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Research Article

GLYCOGEN SYNTHASE KINASE 3B PREDICTION AS PRIMARY CELLULAR TARGET TO MEDIATE ANTI-HEPATITIS C EFFECT OF NITAZOXANIDE

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ABSTRACT

Hepatitis C is a major healthcare problem that launched the quest and the interest of scientists to search for solutions that alleviate its risks. New groups of drugs are developed and repurposed for this reason. One of the drugs that recently repurposed to be used as an anti-HCV drug is Nitazoxanide (NTZ). NTZ is mainly a thiazolide antiparasitic drug that is mainly used for the treatment of cryptosporidiosis and giardiasis. Tizoxanide (TIZ), which is the active metabolite of NTZ, was recently reported to be active against some viruses including hepatitis C virus (HCV). The anti- HCV mode of action of Nitazoxanide is the overproduction of the hyperphosphorylated HCV non-structural protein 5 A (NS5A). However, the exact mechanism of action is not so clear. Some previous works suggested one member of the CMGC Serine/Threonine protein kinase family to be the primary cellular target of NTZ. A more recent work revealed that NS5A is a direct substrate of casein kinase I α (CKIα). However, no direct effect of NTZ or TIZ was reported on CKIα in enzymatic assays. In this work, starting with the chemical structure of NTZ and TIZ, some in-silico approaches were applied to hypothesize the human primary cellular target for NTZ. Accordingly, glycogen synthase kinase 3β (GSK3β), a member of CMGC Serine/Threonine protein kinase family, was retrieved as a proposed target of NTZ that is likely mediating its anti-HCV effect.

Keywords: drug repurposing, Nitazoxanide mode of action, Similarity ensemble approach, pharmacophore mapping.

INTRODUCTION

Nitazoxanide, also known by the brand name Alinia, is a synthetic nitrothiazolyl-salicylamide derivative and an anti-protozoan agent. It is approved for treatment of infectious diarrhea caused by *Cryptosporidium parvum* and *Giardia lamblia* in patients 1 year of age and older. Following oral administration it is rapidly hydrolyzed to its active metabolite, tizoxanide, which is 99 % protein bound. Its mode of action is the inhibition of parasitic pyruvate ferrodoxin oxidoreductase (PFOR) enzyme. NTZ and its active metabolite TIZ were recently reported to exhibit potent antiviral activity against some viruses including multiple genotypes of HCV. NTZ is now used in a triple therapy with peginterferon and ribavirin for the treatment of HCV[1-3,10]. HCV replicon contains a non-structural protein called (NS5A). This protein has two phosphoforms:

- The basal form which is referred to as (p56)
- The hyperphosphorylated form which is referred to as (p58)[4].

It was noticed that the elevated levels of p58 down-regulates HCV replication. Tentatively, the analysis of the effects of a panel of kinase inhibitors on NS5A phosphorylation *in vivo* and *in vitro* has suggested that the kinase responsible for the majority of p58 overproduction may be a member of the CMGC kinase group. This enzyme family includes casein kinase II (CKII) and glycogen synthase kinase 3 (GSK3) subfamilies[5]. In a more recent work, the membrane-associated cellular kinase (previously known as casein kinase) CKIα has been shown to be the enzyme responsible for NS5A hyperphosphorylation in cell culture[6]. However, due to the lack of a demonstration of a direct effect by TIZ on CKIα, its primary cellular target, the mode of action of NTZ remains unknown[7].

MATERIALS AND METHODS

Two in-silico approaches were applied directly to the chemical structures of NTZ and its active metabolite TIZ:

The similarity ensemble approach

In 2009, Keiser *et al*, confirmed new drug-target associations using chemical similarities between drugs[8]. This new approach was featured among top scientific breakthroughs and was referred to as the Similarity Ensemble Approach (SEA). According to this approach, two significant chemically similar ligands are most likely to target the same protein[9]. The similarity tool embedded in the drug bank database[10] was used directly from the NTZ page. The search parameters were by default: (all drugs – Tanimoto similarity – similarity threshold = 0.6).

The pharmacophore mapping approach

The pharmacophore of a ligand is the necessary molecular features causing this ligand to be recognized by the biological targets. Mapping the ligand's pharmacophore is an in-silico method to predict its biological target. The Pharmapper[11] server in-silico tool was used to investigate the biological target of TIZ. TIZ MOL2 file was downloaded from ZINC database[12] and then submitted as a query file at the (submit job) page at the Pharmapper server (http://59.78.96.61 /pharmmapper/submit_file.php). In step 2 of submitting the job, the chosen parameters were:

- Pharmacophore mapping targets set = Human Protein Targets Only (2,241).
- Number of Reserved Matched Targets (Max 1,000) = 50.

At the end of the submission, the job id was (13105055132).

RESULTS

The similarity ensemble approach (SEA) results

The chemical similarity tool of the Drug Bank database retrieved two significant hits. The first is the NTZ itself with Tanimoto co-efficient = 1 and the other is the experimental drug (N-(4-Methoxybenzyl)-N'-(5-Nitro-1,3-Thiazol-2-

Yl)Urea) with Tanimoto co-efficient = 0.653 (Table 1). According to its page in the Drug Bank database, the known biological target of this experimental drug is the GSK3β. This page can be accessed directly from the hyperlink of the experimental drug in the search results page.

The pharmacophore mapping approach results

According to the Pharmapper tool results for the job no. (13105055132) the best 50 human protein targets for TIZ were retrieved and - by default - ranked by fit score in descending order. The GSK3β has the best Z-score (1.10278), the best normalized fit score (0.7677) and the eighth best fit score (3.838) for the human protein targets predicted for TIZ. According to the manual (help document) of the tool, the best significant target is the protein with the best Z-score. In this job the best Z-score was for the GSK3β (Table 2).

Table 1: The two significant drugs retrieved by the similarity tool of the Drug Bank database

Drug bank ID	Name	score	Drug group
DB00507	Nitazoxanide	1	approved
DB01950	N-(4-Methoxybenzyl)-N'-(5-Nitro-1,3-Thiazol-2-Yl)Urea	0.653	experimental

Table 2: The best five PDB proteins retrieved by Pharmapper tool as the best target proteins ranked by Z-score

Rank	PDB ID	Target name	Fit score	Normalized fit score	Z-score
1.	1Q5K	GSK3β	3.838	0.7677	1.10278
2.	1P2S	GTPase HRas	4.52	0.3014	0.78427
3.	1R1H	Neprilysin	4.277	0.4277	0.467359
4.	1MD4	Glutathione S- transferase P	3.871	0.3871	0.316834
5.	1KBN	Glutathione S- transferase P	3.884	0.3844	0.314162

DISCUSSION

It's well settled that the over production of the hyperphosphorylated form of the NS5A protein (p58) down-regulates the HCV replication. The phosphorylation occurs at the c-terminus of the protein which contains serine and proline residues. In 1997, Reed *et al*. work suggested that the kinase responsible for the majority of NS5A hyperphosphorylation is a member of CMGC kinase group. This group of kinases is sub-divided into families including:

- Serine/Threonine kinases like cellular kinases (CK) (previously known as casein kinases).
- Proline-directed kinases like glycogen synthase kinase 3 (GSK3).
- Other kinase families that are out of our interest in this work.

Later, CKIα was reported to be a direct substrate of NS5A that causes hyperphosphorylation at c-terminus serine residues. This finding does not exclude that other kinases may be involved in different types of NS5A phosphorylation. Moreover, no evidence was found about a direct effect of NTZ or TIZ on CKIα. Instead of the retro-grade way used to figure-out the pathway that NTZ causes the hyperphosphorylation of NS5A, this work used a direct way to suggest the human protein that can act as a primary target for NTZ. We succeeded to spot a protein kinase that may act as a primary target for NTZ using two different in-silico approaches. One supporting sign for our result is that GSK3β is a proline-directed kinase. This means that its site of action should be preceded by proline. In fact, the c-terminus of NS5A protein is rich with proline residues[6]. Another supportive point is that we mapped the pharmacophore of the experimental drug (N-(4-Methoxybenzyl)-N'-(5-Nitro-1,3-Thiazol-2-Yl)Urea) with the Pharmapper tool[11]. As expected, the on-label target of NTZ, namely PFOR enzyme, appeared as a possible protein target for the experimental drug (check job ID 131016220415)[11]. The PFOR rank was not as good as the rank of GSK3β as a target for NTZ. However, this may be due to the lack of "non-human proteins only" option in the search parameters of the pharmapper tool. So, the expected human protein targets are mixed with non-human ones. If human protein targets are excluded, the rank of PFOR will get better. A contradicting finding is that CKIα activity in intracellular membrane preparations from NTZ-treated HCV replicon cells was two fold higher than those from untreated cells in enzymatic assays[7]. However, this paradox may be explained by the fact that GSK3β substrate recognition requirements are complex and, in some cases, depend on prior phosphorylation events catalyzed by other kinases[6]. In other words, CKIα activity increment may be the prior phosphorylation activity that paves the way to GSK3β for the hyperphosphorylation of the serine residues at the NS5A c-terminus. At last, some further investigations are needed to be done in wet labs like:

- Is NTZ a direct substrate of GSK3β ? (Wet lab evidence is needed).
- Is NS5A protein a direct substrate for GSK3β?

Also, there is a need to profile the GSK3β activity based on NTZ treatment. Hopefully, this may lead to figuring out the mode of action of NTZ that may reveal a new group of drug to be used for the effective treatment of HCV.

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