.78 μmol/l at 96 h). The inhibitory concentration (IC_{50}) was calculated for each incubation time and is reported in Table 3.

The antiproliferative effect of haloperidol metabolite II has been related to σ_1 antagonist and σ_2 agonist properties [32,33]. Therefore, we evaluated the contribution of each receptor subtypes to antiproliferative activity of (±)-**MRJF4** on LNCaP and PC3 cells using prototypic selective σ_1 agonist (+)-pentazocine (PT) [32] and σ_2 antagonist AC927 (AC) [31,34] (Fig. 3). Results showed that both σ receptor subtypes were involved in the antiproliferative effects of (±)-**MRJF4** but with a prevalence of the σ_2 receptors. Specifically, on LNCaP cells, treatment with (±)-MRJF4 induced a loss of cell viability of about 45% while treatment with (±)-MRJF4 and (+)-pentazocine, which blocks the σ_1 activity of (±)-MRJF4, induced a loss of cell viability of about 40%. Whereas, treatment with (±)-MRFJ4 and AC927, which contrasts the σ_2 activity of (±)-MRJF4, caused a loss of cell viability of about 20%. Similar results were obtained on hormone-refractory PC3 cells but in this case the loss of cell viability was approximately 10%.

The ability of (±)-MRJF4 to inhibit HDAC activity was measured in HeLa extracts *in situ* using a fluorescently-labeled HDAC substrate. Trichostatin A (TSA) was used as a positive control for maximal inhibition. Fig. 4 shows relative HDAC activities measured in triplicate. TSA at a concentration of 1 μM almost completely inhibited HDAC activity, while 4-phenylbutyric acid (4-PHB) at a concentration of 1.5 mM induced a minimum of 60% inhibition compared to the untreated control. (±)-MRJF4 did not inhibit HDAC activity at a concentration of 10 μM.

Table 2
Antiproliferation activity on LNCaP and PC3 cell lines.[a]

Compounds	LNCaP Cells	PC3 Cells
	IC_{50} (μM) ± s.e.	IC_{50} (μM) ± s.e.
(±)-MRJF4	11 ± 1.5	13 ± 3.3
4-Phenylbutyric acid	2324 ± 0.1	2273 ± 0.1
Haloperidol metabolite II	177 ± 7.6	208 ± 1.3
4-Phenylbutyric acid + Haloperidol metabolite II	190 ± 1.7	165 ± 3.0

[a] IC_{50} values ± s.e. of (±)-MRJF4, 4-phenylbutyric acid, haloperidol metabolite II and equimolar pharmacological association of two drugs on LNCaP and PC3 cells for 24 h. IC_{50} values are averaged from multiple determinations ($n \geq 3$).

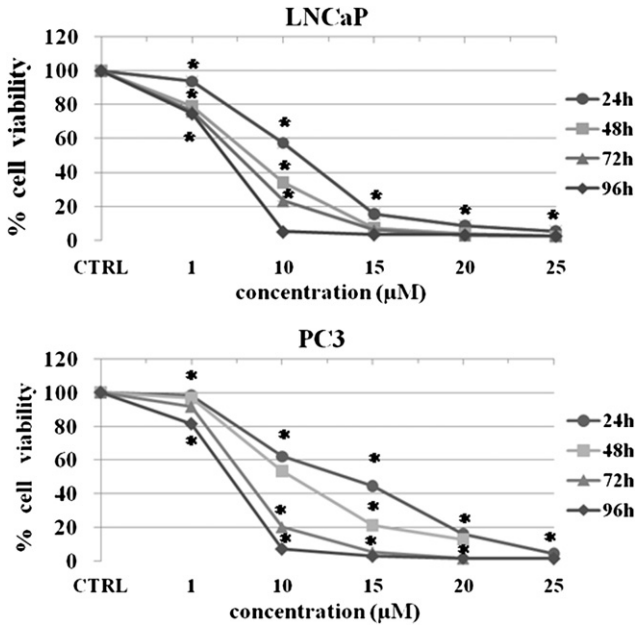


Fig. 2. Percent growth as compared to vehicle treated control (% cell viability) for LNCaP and PC3 cells treated with (±)-MRJF4 for 24, 48, 72, 96 h. (n = 4). *$p < 0.05$ relative to negative controls. Error bars were omitted for clarity; see Table 3 for an indication of reproducibility.

HDAC activity was also tested in LNCaP and PC3 cell lines that were treated for 1 h with TSA (1 μM), 4-PHB (1.5 mM) and (±)-MRJF4 (10 μM) (Fig. 5). The inhibition of HDAC activity in the nuclear extracts after 1 h of treatment was similar to the HDAC inhibition induced by (±)-MRJF4 in the HeLa extracts. These results suggest that, contrary to 4-PHB and the potent inhibitor TSA, (±)-MRJF4 does not have direct inhibitory effects on HDAC enzymes. Rather, the inhibition of LNCaP and PC3 cell proliferation may be related to the high expression of σ receptors in prostate cancer cell lines [20,21], the subsequent enhancement of (±)-MRJF4 uptake and intracellular hydrolysis into 4-phenybutyric acid and haloperidol metabolite II. In fact, several studies [18–24] have confirmed that the overexpression of σ receptors in cancer cells is an important tool to selectively increase the concentration of radiomarker ligands or cytotoxic substances in tumor cells. The appreciable binding affinity of (±)-MRJF4 is in agreement with this hypothesis.

4. Conclusion

In summary, we have reported the synthesis of (±)-MRJF4 a conjugate ester of phenylbutyric acid (HDAC inhibitor) and haloperidol metabolite II (σ_2 agonist/σ_1 antagonist ligand), as a novel tool for treatment of prostate cancer. (±)-MRJF4 has showed good binding affinity and selectivity for σ receptors and

Table 3
Growth inhibition of cell treated with (±)-MRJF4.[a]

(±)-MRJF4	LNCaP Cells	PC3 Cells
	IC_{50} (μM) ± s.e.	IC_{50} (μM) ± s.e.
24 h	10.87 ± 1.54	12.86 ± 3.3
48 h	8.83 ± 3.83	11.02 ± 8.1
72 h	8.13 ± 7.89	7.8 ± 8.8
96 h	6.65 ± 4.04	6.78 ± 3.14

[a] IC_{50} values are averaged from multiple determinations ($n \geq 3$).

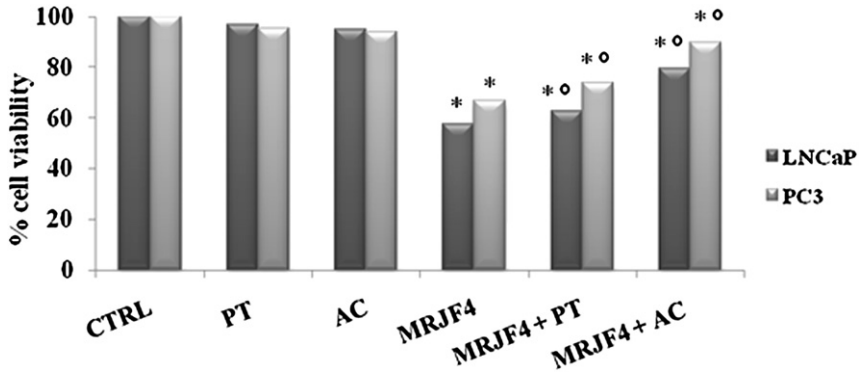


Fig. 3. Effect of (±)-MRJF4 + PT and (±)-MRJF4 + AC927 on LNCaP and PC3 cells after 24 h of exposure. *$P < 0.05$ (ANOVA followed by Fisher's test) vs. CTRL. °$P < 0.05$ (ANOVA followed by Fisher's test) vs. (±)-MRJF4.

antiproliferative activity mainly mediated by σ_2 subtypes. (±)-MRJF4 in comparison with the single compounds or the equimolar mixture of 4-phenylbutyric acid and haloperidol metabolite II acquired an enhanced potency to produce anticancer effects. Consequently, we can assume that the combination in the same molecule of HDAC inhibition activity and σ_2 agonist/σ_1 antagonist properties could be a useful strategy to develop novel anticancer agents. Further studies on (±)-MRJF4 and its enantiomers are in progress in our laboratories and will be reported in the future.

5. Experimental

5.1. Materials and methods

Solvents and reagents were obtained from commercial suppliers and were used without further purification. Flash chromatography purification was performed on a Merck silica gel 60 0.040-0.063 mm (230–400 mesh) stationary phase. Melting points were determined using a Thomas Hoover apparatus and are uncorrected. Nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR recorded at 200 and 50 MHz respectively) were obtained on VARIAN INOVA spectrometers using the $CDCl_3$ or DMSO-d_6 solvents. TMS was used as an internal standard. Coupling constants (*J*) are reported in hertz. Microanalysis (C, H, N) was performed on a Carlo Erba instrument model E-1110, and the results agreed with the theoretical values (±0.4%). Thin-layer chromatography (TLC) was performed on silica gel plates with a fluorescence indicator of F254 (0.2 mm, E. Merck); the spots were visualized by UV light. The following radioactive materials were used: [3H]-(+)-pentazocine (45 Ci/mmol), [^{3}H]DTG [1,3-di-(2-tolyl)guanidine] (31 Ci/mmol), obtained from Perkin–Elmer, [^{3}H]spiperone (20 Ci/mmol) was obtained from NEN and [^{3}H]-7OH-DPAT (139 Ci/mmol) was obtained from Amersham (U.K.).

5.2. Chemical synthesis

5.2.1. (±)-4-Chloro-1-(4-fluorophenyl)butan-1-ol (**2**)

$NaBH_4$ (0.483 g, 12.78 mmol) was added to a solution of (**1**) (5.0 g, 24.92 mmol) in EtOH (30 mL) at 0 °C. The mixture was stirred at room temperature for 12 h. The reaction mixture was quenched with water (20 mL) and evaporated to remove the organic portion. The residue was diluted in saturated Na_2CO_3 solution and extracted using $CHCl_3$ (3 × 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum to obtain (2) (4.69 g, 93% of yield) as a yellow oil, which was used without further purification.

5.2.2. (±)-4-Chloro-1-(4-fluorophenyl)butyl 4-phenylbutanoate (**3**)

4-Chloro-1-(4′-fluorophenyl)-butanol (2) (1.0 g, 4.93 mmol) was dissolved in anhydrous THF (35 mL) and 4-*N*,*N*-dimethylaminopiridin (0.6 g 4.93 mol) was added while stirring continuously. A solution of 4-phenylbutanoyl chloride (0.9 g, 4.93 mmol) in THF was added dropwise at 0 °C and the reaction mixture was stirred at room temperature for 48 h. The reaction was quenched with water (10 mL), the organic solvent was then evaporated under vacuum and the residue was dissolved in AcOEt. The residue was then treated with a solution of 1 N HCl, followed the addition of a $NaHCO_3$ (5%) solution. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to obtain a crude yellow oil product. Purification by flash chromatography

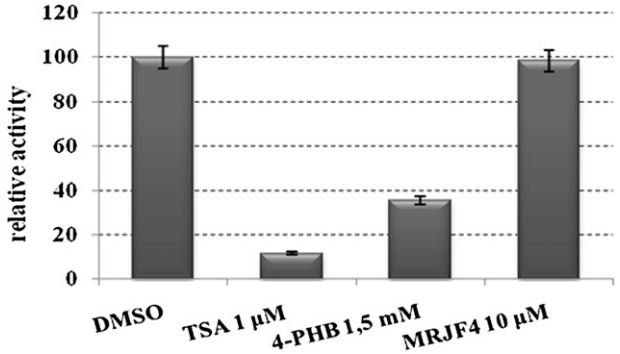


Fig. 4. The HDAC activity of HeLa extracts in the presence of positive control (TSA 1 μM), 4-PHB (1.5 mM) and (±)-MRJF4 (10 μM), relative to untreated control (DMSO). Experiments were performed in triplicate and the bars indicate ± S.D.

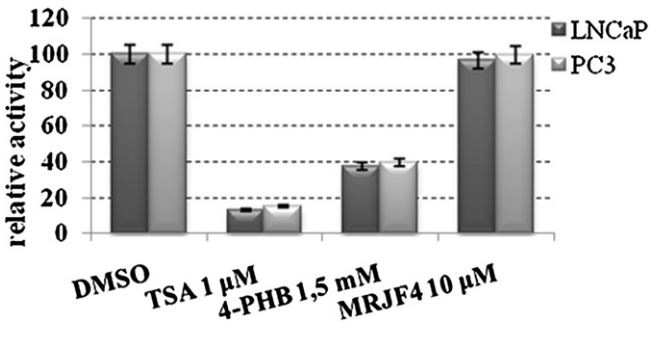


Fig. 5. The HDAC activity of LNCaP and PC3 nuclear extracts after (a) 1 h of treatment with positive control (TSA 1 μM), 4-PHB (1.5 mM) and (±)-MRJF4 (10 μM), relative to untreated control (DMSO). Experiments were performed in triplicate and the bars indicate ± S.D.

(10–90% AcOEt in cyclohexane) yielded (3) (1.1 g, 60% of yield) as colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.33-6.98 (m, 9H), 5.74 (t, J = 6.0 Hz, 1H), 3.52 (m, 2H), 2.61 (t, J = 6.0 Hz, 2H), 2.38-2.30 (m, 2H), 2.06-1.62 ppm (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 172.85, 162.60 (d, J_{CF} = 245.5 Hz), 141.44, 136.26 (d, J_{CF} = 3.65 Hz), 128.67, 128.62, 128.43 (d, J_{CF} = 7.9 Hz), 126.24, 115.69 (d, J_{CF} = 21.9 Hz), 74.64, 44.66, 35.23, 33.94, 33.79, 28.77, 26.63 ppm.

5.2.3. (±)-4-[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butyl 4-phenylbutanoate [(±)-**MRJF4**]

A mixture of 4-(4-chlorophenyl)piperidin-4-ol (0.217 g, 1.026 mmol), $NaHCO_3$ (0.086 g, 1.026 mmol) and compound (3) (0.179 g, 0.513 mmol) in anhydrous DMF was stirred at 75 °C for 12 h. The organic solvent was evaporated under vacuum and the residue was dissolved in water (50 mL) followed by an extraction with CH_2Cl_2 (3 × 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated in a vacuum to obtain a crude yellow oil product. Purification by flash chromatography (20–80% AcOEt in cyclohexane) yielded **(±)-**MRJF4 (0.113 g, 43% of yield) as colorless oil, which was transformed into an oxalate salt: mp: 132.5–135.2 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ = 7.46-7.07 (m, 13H), 6.36 (br s, 3H), 5.67 (t, J = 6.0 Hz, 1H), 3.28-3.03 (m, 6H), 2.53-2.12 (m, 6H), 1.79-1.66 ppm (m, 8H); ^{13}C NMR (50 MHz, [D_6]DMSO): δ 172.35, 164.8, 161.5 (d, J_{CF} = 242.45 Hz), 147.01, 141.35, 136.63, 131.48, 128.49, 128.33, 128.06, 126.71, 125.91, 115.50, 115-07, 73.91, 67.98, 48.03, 34.74, 34.26, 33.04, 32.84, 26.29, 19.97 ppm; Anal. Calcd for $C_{31}H_{35}ClFNO_3 \cdot C_2H_2O_4$: C 64.54, H 6.07, N 2.28, found: C 64.21, H 6.28, N 2.55.

5.3. Radioligand binding assays

Guinea pig membranes were prepared as previously reported by Matsumoto et al.[35] and Mach et al.[36] Sigma-1 binding affinity was determined incubating membrane aliquots (400 μL, 500 μg protein) to equilibrium (150 min at 37 °C) with 3 nM [^{3}H]-(+)-pentazocine (45 Ci/mmol) and increasing concentrations of displacing ligands in 50 mM Tris–HCl (pH 7.4) to a total volume of 1 mL. Nonspecific binding was assessed in the presence of 10 μM haloperidol. For the sigma-2 binding assays, the membranes (300 μL, 360 μg protein) were incubated (120 min at room temperature) with 3 nM [^{3}H]DTG [1,3-di-2-tolylguanidine] (31 Ci/mM) in the presence of 0.4 μM (+)-SKF10,047 to block the σ_1 sites. The incubation was performed in 50 mM Tris–HCl (pH 8.0) to a total volume of 0.5 mL. Nonspecific binding was evaluated in the presence of 5 μM DTG. The rat striatum and rat olfactory tubercle were used for D_2 and D_3 receptors, respectively. Tissue preparations and binding assays were carried out according to Mennini et al. [37]. After incubation, the samples were filtered through Whatman GF/B or GF/C glass fiber filters, which were pre-soaked in a 0.5% polyethylenimine solution, using a Millipore filter apparatus. The filters were washed twice with 4 ml of a suitable ice-cold buffer, and radioactivity was counted in 4 ml of "Ultima Gold MV" using a 1414 Winspectral Perkin Elmer Wallac liquid scintillation counter. Inhibition constants (*Ki* values) for the tested compounds were calculated using the EBDALIGAND program 34 purchased from Elsevier/Biosoft.

5.4. Cell culture

The androgen-sensitive cell line, LNCaP, and the androgen-refractory cell line, PC3, were purchased from the American Type Culture Collection. PC3 cells were maintained in RPMI 1640 medium supplemented with 10% v/v heat-inactivated fetal bovine serum and 50 U/ml penicillin/streptomycin. LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% v/v heat-inactivated fetal bovine serum and 50 U/ml penicillin/streptomycin, 10 nM HEPES, 1 mM sodium pyruvate and 2.5 g/L D-(+)-glucose. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Drug treatments were performed by preparing a 1:1000 dilution from the dimethyl sulfoxide (DMSO) stock solutions in culture medium immediately before use.

5.5. Cell viability assay

Cell viability was measured using an MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) growth assay according to the manufacturer's instructions [38]. This quantitative colorimetric assay is based on the ability of the cells to reduce the MTT tetrazolium salt, a pale yellow substrate, to a dark-blue formazan product. In viable mitochondria, succinate dehydrogenases cleave the tetrazolium ring into formazan crystals. DMSO is added to dissolve the insoluble crystals into a colored solution. Cells were plated in 96-well plates at a density of 4 × 10^4 cells per well and allowed to adhere overnight. The experiments were performed in quadruplicate and began by replacing the medium with fresh medium containing various concentrations of the compounds under investigation (haloperidol metabolite II, 4-phenylbutiric acid, haloperidol metabolite II + 4-phenylbutyric acid, (±)-MRJF4, (+)-pentazocine and AC927). Cells were incubated for different time periods (24, 48, 72 and 96 h) with compounds at the optimal concentrations without refreshing the medium to carry out the tests described. Following the appropriate incubation period, 10 μL MTT (10% total volume) was added to each well, and the cells were incubated for an additional 4 h. The media from culture plates containing adherent cells was then aspirated using a fine-point Pasteur pipette and the crystals were solubilized in DMSO. The absorbance was measured at 570 nm using a spectrophotometer. The ED_{50} values (dose at which 50% of the metabolic activity compared to the vehicle control remains) were derived from the dose effect curves.

5.6. Nuclear protein extraction

Total nuclear protein was extracted using the NEPER extraction kit (Pierce). LNCaP and PC3 cells were seeded in 6 mm^2 dishes at a density of 3 × 10^5 cells per dish and were allowed to adhere overnight. The following day the medium was replaced by medium containing substrate and (±)-MRJF4 (10 μM), Trichostatin A (TSA) (1 μM) or 4-phenylbutiric acid (4-PHB) (1.5 mM). After 1 h and again after 24 h the cells were washed with PBS and harvested in 100 mL of lysis buffer (50 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L phenyl methyl sulfonyl fluoride, 0.5 mg/mL leupeptin, 5 mg/mL aprotinin, and 1 mg/mL pepstatin). The samples were centrifuged at 16,000 × g for 30 min at 4 °C. The resulting supernatants were isolated and protein concentration was determined by the Bradford (1976) method [39]. Subsequently, the separation of cytoplasmic and nuclear proteins was performed. Forty milligrams of cells were spun down into a 1.5 mL microcentrifuge tube at 500 × g for 3 min. After spinning, the supernatant was discarded. Two hundred microliters of ice-cold CER I was then added to the cell pellet. The mixture was vortexed vigorously for 15 s to fully resuspend the pellet. The tube was then incubated on ice for 10 min. Eleven microliters of ice-cold CER II was added, and the samples were vortexed for 5 s. Another 1-min incubation on ice was followed by vortexing for 5 s. Next, the mixture was centrifuged at 16,000 × g for 5 min. The supernatant (cytoplasmic extract) was transferred to a new pre-chilled tube. For the extraction of nuclear proteins, the insoluble pellet was resuspended in 100 μL of ice-cold NER and vortexed for 15 s. The sample was put on the ice for 10 min and vortexed for 15 s. This step was repeated 4 times for a total of

40 min. Afterward, the sample was centrifuged at 16,000 × g for 10 min. The supernatant, which contained the nuclear extract, was transferred into a clean pre-chilled tube and kept at −20 °C further experimentation.

5.7. Histone deacetylase activity assay

HDAC activity was measured using the HDAC Fluorescent Activity assay/Drug Discovery kit according to the manufacturer's instructions (AK-500, BIOMOL International) [40,41]. TSA and the fluorescent substrate were purchased with the Fluor-de-Lys kit. The assay measures the activity of HDACs to deacetylate a substrate that contains acetylated lysine side chains. The HeLa extracts (supplied in the kit) were added to each well in a 96-well plate except wells containing the untreated control samples. The assay buffer, TSA (1 μM), 4-phenylbutyric acid (1.5 mM) and (±)-MRJF4 (10 μM) were added to appropriate wells. Only the assay buffer solution was added to the LNCaP and PC3 extracts (treated as described in the nuclear proteins extraction procedure). The HDAC reaction initiated by adding the *Fluor de Lys* substrate (25 μL) to each well and mixing thoroughly. The assay was performed at 25–37 °C. The reaction was quenced after 10 min with the *Fluor the Lys* developer solution (50 μL) and the plate was incubated at room temperature for 10–15 min. The substrate is deacetylated by the HDAC1, HDAC2, HDAC3, HDAC4, HDAC7, HDAC8, HDAC9 and SIRT1 enzymes. The intensity of the fluorescent signal is indicative of the amount of HDAC activity in the sample. The fluorescence of each reaction was measured on a fluorometric reader (Perkin Elmer Wallac 1420-032 Victor^2 multilaber counter) with the excitation set to 360 nm and the emission set to 460 nm. The percent inhibition for each fluorescence reading of the inhibited reaction was calculated relative to the fluorescence of the control reaction. Experiments were performed in triplicate.

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References

[1] L.C. Li, Front. Biosci. 12 (2007) 3377–3397.
[2] A.I. So, A. Hurtado-Coll, M.E. Gleave, World J. Urol. 21 (2003) 325–337.
[3] K. Iguchi, M. Ito, S. Usui, A. Mizokami, M. Namiki, K. Hirano, J. Androl. 29 (2008) 207–212.
[4] F. Stavridi, E.M. Karapanagiotou, K.N. Syrigos, Cancer Treat. Rev. 36 (2010) 122–130.
[5] B.A. Hadaschik, R.D. Sowery, M.E. Gleave, Curr. Opin. Urol. 17 (2007) 182–187.
[6] K.V. Balakin, Y.A. Ivanenkov, A.S. Kiselyov, S.E. Tkachenko, Anticancer Agents Med. Chem. 7 (2007) 576–592.
[7] H. Hess-Stumpp, Eur. J. Cell Bio. 84 (2005) 109–121.
[8] S. Mei, A.D. Ho, U. Mahlknecht, Int. J. Oncol. 25 (2004) 1509–1519.
[9] R. Somech, S. Izraeli, A.J. Simon, Cancer Treat. Rev. 30 (2004) 461–472.
[10] P. Jones, S. Altamura, R. De Francesco, O.G. Paz, O. Kinzel, G. Mesiti, E. Monteagudo, G. Pescatore, M. Rowley, M. Verdirame, C. Steinkühler, J. Med. Chem. 51 (2008) 2350–2353.
[11] L.C. Boffa, G. Vidali, R.S. Mann, V.G. Allfrey, J. Biol. Chem. 253 (1978) 3364–3366.
[12] M.W. Sung, S. Waxman, Anticancer Res. 27 (2007) 995–1001.
[13] L.H. Camacho, J. Olson, W.P. Tong, C.W. Young, D.R. Spriggs, M.G. Malkin, Invest. New Drugs 25 (2007) 131–138.
[14] R.R. Matsumoto, W.D. Bowen, T.P. Su, Sigma Receptors Chemistry, Cell Biology and Clinical Implications. Springer Science+Business Media, LLC, New York, 2007.
[15] M. Hanner, F.F. Moebius, A. Flandorfer, G. H-Knaus, J. Striessnig, E. Kempner, H. Glossmann, Proc. Natl. Acad. Sci. USA 93 (1996) 8072–8077.
[16] S.B. Hellewell, A. Bruce, G. Feinstein, J. Orringer, W. Williams, W.D. Bowen, Eur. J. Pharmacol. 268 (1994) 9–18.
[17] H. Kashiwagi, J.E. McDunn, P.O. Simon Jr., P.S. Goedegebuure, J. Xu, L. Jones, K. Chang, F. Johnston, K. Trinkaus, R.S. Hotchkiss, R.H. Mach, W.G. Hawkins, Mol. Cancer 6 (2007) 48–60.
[18] K.T. Wheeler, L.M. Wang, C.A. Wallen, S.R. Childers, J.M. Cline, P.C. Keng, R.H. Mach, Br. J. Cancer 82 (2000) 1223–1232.
[19] C.S. John, B.J. Vilner, B.C. Geyer, T. Moody, W.D. Bowen, Cancer Res. 59 (1999) 4578–4583.
[20] C.S. John, M.E. Gulden, J. Li, W.D. Bowen, J.G. McAfee, M.L. Thakur, Nucl. Med. Biol. 25 (1998) 189–194.
[21] R. Banerjee, P. Tyagi, S. Li, L. Huang, Int. J. Cancer 112 (2004) 693–700.
[22] S.D. Li, Y.C. Chen, M.J. Hackett, L. Huang, Mol. Ther. 16 (2008) 163–169.
[23] S.D. Li, L. Huang, Mol. Pharm. 3 (2006) 579–588.
[24] P.J. Brent, G. Pang, G. Little, P.J. Dosen, D.F. Van Helden, Biochem. Biophys. Res. Commun. 219 (1996) 219–226.
[25] B.J. Vilner, W.D. Bowen, J. Pharmacol, Exp. Ther. 292 (2000) 900–911.
[26] W.D. Bowen, E.L. Moses, P.J. Tolentino, J.M. Walker, Eur. J. Pharmacol. 177 (1990) 111–118.
[27] J.C. Jaen, B.W. Caprathe, T.A. Pugaley, L.D. Wise, J. Med. Chem. 36 (1993) 3929–3936.
[28] A. Azzariti, N.A. Colabufo, F. Berardi, L. Porcelli, M. Niso, G.M. Simone, R. Perrone, A. Paradiso, Mol. Cancer Ther. 5 (2006) 1807–1816.
[29] M. Tucci, C. Quartaro, F. Dammacco, F. Silvestris, Curr. Top. Med. Chem. 9 (2009) 218–224.
[30] S. Hauswald, J. Dunque-Anfoso, M.M. Wagner, F.M. Schertl, M. Lubbert, C. Peschel, K. Ulrich, T. Licht, Clin. Cancer Res. 15 (2009) 3705–3715.
[31] N.A. Colabufo, F. Berardi, M. Contino, M. Niso, C. Abate, R. Perrone, V. Tortorella, Naunyn Schmiedebergs Arch. Pharmacol. 370 (2004) 106–113.
[32] B.A. Spruce, L.A. Campbell, N. McTavish, M.A. Cooper, M.V.L. Appleyard, M. O'Neil, J. Howie, J. Sanson, S. Watt, K. Murray, D. McLean, N.R. Leslie, S.T. Safrany, M.J. Ferguson, J.A. Peters, A.R. Prescott, G. Box, A. Hayes, B. Nutley, F. Raynaud, C.I. Downes, J.J. Lambert, A.M. Thomson, S. Eccles, Cancer Res. 64 (2004) 4875–4886.
[33] W.D. Bowen, Pharm. Acta Helv. 74 (2000) 211–218.
[34] R.R. Matsumoto, J. Shaikh, L.L. Wilson, S. Vedam, A. Coop, Eur. Neuropsychopharmacol. 18 (2008) 871–881.
[35] R. Matsumoto, W.D. Bowen, M.A. Tom, V.N. Vo, D.D. Truong, B.R. De Costa, Eur. J. Pharmacol. 280 (1995) 301–310.
[36] R.H. Mach, C.R. Smith, S.R. Childers, Life Sci. 57 (1995) L57–L62.
[37] T. Mennini, P. Bernasconi, M.G. Fiori, Invest. Radiol. 28 (1993) 821–827.
[38] J. Carmichael, W.G. DeGraff, A.F. Gazdar, J.D. Minna, J.B. Mitchell, Cancer Res. 47 (1987) 943–946.
[39] G. Cantarella, G. Di Benedetto, S. Pezzino, N. Risuglia, R. Bernardini, J. Neurochem. 105 (2008) 1915–1923.
[40] T. Liu, G. Kapustin, F.A. Etzkorn, J. Med. Chem. 50 (2007) 2003–2006.
[41] A.J. de Ruijter, S. Kemp, G. Kramer, R.J. Meinsma, J.O. Kaufmann, H.N. Caron, A.B. van Kuilenburg, Biochem. Pharmacol. 68 (2004) 1279–1288.