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Anti-leprosy drug clofazimine inhibits growth of triple-negative breast cancer cells via inhibition of canonical Wnt signaling

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ABSTRACT

Research on existing drugs often discovers novel mechanisms of their action and leads to the expansion of their therapeutic scope and subsequent remarketing. The Wnt signaling pathway is of the immediate therapeutic relevance, as it plays critical roles in cancer development and progression. However, drugs which disrupt this pathway are unavailable despite the high demand. Here we report an attempt to identify antagonists of the Wnt–FZD interaction among the library of the FDA-approved drugs. We performed an *in silico* screening which brought up several potential antagonists of the ligand-receptor interaction. 14 of these substances were tested using the TopFlash luciferase reporter assay and four of them identified as active and specific inhibitors of the Wnt3a-induced signaling. However, further analysis through GTP-binding and β-catenin stabilization assays showed that the compounds do not target the Wnt-FZD pair, but inhibit the signaling at downstream levels. We further describe the previously unknown inhibitory activity of an anti-leprosy drug clofazimine in the Wnt pathway and provide data demonstrating its efficiency in suppressing growth of Wnt-dependent triple-negative breast cancer cells. These data provide a basis for further investigations of the efficiency of clofazimine in treatment of Wnt-dependent cancers.

1. Introduction

Despite multiple efforts in development of novel therapies, bringing novel drugs to the market is a costly and time-consuming process that is coupled with great risks of failure. In many cases the candidate substance does not reach the patients due to its side effects, which are in turn the product of the off–target interactions. Virtually any small molecule has at least several interactors among tens of thousands of macromolecules present in our bodies. Normally undesirable, such interactions of approved drugs with known toxicity profiles are sometimes of a potential great value as they might be useful for treatment of other diseases, resulting in quick and low-cost repositioning of the drug. Consequently, many researchers are searching for novel applications of substances that have already passed the filter of clinical trials. To-date these efforts resulted in the "second life" of 22 drugs [1]. In many cases novel fields of drug application are unexpected, such as the notorious case of sildanefil (Viagra) [2] promoted from a poor remedy against hypertension and angina to the rescue for erectile dysfunction; or a less known case of thalidomide, leaping from the dangerous (and later prohibited) drug against morning sickness in pregnant women to the novel and effective anti-myeloma treatment [3].

Wnt-initiated signal transduction cascades play major roles in the control of cell fate, proliferation and migration during developmental stages of organisms but remain relatively silent in healthy adults. Improper re-activation of the Wnt signaling underlies multiple disorders, most notably cancers [4]. These factors make the Wnt-controlled cascades pharmacologically relevant focusing the efforts of many researchers on the development of novel therapies targeting their components.

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The Wnt signaling is the unifying term for all the numerous events induced in cells by these ligands. It is divided into at least three main branches—the β-catenin (canonical), the planar cell polarity (PCP), and the Ca^{2+}-related signaling. All these events seem extremely diverse, and indeed their major intersection occurs at level of their initiation, *i.e.* interaction of Wnt ligands with the receptor of the Frizzled (FZD) family. Many cancers, including the breast cancer, depend on overactivation of the Wnt/FZD pathway, in many cases—at the level of ligand/receptor interaction through overproduction of a Wnt or a FZD or both [5]. This is especially true for the most aggressive and therapeutically difficult triple-negative breast cancer [6]. Thus, interruption of the WNT/FZD cascade at this level might be an efficient anti-cancer therapy. Despite this potential, presently small molecules affecting the Wnt-FZD ligand-receptor pair are unknown.

In the present work we describe an attempt to find such compounds among those small molecules that are already marketed as drugs. Recent breakthrough in understanding of the molecular determinants of Wnt signaling – the disclosure of the crystal structure of the *Xenopus* Wnt8 in complex with the ligand-binding domain of mouse FZD_8 [7] – provided us with a scaffold to perform *in silico* docking of the 1100-large FDA approved drugs library. Fourteen candidate compounds with higher scores were selected for further *in vitro* testing using combination of the conventional TopFlash transcriptional readout assay [8] and the GTP-binding assay, the latter especially suitable for monitoring of the early steps in Wnt signaling [9].

Out of 14 candidates, we found four that specifically inhibited the Wnt signal transduction. However, in all cases suppression occurs at the levels of the cascade lower than the ligand–receptor interaction. We continued to work with one of these substances, the riminophenazine dye clofazimine which is marketed as an anti-leprosy, anti-tuberculosis and anti-inflammatory agent [10], but has never been shown to affect Wnt signaling before. We further report the specific anti-cancer activity of clofazimine affecting Wnt-dependent triple-negative breast cancer cells.

2. Materials and methods

2.1. In silico *screening*

As the basis of the *in silico* screening of potential ligands for Wnt and FZD proteins, we used the newest X-ray structure of the protein complex from the RCSB protein data bank (PDB id 4F0A, [7]). There are two binding interfaces of Wnt and FZD proteins in the complex which prompted us to select four potential binding sites, two for each protein. The target structures were prepared for docking using the protein preparation wizard incorporated in the AutoDockTools software package [11]. Sites for potential ligands were defined by the $25 \times 25 \times 25$ Å box centered on the proteins connecting residues. The docking process was performed with the Vina AutoDock package at default settings. The selection of drugs with the best scores is described in Section 3.

2.2. Luciferase-based assay of the Wnt-dependent transcriptional activity

HEK293T cells were stably transfected with M50 Super 8x TOPFlash plasmid (Addgene, Cambridge, USA, plasmid 12456) at the 20:1 ratio with the pcDNA3.1 to provide antibiotic resistance. The best responding clone was selected and expanded; this line is referred to as HEK293-Tf throughout the text. The assay was performed in white tissue-culture treated 96-well plates (Greiner, cat. no. 655073). The HEK293-Tf cells were seeded in 100 μl DMEM medium containing 10% FCS at ~5000 cells/well and subsequently stimulated by 0.5 μg/ml mouse Wnt3a purified as described [12] or 20 mM LiCl in the medium in presence or absence of the drug compound for 12 h. The drugs suppliers are as follows: imatinib, nicergoline, sorafenib, bexarotene (Santa Cruz Biotechnology, Heidelberg, Germany), adapalene, risperidone, glimepiride (Tocris Bioscience, Bristol, UK), quinestrol, atovaquone, clofazimine, astemizole, sertindole, ethynylestradiol, vitamin D3(Sigma, St. Gallen, Switzerland). If indicated, the cells were additionally transfected by the pCMV-RL plasmid for constitutive expression of *Renilla* luciferase (kindly provided by Konrad Basler [13]) using X-tremeGENE 9 reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol. The medium was subsequently removed, and 15 μl of the lysis buffer (25 mM Glycylglycine pH 7.8, 1% Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT) were pipetted into each well. After incubation for 5 min at room temperature the 96-well plate was analyzed in the Victor^3 Multilabel Counter (PerkinElmer) with two-channel dispensing unit primed with the buffer solutions for firefly and, if necessary, *Renilla* luciferase activity measurements (prepared as described in [14]). The final volumes dispensed per well were 50 μl of firefly and 50 μl of *Renilla* solutions.

2.3. GTP-binding assay

The GTP-binding assay was performed as described earlier [12] in presence of 30 nM Wnt3a. The expression construct for human FZD_7 was obtained by its subcloning in pcDNA3.1 from I.M.A.G.E. clone IRAUp969F0465D (Source Bioscience, Berlin, Germany).

2.4. β-*Catenin stabilization assay and anti-*β-*catenin staining*

Analysis of β-catenin stabilization in response to the Wnt3a protein was modified from [15]. The L-cells were seeded at ~50,000 cell/well in 48-well plates and the next day the old medium was removed and replaced by either vehicle or drug-containing fresh one. After 1 h pre-incubation at 37 °C, purified Wnt3a or vehicle buffer were directly added in the wells to the final concentration of 1 μg/ml and incubated at 37 °C for 2 more hours. Subsequently the medium was removed, the cells in each well were washed once with 500 μl of 1x PBS (Biochrom AG, Berlin, Germany) and lysed directly in the well by addition of 50 μl of ice-cold RIPA buffer for 10 min on ice. The cells were then resuspended, the debris was removed by 10 min centrifugation at 16,000 g, 4 °C, the probes were further analyzed by Western blot with antibodies against β-catenin (BD, Allschwil, Switzerland, cat.# 610153) and α-tubulin (Sigma, St. Gallen, Switzerland, cat. no. T9026). For staining, the cells were seeded on the glass coverslips and after overnight attachment were stimulated in presence of indicated substances for 12 h. Subsequently, they were fixed in 4%PFA in 1x PBS, washed, blocked and stained with anti-β-catenin antibodies in 1%BSA in 1x PBS. After subsequent staining with DAPI and secondary anti-mouse antibody they were mounted and imaged using Zeiss Axio Imager M2 microscope (Carl Zeiss AG, Feldbach).

2.5. Drug treatment and growth dynamics of HTB19 and HMEC cells

For growth analysis, HTB19 or HMEC cells were seeded at the initial concentration of 10^5 cells/well in the 12-well plates. HTB19 were grown in DMEM (Invitrogen, Lucerne, Switzerland) supplemented with 10% FCS (PAA, Cölbe, Germany); the HMEC were seeded in MEGM (MEBM supplemented with 5 ng/ml EGF, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 70 μg/ml BPE (all from Lonza, Bettlach, Switzerland). In case of HTB19 the next day after seeding the medium was replaced with a fresh batch containing indicated concentrations of drug or vehicle. In case of HMEC cells the drug was provided in the serum-containing MM medium (1:1 mixture