17β-Hydroxysteroid Dehydrogenase Type 1 Inhibition: A Potential Treatment Option for Non-Small Cell Lung Cancer

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ABSTRACT: In the face of the clinical challenge posed by non-small-cell lung cancer (NSCLC), the present need for new therapeutic approaches is genuine. Up to now no proof existed that 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) is a viable target for treating this disease. Synthesis of a rationally designed library of 2,5-disubstituted furan derivatives, followed by biological screening led to the discovery of 17β-HSD1 inhibitor **1**, capable of fully inhibiting human NSCLC Calu-1 cell proliferation. Its pharmacological profile renders it eligible for further *in vivo* studies. The very high selectivity of **1** over 17β-HSD2 was investigated, revealing a rational approach for the design of selective inhibitors. 17β-HSD1 and **1** hold promise in fighting NSCLC.

KEYWORDS: 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1); Non-Small Cell Lung Cancer (NSCLC); Steroidogenic Enzyme Inhibition; Drug Design; Structure-Activity Relationship (SAR); Molecular Docking

Lung cancer is the leading cause of death from cancer worldwide,[1] and in particular non-small-cell lung cancer (NSCLC), which accounts for more than 85% of the cases, shows only 15.9% and 49% 5-years predicted survival rate for all and early stages of lung cancer, respectively.[2] Thus, finding more efficient drugs with novel modes of action is an urgent necessity.

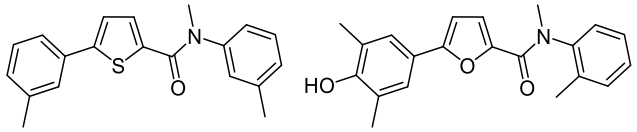
The recognition of the great heterogeneity of lung cancer constitutes the most important advance in the field made in recent years,[3] suggesting the need for exploring different therapeutic targets and leading until today to the discovery of a few novel targets and therapies.[4] We disclose in this report that compound **1**, a highly selective inhibitor of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) inhibits NSCLC cell proliferation at low nanomolar concentrations, providing the first proof of principle of 17β-HSD1 as a target for NSCLC treatment. We document that **1** presents suitable *in vitro* properties, and shows an acceptable bioavailability and toxicological profile. Finally, we provide a rationale for its very high selectivity over 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2).

Over the last twenty years, increasing evidence has demonstrated the pivotal role of estrogens in lung tumorigenesis, both in women and men.[5-7] Different strategies to target the estrogen signaling pathway, such as the use of the down-regulator of estrogen receptors (ERs) function. Fulvestrant[8] and aromatase inhibitors, such as Anastrazole[9] and Exemestane,[10] have shown promising

results in preclinical studies. However, 17β-HSD1 and 17β-HSD2, which are key local regulators of the estradiol/estrone (E2/E1) ratio,[11] have remained unexplored targets for the treatment of NSCLC. 17β-HSD1 catalyzes the conversion of the weakly active E1 to the potent E2 and 17β-HSD2 is its biological counterpart. The expression levels of these enzymes were found to be altered in NSCLC cells compared to healthy tissue, providing a significant prognostic factor and contributing to tumor progression in a stimulatory fashion – probably by increasing the E2/E1 ratio.[12-15] Selective inhibition of 17β-HSD1 seems therefore a potential approach for the treatment of NSCLC and might be superior to aromatase inhibition in terms of potential side effects: 17ß-HSD1 inhibition would result in only a local, intracellular drop in estradiol levels in the target cells while aromatase inhibition would decrease systemic circulating estradiol levels.

We have reported about the synthesis of different classes of 17β-HSD1 inhibitors for the treatment of breast cancer and have demonstrated their antitumor activity *in vitro*.[16,17] In light of the higher expression of 17β-HSD2 mRNA in NSCLC cells than in breast carcinoma cells and given the positive correlation between 17β-HSD2 expression and NSCLC survival rate,[12] we reasoned that a 17β-HSD1 inhibitor should display very high selectivity over 17β-HSD2, in order to be effective in NSCLC.

Applying our experience in the SARs of 17β-HSD1 and 17β-HSD2 inhibitors, we synthesized around 50 furan analogues of compound **A**[18] and screened them for inhibitory activity toward 17β-HSD1 and 17β-HSD2, affinity for the estrogen receptors α and β (ERs), metabolic stability and cytotoxicity.



A **1**

Table 1 shows the inhibitory data of the ten most interesting compounds which resulted. Inhibitor **1** emerged from this study with the desired attributes, including very high selectivity over 17β-HSD2.

Table 1. Inhibition of 17β-HSD1 and 17β-HSD2 by 2,5-disubstituted furans 1-10.

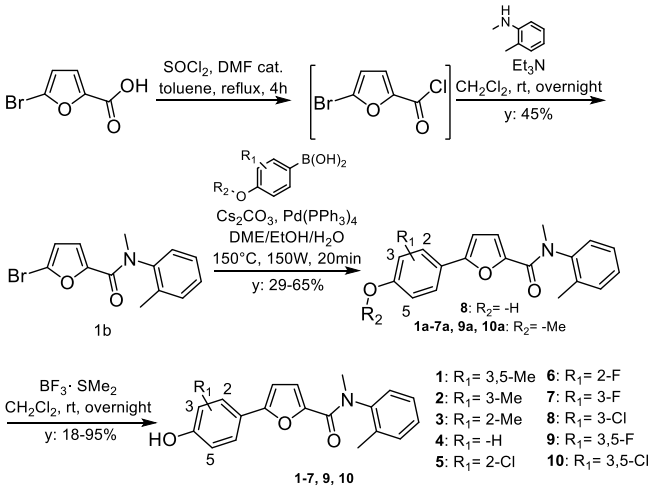
		IC_{50} (nM)[a]		
Cmpd	R_1[b]	17β-HSD1[c]	17β-HSD2[d]	s.f.[e]
1	3,5-Me	5.6	3155	563
2	3-Me	8.1	1171	145
3	2-Me	31.0	1077	35
4	H	55.2	2786	50
5	2-Cl	31.5	426	14
6	2-F	22.4	928	41
7	3-F	11.6	927	80
8	3-Cl	2.7	203	75
9	3,5-F	18.0	56	3
10	3,5-Cl	2.9	71	25

[a]Mean value of at least two determinations, standard deviation less than 20%, [b]cf. Scheme 1, [c]Human placental, cytosolic fraction, substrate E1 [500 nM], cofactor NADH [500 µM]. [d]Human placental, microsomal fraction, substrate E2 [500 nM], cofactor NAD^+ [1500 µM]. [e]s.f.: selectivity factor = IC_{50}(17β-HSD2) / IC_{50}(17β-HSD1).

Compounds **1-10** were synthesized as depicted in Scheme 1. The intermediate 5-bromofuran-2-carboxylic acid chloride was obtained from the corresponding carboxylic acid by reaction with $SOCl_2$. Subsequent reaction with *N*,2-dimethylaniline afforded the amide **1b** . The latter was subjected to a Suzuki coupling reaction with the appropriate boronic acid, under microwave irradiation (150 °C, 150 W, 20 min), providing the desired 2,5-disubstituted furans. The cleavage of the methoxy group was performed using boron trifluoride dimethyl-sulphide complex.

Compound **1** displayed a half-life of 50 minutes in human liver preparation (S9 fraction; phase I and II metabolism), a relative binding affinity (RBA) towards ERs lower than 0.1% and no detectable toxic effect on HEK293 cells at a concentration 1000-fold higher than the human 17ß-HSD1 IC50 value (for details, see Supporting Information). Human NSCLC Calu-1 cells convert E1 to E2, which in turn promotes Calu-1 cell proliferation.[14] We investigated the effect of inhibitor **1** on this stimulation (Figure 1).

Scheme 1. Synthesis of the 2,5-disubstituted furans 1-10.



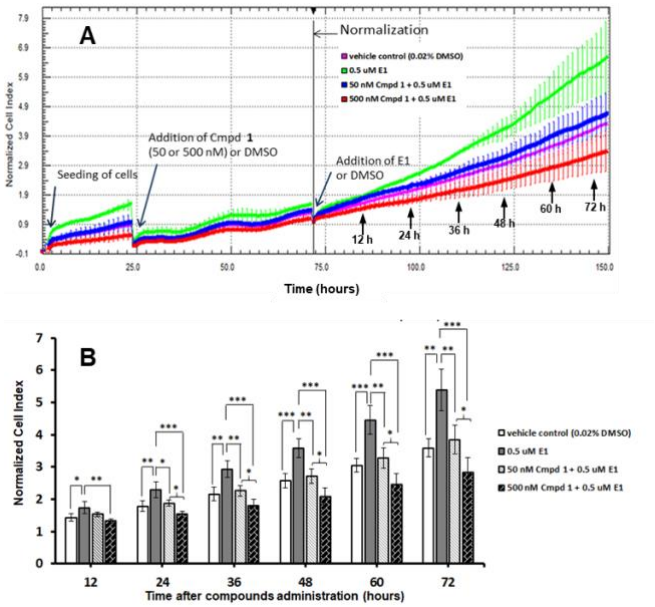


Figure 1.**Effect of compound 1 on Calu-1 cell proliferation in the presence of E1 in real-time conditions. (A)** Proliferation of Calu-1 cells was monitored in real-time by the xCELLigence RTCA DP System. Cells were seeded and incubated overnight in phenol red-free RPMI 1640 medium, supplemented with 10% charcoal-dextran-stripped FBS (Phase 1). After 24 h, cells were cultured for 48 h (phase 2) in the following experimental groups: control group – cells cultured in phenol red-free RPMI 1640 alone (green curve), cells cultured in phenol red-free RPMI 1640 plus 50 nM compound **1** (dark blue curve), cells cultured in phenol red-free RPMI 1640 plus 500 nM compound **1** (red curve), and vehicle control group – cells cultured in phenol red-free RPMI 1640 plus DMSO (final conc. 0.02%) (purple curve). At 72 h, media were changed once again and cells were cultured further in the presence of following compounds (Phase 3): 0.5 µM E1 (green curve), 0.5 µM E1 + 50 nM compound **1** (dark blue curve), 0.5 µM E1 + 500 nM compound **1** (red curve), and DMSO (0.02%) as a vehicle control (purple curve). Cell Index values were normalized to the starting time point of phase 3.(black vertical line). Four replicates at each investigated time point were used and the mean normalized cell index values with standard deviation for each time point for each group are shown. The electrical impedance was measured at 15-minutes intervals throughout the cultivation period (total time: 150 hrs). There was no statistical significance between the data groups at 4, 12 and 20 h after seeding (phase 1) or between the data groups after 12, 24, 36 and 45 h of phase 2 treatment as assessed by ANOVA analysis. **(B)** Statistical analysis of Phase 3. Effect of inhibitor **1** treatment on Calu-1 cell proliferation in the presence of 0.5 µM E1. Mean values of normalized cell index ± SD for each group after 12, 24, 36, 48, 60, 72 hrs of treatment (see Figure 1, A) are shown. All experiments were performed in four replicates. Data groups were assessed by ANOVA to evaluate whether there was significance ($p < 0.05$) between the groups. Individual comparisons were made by post hoc Tukey's HSD (honestly significant difference) test. Statistical significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. For more details see supporting information.

The proliferation of Calu-1 cells was monitored in real-time (Figure 1, *phase 1-3*). After seeding (*phase 1*), Calu-1 cells were preincubated with medium, 50 nM compound **1**, 500 nM compound **1** or DMSO (vehicle control) for 48 hrs (*phase 2*). There was no effect of 50 nM or 500 nM compound 1 on cell proliferation (see Figure 1 legend for details). Then, cells were exposed to the compounds: 0.5 µM E1 (green curve), 0.5 µM E1 + 50 nM compound **1** (dark blue curve), 0.5 µM E1 + 500 nM compound **1** (red curve) or DMSO alone (purple curve) as a vehicle control (*phase 3*). Addition of E1 to the incubation medium during a total of 75 h strongly increased cell proliferation compared to vehicle control (Fig. 1 and Fig. S1). Co-incubation of E1 with 50 nM of compound **1** reduced the cell profileration to the vehicle control level at all time points (Figure 1 and Figure S1). There was no statistical difference in cell proliferation between co-incubation between either 50 or 500 nM compound 1 (together with E1) and vehicle control, as measured at 12, 24, 36, 48, 60 and 72 h after initiation of *phase 3* (Tukey HSD test, $P>0.05$, Figure S1).

Preclinical proof of principle is to be demonstrated *in vivo* in an animal model of cancer, usually nude mouse or rat xenograft models inoculated with human cancer cells. We therefore performed a preliminary pharmacokinetic study with inhibitor **1** administered subcutaneously in Sprague-Dawley rats at a dose of 200 µmol/kg (= 67,0 mg/kg). Successive administration at 0 h, 24 h, 48 h and 72 h resulted in plasma concentrations more than sufficient to block human 17β-HSD1 (i.e. 51 nM, 82 nM, 119 nM and 156 nM at 23.5 h, 47.5 h, 71.5 h and 95.5 h, respectively). Half-life of **1** was determined in rat liver S9 fraction to be 19 min.

Among known non-steroidal 17β-HSD1 inhibitors, compound **1** displays the highest selectivity over 17β-HSD2, whose crystal structure, contrary to 17β-HSD1 is not yet available. In order to provide a structure-based hypothesis for this remarkable selectivity, docking simulations of **1** were performed by GLIDE v6.8[19] on the 17β-HSD1 crystal structure (PDB 3HB5).[20] The top score pose is shown in Figure 2. As displayed, the phenolic hydroxy group engages an H-bond interaction with the carboxylate group of E282, while the benzamide moiety establishes π-π interactions with two close aromatic side chains, namely Y155 and F192.

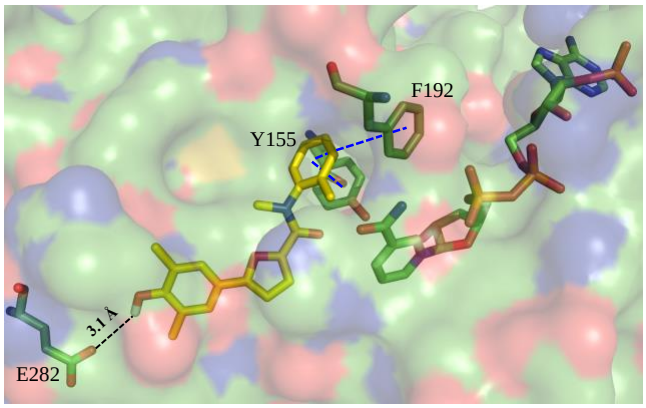


Figure 2. Top-scored docking pose of **1**. Important amino acid residues of 17β-HSD1, co-factor NADPH and compound **1** are rendered as sticks while the protein is shown as a surface. For the sake of clarity, the non-polar hydrogen atoms of the ligand are not displayed. H-bond interaction is depicted by a black dotted line, π- π interactions by a blue dotted line.

Comparison between the primary sequences of binding sites of the two 17β-HSD enzymes, although appearing highly conserved, revealed an interesting difference: 17β-HSD2 shows an arginine (R364) in place of the glutamate (E282) of 17β-HSD1. Therefore, these two residues may play a pivotal role in addressing ligand protein interactions and, even in explaining the molecular selectivity. In fact, the side chains of arginine and glutamate are very much diverse, with the result that replacement of the negative charge of E282 in 17β-HSD1 by the positively charged R364 in 17β-HSD2 should lead to a stronger binding of negatively ionized species to 17β-HSD2.

In order to prove the validity of this interaction model, we synthesized and tested a series of derivatives of compound **1**. The methyl groups next to the hydroxy function were exchanged by different substituents, leading to inhibitors **2-10** (Table 1), with diverse hydroxy-associated pKa values. The new molecules were obtained using the same synthetic strategy as applied for **1** (Scheme 1).

It is acknowledged that the C-O bond distance ($d_{C\text{-}O}$) is an appropriate measure to explain the electronic effects of substituents on physicochemical properties like pK_a of phenols and it has been successfully correlated with the empirical Hammett constant.[21,22] Consequently, the relationship between the $d_{C\text{-}O}$ values, measured using Density Functional Theory (DFT) optimized structures and the pIC_{50}s observed for 17β-HSD2 inhibition was investigated for **1-10**. As shown in Figure 3, a very good linear correlation was found: the lower the $d_{C\text{-}O}$ value (thus, lower pK_a), the higher is 17β-HSD2 inhibition. On the contrary, such correlation is not found with pIC_{50} values observed for 17β-HSD1 inhibition.

The robustness of this relationship was further challenged performing an intensive y-randomization analysis to avoid the risk of chance correlations.[23]

In this respect, we performed 1 million of y-scrambling runs to assess the reliability and goodness of the correlations (Figure S3) and satisfactorily, all the scrambled r^2 values were far from that reported in Figure 3.

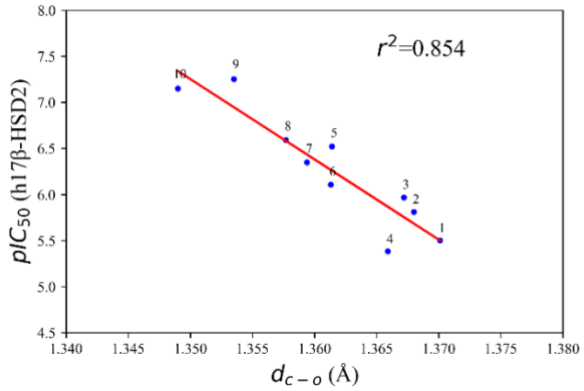


Figure 3. Relationship between pIC_{50} values towards 17β-HSD2 and $d_{C\text{-}O}$ distance calculated with the DFT optimized structures.

The obtained correlation supports the hypothesis whereby the electronic structure of the phenolic ring is a key element for addressing molecular selectivity.

The discovery that the inhibitory activity on 17β-HSD2 is dependent on the pK_a of the phenolic group is an important finding which can be further exploited for the rational development of additional selective 17β-HSD1 inhibitors.

In summary, we validated 17β-HSD1 as new therapeutic target for the treatment of NSCLC. Compound **1** fully inhibits the E1-dependent Calu-1 cell proliferation at low nanomolar concentrations. In addition, its pharmacological profile renders it a highly suitable candidate for further *in vivo* studies in animal models to establish a novel strategy for the treatment of NSCLC which is urgently needed.

ASSOCIATED CONTENTS

Supporting Information

Biological experimental details, detailed synthesis, molecules spectral data, docking simulations, density functional theory calculations and model validation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

A.S.A, C.J.v.K, S.M.-O., R.W.H and M.F. are inventors in a US-patent covering compounds **1-10** (US9884839 (B2)).

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Abbreviations

17β-HSD1: 17β-hydroxysteroid dehydrogenase type 1; 17β-HSD2: 17β-hydroxysteroid dehydrogenase type 2; NSCLC: non-small-cell lung cancer; E1: estrone; E2: 17β-estradiol; ER: estrogen receptor; s.f.: selectivity factor; IC_{50}: inhibitor concentration resulting in 50% enzyme inhibition; pIC_{50}: negative logarithm of IC_{50}; RBA: relative binding affinity (relative to the binding affinity of E2 which was set at 100%); DFT: density functional theory.

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