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Analogs of the RSK inhibitor SL0101: Optimization of in vitro biological stability

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ABSTRACT

The Ser/Thr protein kinase, RSK, is important in the etiology of tumor progression including invasion and motility. The natural product kaempferol-3-*O*-(3″,4″-di-*O*-acetyl-α-L-rhamnopyranoside), called SL0101, is a highly specific RSK inhibitor. Acylation of the rhamnose moiety is necessary for high affinity binding and selectivity. However, the acetyl groups can be cleaved by esterases, which accounts for the poor in vitro biological stability of SL0101. To address this problem a series of analogs containing acetyl group replacements were synthesized and their in vitro stability evaluated. Monosubstituted carbamate analogs of SL0101 showed improved in vitro biological stability while maintaining specificity for RSK. These results should facilitate the development of RSK inhibitors derived from SL0101 as anticancer agents.

The members of the p90 ribosomal S6 kinase (RSK) family of Ser/Thr protein kinases have been shown to play a role in a number of different cancers as key drivers of proliferation and metastasis.[1–8] These discoveries have been enabled in part by our report of the identification and isolation of the RSK inhibitor SL0101 (**1**, Fig. 1).[9] SL0101 is a flavonoid glycoside (kaempferol 3-*O*-(3″,4″-di-*O*-acetyl-α-L-rhamnopyranoside)) isolated from *Forsteronia refracta*, a variety of dogbane found in the South American rainforest. SL0101 is highly specific for RSK, inhibiting RSK1/2 but not unrelated kinases nor the closely related kinases MSK1 and p70S6K1.[2,9,10] SL0101 inhibits the proliferation of breast and prostate cancer lines but not their normal counterparts even though it inhibits RSK activity in all the lines.[1,5,9] Thus it appears that some cancer cells have become addicted to RSK, which suggests that RSK may be a potential new target for cancer therapeutics. SL0101, owing to its exquisite specificity, is a compelling lead compound from which to begin the process of identifying drug-like RSK inhibitors.

We and others have reported the total synthesis and biological evaluation of SL0101 and a number of analogs, with the ultimate goal of developing an anticancer drug that targets RSK.[11–15] These analogs have provided key information about the SAR of both the aglycone and carbohydrate portions of the natural product. In the course of this work we discovered that the 3″ and 4″ acetyl groups of the carbohydrate are critical for potency and specificity for RSK.[13] TriOH-SL0101 (**2**), lacking these acetyl groups, is 12-fold less potent for inhibition of RSK in vitro and does not inhibit the growth of cancer cell lines, likely due to poor membrane permeability.[13] These results indicate that SL0101 is not a suitable candidate for in vivo evaluation, as hydrolysis of the acetates by esterases would generate a less potent inhibitor.

An analog that replaces these acetates with ethyl ethers (**3**) inhibits RSK with potency roughly equivalent to SL0101.[13] We previously determined that the specificity of SL0101 and its analogs for RSK could be evaluated by their preferential ability to inhibit the growth of the human breast cancer line, MCF7, compared to the normal human breast line, MCF-10A.[12] Unexpectedly, we observed that the ethyl ether analog **3** inhibited both lines to a similar extent, which indicates that it has a decreased specificity for RSK.[13] These results demonstrate that the acetates are a key modulator of specificity and thus a more carefully considered approach is necessary to identify suitable replacements. Accordingly, we have focused our efforts on identifying analogs bearing replacements for the acetates that confer greater biological stability without decreasing potency or specificity for RSK. Herein we present our approach, which has led to the identification of SL0101 analogs that are both specific for RSK and more biologically stable in vitro than the parent compound.

The only structural difference between the diethyl analog **3** and the diacetyl parent compound is the replacement of two methylenes with two carbonyl groups. It is surprising that such a seemingly small structural feature can regulate specificity for RSK. To recover this specificity, in the design of new analogs we sought to better mimic the acetates and particularly the acetate carbonyls, sterically and electronically, in a way that would confer a greater resistance to metabolism by esterases. In one approach we investigated the dependence of potency and specificity on the relative

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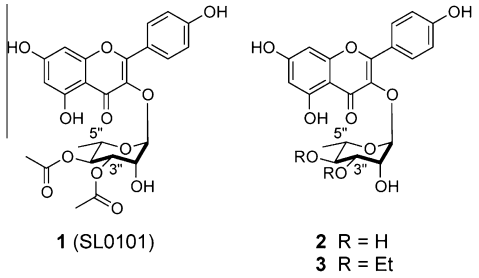
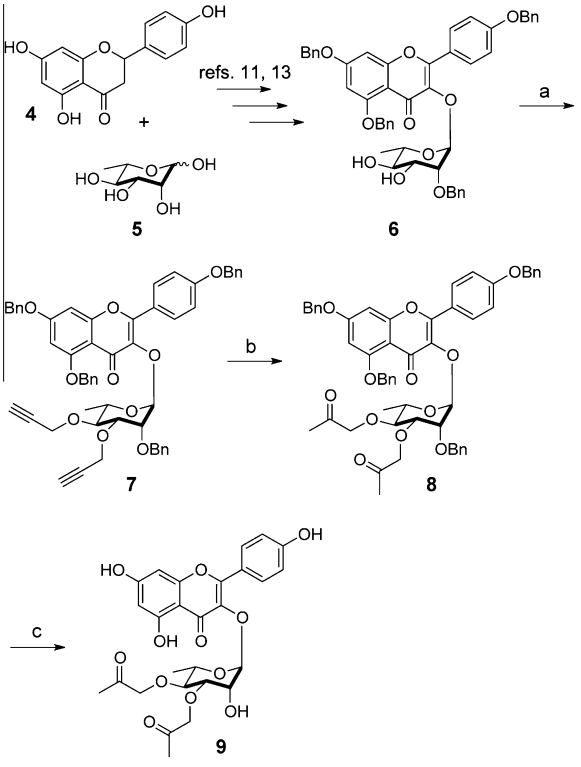


Figure 1. The RSK inhibitor SL0101 and two previously reported analogs.[13]

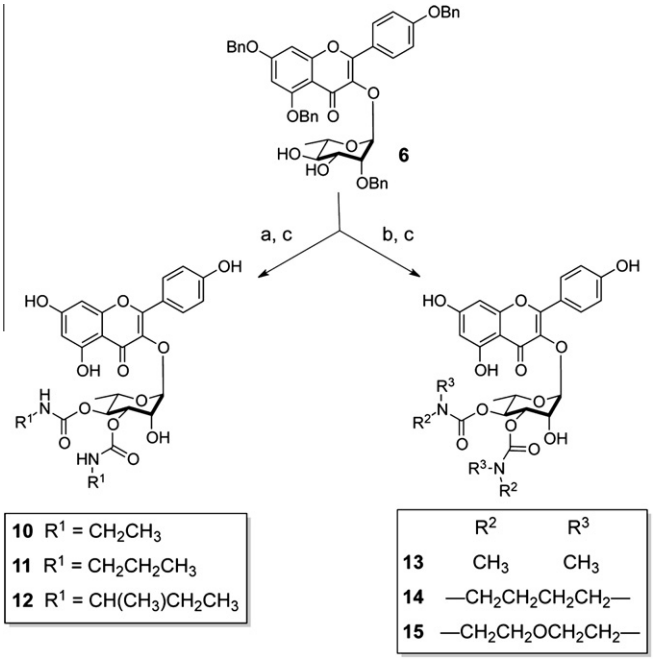
position of the carbonyl group. To this end we prepared an analog **9** in which the acetates are replaced by alkoxyacetones (Scheme 1), moving the carbonyl group one carbon further from the carbohydrate ring and replacing the labile ester bond with an ether bond. The desired functionality could be installed at a late stage in the synthesis of the analog. Alkylation of known diol **6**[13] with propargyl bromide provided bis-alkyne **7**, which after mercury-catalyzed hydration provided bis-ketone **8**. Removal of the benzyl ether protecting groups by hydrogenolysis using Pearlman's catalyst provided the completed analog **9**.

In a second approach we retained the acetate carbonyl in the correct position but in a more biologically stable form in a series of analogs in which we replaced the acetates with bioisosteric mono- or disubstituted carbamates. Late-stage installation of the carbamate was desirable for maximum synthetic efficiency. Thus, carbamoylation of diol **6** with the appropriate isocyanate or diaklycarbamoyl chloride followed by hydrogenolysis of the benzyl ethers provided mono- or dialkylated carbamates **10**–**15** (Scheme 2).

The ability of all new analogs to inhibit RSK activity was determined in an in vitro kinase assay and compared with the parent



Scheme 1. Synthesis of a bis-ketone analog of SL0101. Reagents and conditions: (a) NaH, propargyl bromide, THF, 0 °C to rt, 66%; (b) $Hg(OAc)_2$, PPTS, water, acetone, rt, 62%; (c) H_2, $Pd(OH)_2/C$, MeOH, EtOAc, 50%. Yields are unoptimized.



Scheme 2. General scheme for the preparation of carbamate analogs of SL0101. Reagents and conditions: (a) R_1NCO, Et_3N, DMF, 45 °C, 44–66%; (b) R_2R_3NCOCl, NaH, DMF, 0 °C to rt, 26–69%; (c) H_2, $Pd(OH)_2/C$, MeOH, EtOAc, rt, 46–94%. Yields are unoptimized.

Table 1
Potency of analogs in in vitro kinase and MCF7 cell-based assays

Compound	RSK2 IC_{50} (μM)	MCF7 IC_{50} (μM)
1	0.583 (0.489–0.696)	45.6 (42.7–48.8)
9	0.252 (0.189–0.336)*	34.1 (30.1–38.5)*
10	1.13 (0.876–1.46)*	77.0 (71.6–82.7)*
11	0.869 (0.649–1.16)	46.4 (43.2–50.0)
12	1.92 (1.29–2.86)*	53.3 (50.6–56.2)*
13	0.493 (0.355–0.684)	PS
14	0.356 (0.255–0.496)	PS
15	1.43 (1.09–2.04)*	>100

IC_{50} is concentration needed for 50% inhibition; the 95% CI is shown in parentheses; $n = 3$ in triplicate.
* $p < 0.05$. PS; partially soluble.

compound **1** (Table 1). The ketone analog **9** was twofold more potent than **1** at inhibiting RSK2. Analogs **11**, **13**, and **14** were as potent as SL0101, and analogs **10**, **12**, and **15** were slightly (two to threefold) less potent. Overall, we found that the ability of an analog to inhibit RSK was not greatly influenced by the structure of the acetate replacement, which is consistent with previous observations.

We also determined the ability of all new analogs to inhibit MCF7 cell proliferation (Table 1). The ketone analog **9** was again the most potent of the new analogs. The three monosubstituted carbamates, analogs **10**–**12**, were similarly potent to the parent compound, with a trend toward improved potency with increasing lipophilicity of the carbamate substituent, presumably due to improved membrane permeability. In the disubstituted carbamate series, the dimethyl analog **13** and 1-pyrrolidinyl carbamate analog **14** exhibited poor solubility in cell culture media and therefore their ability to inhibit cell growth was not determined. The morpholino bis-carbamate **15** showed improved solubility but was unable to inhibit cell proliferation despite its ability to inhibit RSK in the in vitro kinase assay, most likely due to poor membrane permeability.

Analogs that inhibited MCF7 cell proliferation were evaluated along with **1** for their stability in a MCF7 cell-based assay. The inhibitor was added when the cells were plated and proliferation analyzed at various time points to determine the persistence of the inhibitory effect. SL0101 (**1**) was able to inhibit MCF7 proliferation for 48 h (Fig. 2). However, at longer time points the cells began to proliferate indicating that SL0101 was no longer effective, which we hypothesize is due to degradation of the inhibitor by esterases to the inactive triol **2**. Treatment of cells with either the bis-ketone analog **9** or the ethyl carbamate analog **10** did not result in sustained growth inhibition, indicating poor in vitro stability of these analogs. As the 3″ and 4″ substituents of analog **9** are non-hydrolyzable, its poor stability was initially surprising. However, MCF7 cells express aldo-keto reductases (AKRs), well known to be Phase I metabolizing enzymes for a variety of drugs bearing carbonyl groups.[16,17] Thus an alternative metabolic pathway is available to analog **9** whereby one or both ketones could be reduced by AKRs to secondary alcohols, leading either directly to a less potent RSK inhibitor or indirectly as the secondary alcohols could be further metabolized by conjugation.[17]

Encouragingly, the more lipophilic monosubstitued carbamate analogs **11** and **12** demonstrated improved in vitro stability, as cells treated with these compounds did not proliferate over the full time course (Fig. 2). We further examined the stability of analogs **11** and **12** by determining whether cyclin D1 levels were inhibited (Fig. 3). Previously, we found that SL0101 inhibits proliferation in breast cancer cell lines by inducing a cell cycle block in G1, which is due to RSK regulation of cyclin D1 levels.[1,18] In agreement with the MCF7 stability results we observed that SL0101 decreased the levels of cyclin D1 at 48 h compared to the control, but that cyclin D1 levels began to increase at later time points, indicating degradation of the inhibitor. However, cyclin D1 levels remained low in cells treated with **11** or **12**, indicating persistent inhibition of RSK and therefore improved biological stability of the carbamate analogs over the parent compound. Taken together, these results indicate that analogs **11** and **12** have improved stability over SL0101 (**1**).

We then investigated whether our strategy of reintroducing the carbonyl group improved the specificity of **11** and **12** relative to the diethyl analog **3**.[13] We have previously shown that the specificity of an analog for RSK can be evaluated by determining its antiproliferative activity in both MCF-10A and MCF7 cells, with the most specific analogs showing no inhibition of MCF-10A but substantial inhibition of MCF7 proliferation, due to the differential dependence of the growth of these cell lines on RSK.[9,12] We have also previously shown that while SL0101 does not inhibit the growth of MCF-10A cells up to a concentration of 100 μM, the diethyl analog **3** significantly inhibits the growth of MCF-10A cells, indicating reduced

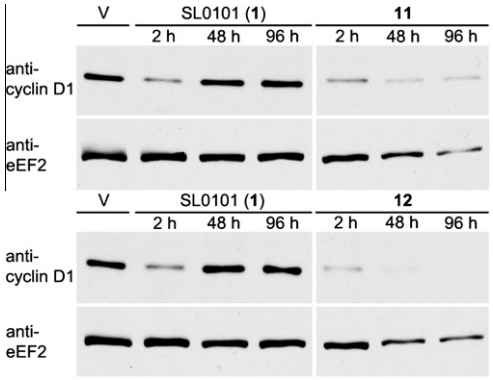


Figure 3. Persistence of RSK inhibition. MCF7 cells were treated with SL0101 or the more stable analogs **11** and **12** (100 μM). At the indicated time in hours (h) the cells were lysed and the lysates immunoblotted. Each analog was analyzed on a single membrane with SL0101. White space indicates sections of the membrane that were cropped to remove unnecessary lanes.

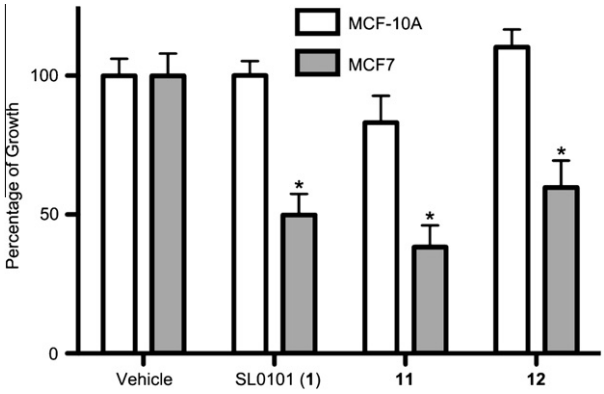


Figure 4. Inhibition of growth of MCF-10A versus MCF7 cells by SL0101 and select analogs. The inhibitor concentration was 50 μM. ($n = 3$ in quadruplicate, $^{*}p \leqslant 0.05$ when compared to vehicle).

specificity for RSK.[13] We found that analogs **11** and **12**, like SL0101, significantly inhibited the growth of MCF7 cells but did not significantly inhibit the growth MCF-10A cells (Fig. 4). These results suggest that analogs **11** and **12**, like SL0101, specifically inhibit RSK.[2,9,10] The only significant differences in biological activity between the two compounds are slightly improved potencies for **11** versus **12** in both the kinase and MCF7 cell proliferation assays. As these small differences are unlikely to be physiologically

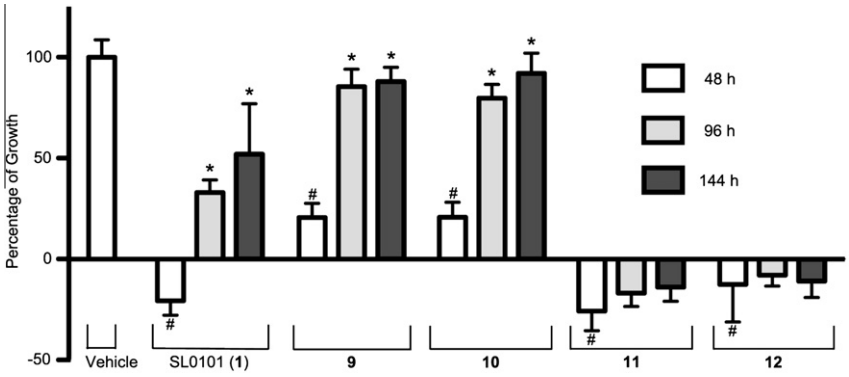


Figure 2. In vitro determination of analog stability. The inhibitor (100 μM) was added to MCF7 cells at time 0 and percentage of growth determined for the indicated time points. ($n = 3$ in quadruplicate, $^{\#}p \leqslant 0.05$ at 48 h when compared to vehicle at 48 h, $^{*}p \leqslant 0.05$ when compared to 48 h treatment with the same analog).

important, either carbamate modification should render an analog suitable for in vivo evaluation.

In summary, the C3″ and C4″ acetates on the carbohydrate moiety of SL0101 are required for both potent and specific inhibition of RSK but we predict that they would be metabolized rapidly by esterases in vivo, a fact which is supported by the poor biological stability of the natural product in vitro. Thus, SL0101 is not suitable for in vivo evaluation and analogs with improved stability are needed. The number of suitable replacements for these acetates that would confer greater biological stability is surprisingly limited as a simple change from acetyl to ethyl leads to a reduction in specificity for RSK. As a solution to this problem, bioisosteric replacement of the acetates by carbamates provided analogs that are more biologically stable than SL0101 in vitro and are as specific as SL0101 for RSK. These modifications along with others aimed at further improving the stability and potency of SL0101 analogs are currently being investigated in our laboratory with the goal of identifying a RSK inhibitor that could be advanced to preclinical testing.

Acknowledgments

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Supplementary data

Supplementary data (experimental procedures and compound characterization for all new compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.033.

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