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Original article

Evaluation of potential Myt1 kinase inhibitors by TR-FRET based binding assay

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ABSTRACT

In the human cell cycle, the Myt1 kinase is a crucial regulator of the G2/M transition. Because this membrane-associated kinase is hard to obtain and assay, there is a distinct lack of data so far. Here we report the derivatization of a glycoglycerolipid which was shown previously to be active in a Myt1 activity assay. These compounds were tested in a binding assay together with a set of common kinase inhibitors against a full-length Myt1 expressed in a human cell line. Dasatinib exhibited nanomolar affinity whereas broad coverage inhibitors such as sunitinib and staurosporine derivatives did not show any effect. We also carried out docking studies for the most potent compounds allowing further insights into the inhibitor interaction of this kinase. The glycoglycerolipids showed no significant effects in the binding assay, endorsing the idea of a mechanism of action distant from the active site.

1. Introduction

The cell cycle includes two checkpoints which provide the cell with opportunities to monitor genomic integrity. These checkpoints can delay the cell cycle progress and allow cells to repair damaged DNA in order to prevent it from being passed on to daughter cells. Many cancer cell lines show deficient G1 checkpoint mechanisms, thus relying on the G2 checkpoint far more than normal cells [1,2]. For this reason, G2 checkpoint abrogation is a promising concept to damage cancerous cells in a preferential way [3]. The main feature influencing the decision to enter mitosis is a complex composed of Cdk1 and cyclin B. Cdk1 exists in a monomeric form and is inactive as a kinase. Association with cyclin B in S- and G2-phase renders the heterodimeric kinase active. Once activated, the Cdk-cyclin complex can phosphorylate hundreds of substrate proteins, finally leading to mitosis [4,5]. Cdk1/CycB is regulated by various feedback mechanisms of which inhibitory phosphorylations at Thr14 and Tyr15 by the Wee family kinases are considered essential [6]. Wee1 phosphorylates Cdk1 specifically at Tyr15 whereas Myt1 is dual-specific for Tyr15 as well as Thr14. Abrogation of the G2 checkpoint by selective inhibition of Wee1 resulted in a sensitization of p53-deficient tumor cells to DNA-damaging agents [7] and was also shown to have single-agent anti-tumor activity against sarcoma cells [8]. Indeed, pushing the cell cycle forward into mitosis might be a more effective strategy to treat cancer than stagnation of the cell cycle, though the optimal target, be it Wee1, Myt1 or another, is not yet clear [9]. Myt1 downregulation by RNA interference did not lead to a significant augmentation of doxorubicin effects. However, inhibition by a small molecule inhibitor is proposed to be a promising option because Myt1 binds Cdk1, altering equilibria and, thus, affecting G2/M transition [10]. In addition, selective Myt1 inhibitors could help analyze contributions of the various components involved in the mitotic entry network [6]. Wee1 as a soluble protein is well investigated and has been established in many kinase panels for tests of potential inhibitors (e.g. Bruyère et al. [11]). In contrast, Myt1 as a membrane associated kinase [12] is much more difficult to prepare and test.

To the best of our knowledge, full-length Myt1 has never before been tested against a set of common kinase inhibitors. Using a coupled Cdk1 – Wee kinase activity assay, Wang et al. reported the G2 checkpoint abrogator PD0166285 to be a potent ATP-competitive inhibitor [13]. This compound proved to have strong nanomolar effects on Wee1 and Myt1 in vivo and in vitro, but the inhibitor is three times more selective for Wee1 and shows, moreover, inhibitory properties against many other tyrosine kinases such as c-Src, epidermal growth factor receptor, and

Abbreviations: BCA, bicinchoninic acid; Cdk1, cyclin dependent kinase 1; CycB, cyclin B; DMSO, dimethylsulfoxide; DTT, dithiothreitol; GGL, glycoglycerolipid; HEK, human embryonic kidney; HR-MS, high resolution mass spectrometry; TR-FRET, time-resolved fluorescence resonance energy transfer.

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platelet-derived growth factor receptor [14,15]. A natural product originally derived from marine algae was reported to have strong inhibitory effects on Myt1 (IC_{50} 0.12 μg/ml) and, additionally, showed high selectivity over other kinases such as Akt and Chk1 [16]. Chemically, the isolated product is a glycoglycerolipid (GGL), whose scaffold is totally different from that of conventional inhibitors. We synthesized the respective GGL and performed a systematic derivatization to exclude unspecific effects and to investigate the actual mechanism.

In addition to the unknown inhibition pattern, Myt1 assay development is hindered by a restrictive substrate acceptance [17]. Therefore, we used a TR-FRET based kinase binding assay [18] to evaluate potential inhibitors.

2. Chemistry

2.1. Organic synthesis

2.1.1. Strategy

A collection of GGLs based on the reported Myt1 inhibitor was synthesized. Previously, we described the neosynthesis of 1,2-dipalmitoyl-3-(*N*-palmitoyl-6′-amino-6′-deoxy-α-D-glucosyl)-*sn*-glycerol (**1α**) in a multistep strategy starting from α-methylglucopyranoside [19]. Based on these results, we altered the hexopyranose core from the glucoside to the epimeric mannoside and galactoside with respect to the anomeric configuration. Furthermore, using the glucoside as a starting point, the palmitoyl chains were shortened to octanoyl chains. Synthesis route and conditions are displayed in Scheme 1. Briefly, the starting material was blocked in position 6 by tritylation of the primary hydroxyl group. After benzylation of the remaining secondary hydroxyl groups we removed the trityl residue to convert the 6-OH group into an azide after activation with methanesulfonyl chloride. Demethylation in position 1 led to a glycosyl donor which reacts after activation with (*S*)-1,2-isopropylideneglycerol. After hydrolysis of the ketal, the characteristic hydrophobic chains can be inserted by acylation. Reduction of the azido group yielded the respective amine that was *N*-acetylated to realize the third acyl chain. Finally, catalytic hydrogenation gave the aspired products. Identities were confirmed by NMR spectroscopic analysis and high resolution mass spectrometry (HR-MS).

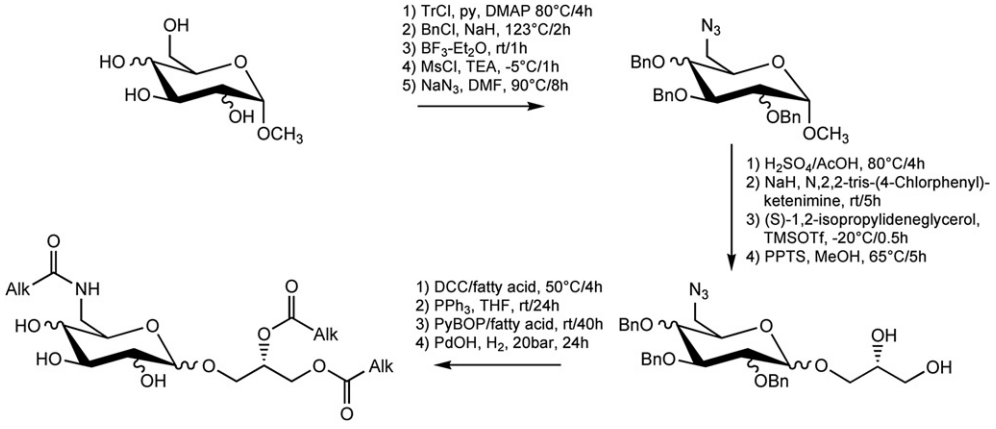
A general illustration of the compounds synthesized is shown in Table 1. Characterization of intermediate products for **1α** is already described in Ref. [19]. In terms of the other final products, characterization of intermediate compounds was carried out analogously. Due to reasons of data compression we herein report data for final products only.

Mass spectra (MS) were recorded off-line with nano-ESI (Proxeon emitters, Odense, Denmark) on a LTQ-Orbitrap XL (Thermo Fisher Scientific, San Jose, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 and a Varian Inova 500; chemical shifts were referenced to residual solvent signals and reported in ppm (δ). Chromatography was performed on silica gel (Merck silica gel 60, 40–63 mesh) by MPLC. Therefore, columns were prepared with a Cartridger C-670 (Büchi). Fractions were sampled with a *Fraction Collector C*-660 (Büchi) by discontinuous enhancement of polarity, for pressure we used a Pump Module C-601 and a Pump Manager C-615 (Büchi). TLC was carried out on silica gel plates (E. Merck 60 F_{254}); spots were detected visually by ultraviolet irradiation (254 nm) or by spray detection (solution of 0.5 g thymol and 5 ml conc. sulfuric acid in 100 ml ethanol) and heated to 130 °C for 10 min. All reagents were used as purchased unless stated otherwise. Solvents were dried according to standard procedures. All reactions were carried out under an atmosphere of dry argon. All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany).

2.1.2. Characterization of final products

*2.1.2.1. 1,2-Dipalmitoyl-3-(N-palmitoyl-6′-amino-6′-deoxy-α-D-glucosyl)-sn-glycerol (**1α**).* ^{1}H NMR: 500 MHz, $CDCl_3$: δ = 5.85–5.89 (m, 1H, –N*H*–CO–), 5.19–5.23 (m, 1H, *sn*2), 4.78 (d, 1H, $^3J_{H\text{-}1/H\text{-}2}$ = 3.7 Hz, *H*-1), 4.36 (dd, 1H, $^2J_{sn1/sn1'}$ = 11.8 Hz, $^3J_{sn1/sn2}$ = 4.0 Hz, *sn*1), 4.11 (dd, 1H, $^2J_{sn1/sn1'}$ = 11.8 Hz, $^3J_{sn1'/sn2}$ = 5.7 Hz, *sn*1′), 3.97–4.03 (m, 1H, *H*-6), 3.77 (dd, 1H, $^2J_{sn3/sn3'}$ = 10.7 Hz, $^3J_{sn2/sn3}$ = 4.4 Hz, *sn*3), 3.71 (t, 1H, ^3J = 9.4 Hz, *H*-3), 3.61 (dd, 1H, $^2J_{sn3/sn3'}$ = 10.7 Hz, $^3J_{sn3'/sn2}$ = 6.0 Hz, *sn*3′), 3.54–3.57 (m, 1H, *H*-5), 3.46 (dd, 1H, $^3J_{H\text{-}1/H\text{-}2}$ = 3.7 Hz, $^3J_{H\text{-}2/H\text{-}3}$ = 9.4 Hz, *H*-2), 3.08 (t, 1H, ^3J = 9.4 Hz, *H*-4), 3.00–3.04 (m, 1H, *H*-6′), 2.27–2.30 (m, 4H, 2× (–O–CO–CH_2–)), 2.21–2.24 (m, 2H, –NH–CO–CH_2–), 1.55–1.63 (m, 6H, 3× (–CO–CH$_2$–CH_2–(CH$_2$)$_{12}$–CH$_3$)), 1.21–1.31 (m, 72H, 3× (–CH$_2$–CH$_2$–(CH_2)$_{12}$–CH$_3$)), 0.86 (t, 9H, *J* = 7.0 Hz, 3× (–CH_3)); ^{13}C NMR: 100 MHz, $CDCl_3$: δ = 13.4, 23.1, 24.7, 25.3, 25.7, 28.5–29.8, 32.1, 33.7, 34.7, 36.9, 39.9, 62.2, 66.6, 69.8, 70.3, 71.3,72.6, 73.6, 76.8, 99.7, 172.8, 173.3, 175.4; MALDI TOF mass spectrum: *m*/*z* 990 (M + Na^+). HRFABMS *m*/*z* 968.8121 [M + H^+]; (calcd for $C_{57}H_{110}NO_{10}$ 968.8130).

*2.1.2.2. 1,2-Dipalmitoyl-3-(N-palmitoyl-6′-amino-6′-deoxy-β-D-glucosyl)-sn-glycerol (**1β**).* ^{1}H NMR: 500 MHz, $CDCl_3$: δ = 6.68–6.72 (m, 1H, –N*H*–CO–), 5.32–5.36 (m, 1H, *sn*2), 4.58 (dd, 1H, $^2J_{sn1/}$



Scheme 1. General scheme of organic synthesis for the glycoglycerolipids starting from the α-methylglycoside in a 13 step synthesis route.