De Novo Synthesis and Biological Evaluation of C6′′-Substituted C4′′-Amide Analogues of SL0101

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Supporting Information

ABSTRACT: In an effort to improve upon the in vivo half-life of the known ribosomal s6 kinase (RSK) inhibitor SL0101, C4′′-amide/C6′′-alkyl substituted analogues of SL0101 were synthesized and evaluated in cell-based assays. The analogues were prepared using a de novo asymmetric synthetic approach, which featured Pd-π-allylic catalyzed glycosylation for the introduction of a C4′′-azido group. Surprisingly replacement of the C4′′-acetate with a C4′′-amide resulted in analogues that were no longer specific for RSK in cell-based assays.

The ribosomal s6 kinases (RSKs) are a family of Ser/Thr kinases, which are downstream effectors of the extracellular signal-regulated kinase 1/2 pathways.1 RSK appears to be involved in the etiology of a number of different cancers and, importantly, regulates a motility/invasive gene program.2 RSK is a dual kinase domain protein with the N-terminal kinase domain (NTKD) responsible for phosphorylation of target substrates.3 In a screen of botanical extracts N-terminal kinase domain (NTKD) responsible for phosphor-ylation of target substrates.3 In a screen of botanical extracts SL0101 (1), a flavonoid glycoside, was identified as an inhibitor of the NTKD of RSK.4 SL0101 (1) is a relatively selective inhibitor for RSK with a Ki of 1 µM. From the crystal structure of SL0101 (1) complexed with the NTKD isoform of RSK25 and de novo synthetic studies,6 we identified analogues (2 and 3) with C6′′-substitutions of the rhamnose that showed improved efficacy in the in vitro kinase assays.7,8

SL0101 (1) has a short biological half-life in vivo,7 which is presumably due to hydrolysis of the C3′/C4′-acetates which are necessary for high affinity.9 To identify less labile groups that could replace the ester without loss of affinity, we investigated replacing the C4′′-acetate with a C4′′-acetamide in combination with the C6′′-alkyl substitution that we previously identified.7 Specifically, we targeted six C4′′-acetamide analogues 4a–d and 5a–b (Figure 1).

Retrosynthetically, we envisioned that C4′′-acetamide substituted analogues 6 could arise from C4′′-azido sugar 7a, which could be prepared from enone sugar 7c via allylic carbonate 7b (Scheme 1). Previously we have shown that C4 allylic azides such as 7a could be prepared from C4 allylic carbonates like 7b via Pd-catalyzed allylic alkylation.10 However, this approach was not compatible for pyran rings with a C1 kaempferol group. To address this issue, a Pd-

glycosylation method was developed for the direct incorporation of a C4′ azido sugar.

Our synthesis started with exposure of flavonol 9 and Boc-pyranoane 13 to our typical glycosylation conditions (2.5 mol % Pd[(DBA)3]·CHCl3 and 10 mol % of PPh3 in CH2Cl2 at 0 °C; 95%), which produced glycosylated pyranone 14 with complete α-selectivity (Scheme 2). Reduction of the enone 14 (NaBH4/CeCl3, −78 °C in CH2Cl2/Methanol, 72%) resulted stereoselectively in allylic alcohol 15.6 A methyl carbonate leaving group was installed on the allylic alcohol by reaction of 15 with methyl chloroformate to form the C4′′-carbonate 16 in 75% yield. Unfortunately, exposure of carbonate 16 to the Sinou conditions ([TMSN3]·[Pd(allyl)Cl]2·1,4-bis(diphenylphosphino)butane) failed to afford the desired regio- and stereoisomeric allylic azide 17. The C-1 kaempferol proved to be the better leaving group, as only products consistent with the hydrolysis at the anomeric position were observed.11

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To solve this problem, we decided to try reversing the sequence of the two Pd-π-allyl substitution reactions, which required the synthesis of allylic azides 29 and 30 (Scheme 3).

This began with a palladium-catalyzed glycosylation (Pd(0)/PPh3, 1:2) of p-methoxybenzyl alcohol with Boc-pyranones 13 and 18 which stereoselectively afforded PMB-pyranones 19 and 20 (95% and 92% respectively). Diastereoselective reduction of the two enones (NaBH4/CeCl3, −78 °C in CH2Cl2/MeOH; 92% and 84%) gave allylic alcohols 21 and 22. Treatment of the two allylic alcohols with methyl chloroformate in the presence of a catalytic amount of DMAP gave the allylic carbonates 23 and 24 (94% and 90%). Exposure of the carbonates to the Sinou conditions (TMSN3, [Pd(allyl)Cl]2/1,4-bis(diphenylphosphino)butane) regio- and stereospecifically afforded the desired allylic azides 25 and 26 (73% and 75%). An oxidative PMB deprotection (DDQ/H2O) of 25 and 26 provided anomeric alcohols 27 and 28 as a 13:1 mixture of anomers in 73% and 78% yields. The following Boc-protection of the two alcohols produced the key azido containing intermediates 29 and 30 in 84% and 82% yields with excellent diastereoselectivity.

To our delight, exposure of sugar donor Boc-allylic azides 29 and 30 and acceptor 9 to our typical Pd-catalyzed glycosylation conditions provided our desired glycosylated allylic azides 17 and 31 in excellent yield (98% and 90%) with complete α-selectivity and no sign of hydrolysis at the anomeric position. Exposure of the two allylic azides to Upjohn conditions (OsO4/NMO; 91% and 87%) stereoselectively converted them into the two rhamno-diols 32 and 33, which are poised for further manipulation into the desired SL0101 analogues (Scheme 4).

We next investigated the reduction and acylation of azidodiols 32 and 33 (Scheme 5). Fortuitously, both the C4 acylated amides 36 and 37 and C3/C4 bis-acylated products 34 and 35 were generated in an ~1:1 mixture in one pot from the reduction of 32 and 33 with zinc dust in the presence of acetic anhydride and acetic acid. Thus, the reduction acylation of 32 gave the desired C4″-acetamides 36 (29%) and 34 (27%), whereas the reduction acylation of 33 gave the desired C4″-acetamides 37 (36%) and 35 (32%).

The intermediates 34−37 were globally deprotected by an exhaustive hydrogenolysis, which produced four of the desired analogues. Thus, exposure of 34 and 35 to typical hydrogenolysis conditions (1 atm of hydrogen with Pd/C) furnished 4b and 5b in good yields (86% and 88%, respectively). Exposure of 36 and 37 to similar hydrogenolysis conditions furnished 4a and 5a in good yields (90% and 86%, respectively). Finally the last two analogues 4c and 4d were prepared by an acylation deprotection sequence. The peracylated product 4d was prepared from 34 in 91% overall yield by bis-acylation (Ac2O, DMAP/Py; 97%) and exhaustive hydrogenolysis. Similarly, the C2 acylated product 4c was prepared from 36 via an ortho-ester mediated C2-acylation (CH3C(O)Me)3, 10% p-TsOH in CH2Cl2; then excess 90%
AcOH/H₂O; 85%) and per-hydrogenolysis (94% overall yield).

The efficacy of the analogues 4a–d and 5a–b to inhibit RSK2 activity was determined in an in vitro kinase assay using purified recombinant RSK2 (Table 1). The data were fit using nonlinear regression analysis. In the n-Pr series, 4b and 4c with a single acetate at the C3°- or C2°-position had significantly lower (~5-fold) IC₅₀s compared to SL0101. However, when compared with our best analogue 2a (C3°/C4°-diacetate, Figure 1), the related C4°-acetamide 4b had a 10-fold increase in IC₅₀. The IC₅₀s for 4a with no C2°- or C3°-acetate and 4d with two acetates were not statistically different from that of SL0101. These results are similar to those obtained in the series in which the acetyl group was at the C4°-position. In the isobutyl series the C3°-acetate 5b had a 3-fold improved IC₅₀ compared to that of SL0101, whereas 5a with no C2°- or C3°-acetate had a much poorer IC₅₀ than SL0101. This suggests that, in the n-Pr-series, the C4°-acetamide can replace the C4°-acetate without dramatically compromising the affinity of the analogues for RSK2.

The six analogues were evaluated for their ability to decrease proliferation of the breast cancer cell line, MCF-7 (Table 1). Initially, each analogue was tested at a dose of 100 μM and compared to SL0101 (1). Analogue 4d was the only analogue that inhibited proliferation to a greater extent than SL0101 (1). A dose response curve with 4d showed that cytotasis occurred at ~35 μM and substantial cell death occurred at ~50 μM (see Supporting Information (SI)). For comparison SL0101 (1) at 100 μM (maximum concentration) induces a reduction in proliferation (~60%). To evaluate whether 4d was specific for RSK, we compared its antiproliferative effects in MCF-7 cells versus MCF-10A, an immortalized nontumored human breast cell line. We previously found that a preferential ability to inhibit MCF-7 compared to MCF-10A proliferation correlates with specificity for RSK inhibition. At 25 μM 4d inhibited proliferation of MCF-7 cells by 50% and marginally inhibited MCF-10A proliferation (see SI). However, at 50 μM of 4d, a cytotoxic dose in MCF-7 cells, proliferation of MCF-10A cells was inhibited by 70%. Thus, 4d shows a very limited ability to preferentially inhibit MCF-7 proliferation and survival compared to MCF-10A cells. These results suggest that 4d is not a specific RSK inhibitor in intact cells.

To further evaluate the specificity of 4d at inhibiting RSK, we compared the efficacy of SL0101 (1) and 4d to alter the phosphorylation of known RSK substrates. We chose to test 4d at both cytostatic (25 μM) and cytotoxic (50 μM) concentrations. To increase the phosphorylation of substrates MCF-7 cells were stimulated with the mitogen, phorbol myristate acetate (PMA), after a pretreatment with inhibitor or vehicle. RSK phosphorylates and inhibits the activity of eukaryotic elongation factor 2 (εEF2) kinase. Thus, inhibition of RSK relieves the inhibition of εEF2 kinase, which results in an increase in p-εEF2. As expected activation of RSK by PMA led to a decrease in p-εEF2 and inhibition of εEF2 kinase. Thus, we compared the εEF2 phosphorylation of MCF-7 cells by 50% and marginally inhibited MCF-10A proliferation (see SI). However, at 50 μM of 4d, a cytotoxic dose in MCF-7 cells, proliferation of MCF-10A cells was inhibited by 70%. Thus, 4d shows a very limited ability to preferentially inhibit MCF-7 proliferation and survival compared to MCF-10A cells. These results suggest that 4d is not a specific RSK inhibitor in intact cells.

Ribosomal protein S6, a component of the 40S ribosomal subunit, is phosphorylated by RSK, and in agreement with these data SL0101 (1) inhibits PMA-induced phosphorylation of S6. We have also found that RSK regulates the levels of the oncogene, cyclin D1, in MCF-7 cells. Consistent with these observations SL0101 (1) inhibited cyclin D1 levels. In contrast with our observations with SL0101 the analogue 4d did not alter the phosphorylation status of εEF2, S6 or the levels of cyclin D1. To further investigate the ability of 4d to inhibit RSK in intact cells, we immunoblotted the lysates with an antibody against the phosphorylation motif that is

Table 1. In Vitro Potency of SL0101 (1) and Analogue**

| compd name | RSK2 IC₅₀ [μM] | RSK2 IC₅₀ p(1) | MCF-7 proliferation [% control] | MCF-7 proliferation p(DMSO) | MCF-7 proliferation p(1) |
|------------|----------------|----------------|-------------------------------|----------------------------|--------------------------|
| SL0101 (1) | 1.04 ± 0.60    | 0.17           | 38.6 ± 14.6                   | <0.01                      | 0.91                     |
| 4a         | 0.76 ± 0.43    | 0.17           | 94.5 ± 21.6                   | 0.41                       |                          |
| 4b         | 0.23 ± 0.07    | <0.01          | 39.2 ± 7.2                    | <0.01                      | 0.16                     |
| 4c         | 0.11 ± 0.09    | <0.01          | 47.8 ± 10.6                   | <0.01                      | <0.01                    |
| 4d         | 0.44 ± 0.39    | 0.02           | −80.0 ± 6.6                   | <0.01                      | 0.21                     |
| 5a         | 2.33 ± 0.88    | <0.01          | 47.5 ± 19.1                   | <0.01                      | 0.03                     |
| 5b         | 0.32 ± 0.18    | <0.01          | 24.6 ± 10.4                   | <0.01                      |                          |

**RSK2 IC₅₀: concentration needed for 50% RSK2 inhibition (n > 2; quadruplicate: mean, S.D.); p(1) Student’s t test compared to SL0101(1). MCF-7 proliferation: (n > 2; triplicate: mean, S.D.; p(DMSO) Student’s t test compared to control; p(1) Student’s t test compared to SL0101 (1)). p < 0.01 significant.
recognized by numerous kinases, including RSK. Treatment with SL0101 increased the phosphorylation of a band at ~60 kDa and decreased the intensity of a band at ~27 kDa. Analogue 4d did not alter the phosphorylation pattern as compared to PMA. Consistent with these results we observed that analogue 4b (100 μM) did not alter the phosphorylation of RSK biomarkers or cyclin D1 levels in intact cells (data not shown). These results suggest that the amide analogues of SL0101 (1) are not specific for RSK.

In conclusion, using de novo synthesis C4’-acetamide analogues of SL0101 with a C6’ substitution were prepared and evaluated as RSK inhibitors. Analogues with improved in vitro kinase inhibitory activities were identified; however, this increase in activity came at a loss of selectivity for RSK. Further studies aimed at defining the requirements for a specific-RSK inhibition are ongoing and will be reported in due course.

**ASSOCIATED CONTENT**

2 Supporting Information
Experimental procedures and spectral data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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