

Targeting RSK: An Overview of Small Molecule Inhibitors

Tam Luong Nguyen*

Target Structure-based Drug Discovery Group, SAIC-Frederick, Inc., National Cancer Institute Frederick, Frederick, MD 21702, USA

Abstract: Ribosomal S6 kinase (RSK) is a family of serine/threonine kinases that has been identified as a promising anti-cancer target. While a number of protein kinase inhibitors that have potent activity against other serine/threonine kinases were shown to also inactivate RSK, there is keen interest in the three different inhibitor chemotypes that were shown to be RSK specific, since these compounds have tremendous utility as chemical probes in elucidating the biochemistry of the RSK signaling cascade and unraveling the molecular basis of cancer. Because each compound may have therapeutic potential, the nonspecific kinase inhibitors as well as the RSK specific inhibitors will be discussed.

Key Words: Serine/threonine kinase, inhibitor, RSK specific.

INTRODUCTION

The p90 ribosomal s6 kinases (RSKs) are a group of serine/threonine kinases that are constituents of the AGC subfamily in the human kinome. There are four RSK isoforms (RSK1-4) and each is a product of a separate gene. The RSK isoforms are characterized by 75% - 80% sequence identity [1-4]. Although the RSK isoforms are broadly distributed in human tissue, they exhibit variable tissue expression, which is an indication that they may be involved in different functions. The RSK isoforms are activated by extracellular signaling molecules that stimulate the Ras-ERK pathway (Fig. (1)). These molecules include a variety of different growth factors, cytokines, peptide hormones and neurotransmitters [5,6].

The RSK isoforms play an important role in the MAPK signaling cascade [7], which is responsible for regulating cellular growth and differentiation. In terms of human diseases, RSK was found to be overexpressed in human breast and prostate cancer cells [8]. RSK has also been linked to Coffin-Lowry syndrome (CLS), which is a rare disorder characterized by mental retardation and dysmorphisms [9]. The cause of CLS is associated with genetic defects in the RSK2 gene [10]. Furthermore, there is compelling evidence for a link between RSK2 and HIV infection. RSK2 was shown to have a reciprocal relationship with HIV-1 Tat. RSK2 is recruited and activated by HIV-1 Tat, and is itself also important to normal Tat function [11].

Stimulation of the Ras-ERK signaling pathway results in activation of RSK by ERK1/2 and PDK1 (Fig. (1)). While the precise mechanism of RSK activation has not been fully elucidated, the current model involves a series of sequential events (Fig. (2)). First, ERK1/2 binds to the carboxy-terminal of RSK and is activated by a stimulating signal such as mitogen. After which, activated ERK1/2 phosphorylates RSK at Thr365 (numbering based on murine RSK2) and Ser369 in the linker region, and Thr577 in the activation loop of the carboxy-terminal kinase domain (CTKD) [7,12]. Subsequently, the CTKD autophosphorylates Ser386 in the linker region of RSK2 to generate a binding site for PDK1 [13], which then binds and phosphorylates RSK at Ser227 in the activation loop of the amino-terminal kinase domain (NTKD). Once Ser227 is phosphorylated, RSK is activated and is capable of phosphorylating downstream targets [14].

RSK phosphorylates a wide range of cytosolic and nuclear proteins preferentially at serine/threonine residues that lie in

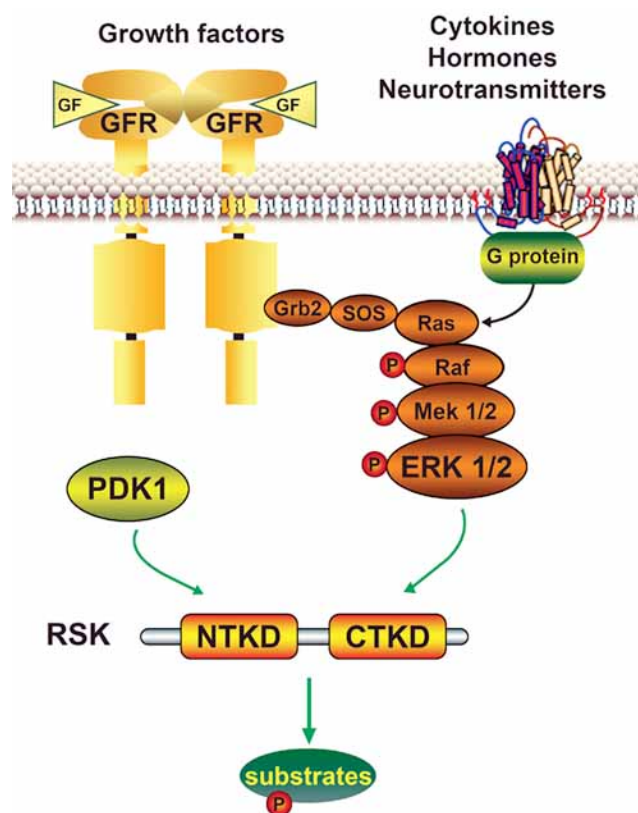


Fig. (1). RSK signaling pathway. RSK is associated with the Ras-ERK pathway and is activated by ERK1/2 in response to growth factors, cytokines, hormones and neurotransmitters. PDK1 is also needed for RSK activation. RSK phosphorylates a wide range of cytosolic and nuclear proteins, and is characterized by two functional catalytic domains, an amino-terminal kinase domain (NTKD) and a carboxy-terminal kinase domain (CTKD).

RRRXXS/T or RRXS/T motifs [15]. RSK substrates include c-Fos, cAMP response element-binding protein (CREB), estrogen receptor- α (ER α), and I κ B α /NF κ B [16-19]. A number of the RSK substrates are in themselves of interest as anticancer targets. For example, RSK phosphorylates and inhibits myelin transcription factor 1 (Myt1), which is an important cell cycle regulator [20]. RSK was also shown to phosphorylate glycogen synthase kinase-3 (GSK-3) [21], which is an important regulator of multiple signaling pathways, and is associated with a number of different human diseases such as cancer, neurodegenerative diseases, and inflammation [22].

*Address correspondence to this author at the Target Structure-based Drug Discovery Group, SAIC-Frederick, Inc., National Cancer Institute Frederick, Frederick, MD 21702, USA; Tel: 301-846-6035; Fax: 301-846-6106; E-mail: nguyent@ncifcrf.gov

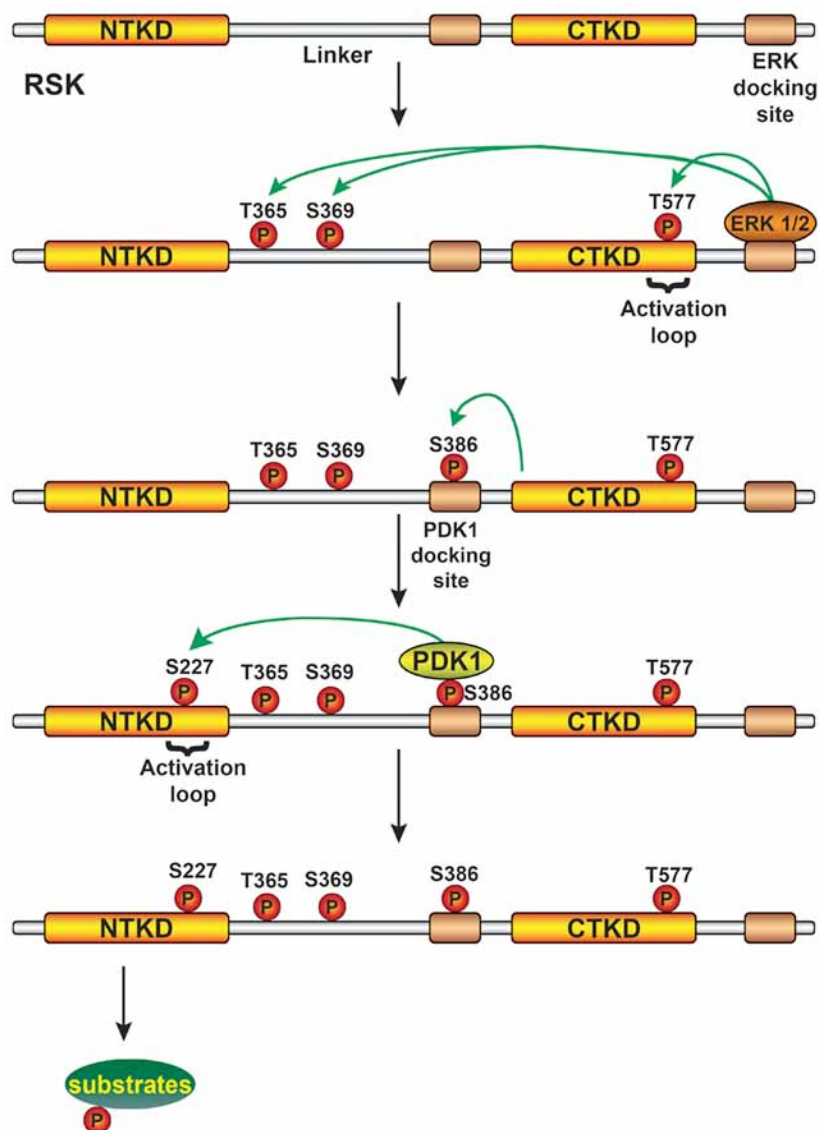


Fig. (2). Molecular mechanism of RSK activation. A sequential sequence of events is involved in RSK activation. ERK1/2 binds to the carboxy-terminal of RSK and is activated. Activated ERK1/2 phosphorylates RSK at Thr365 (numbering based on murine RSK2) and Ser369 in the linker region, and Thr577 in the activation loop of the CTKD. The CTKD autophosphorylates Ser386 in the linker region. PDK1 binds to RSK at the newly generated PDK1 binding site near Ser386, and phosphorylates RSK at Ser227 in the activation loop of the NTKD. Activated RSK then phosphorylates downstream target.

RSK contributes to antiapoptosis signaling by phosphorylating Bcl-2/Bcl-X_L-associated death promoter (BAD) [23], CCAAT/enhancer binding protein β (C/EBP β) [24], and death-associated protein (DAP) kinase [25]. Lastly, it was recently shown that RSK is key mediator of fibroblast growth factor receptor 3 (FGFR3) signaling. FGFR3 is a tyrosine kinase that plays a role in hematopoietic malignancies such as T cell lymphomas, and phosphorylates RSK2 at Y488 and Y529. In addition, RSK2 is a regulator in cell transformations induced by tumor promoters [26].

Structurally, the RSK isoforms are very similar. Each of the isoforms contains two functional catalytic domains that are separated by a large ~100 amino acid linker region. The two kinase domains are distinct and contain nonidentical ATP-binding site. The NTKD is similar to p70 S6 kinase (p70 S6K1), and the CTKD is similar to the calcium/calmodulin protein kinases [1]. Because of its molecular architecture, RSK offers two logical sites for inhibition: the ATP-binding site in the NTKD and the one in the CTKD. Site specific inhibitors for both ATP pockets have been identified.

At present, the three-dimensional structure of full length RSK has not been determined. However, two structures of RSK NTKD have been reported. The first is a molecular model of RSK2 NTKD (residues 68-323) that was used to virtually screen the National Cancer Institute (NCI) chemical repository in order to identify two novel inhibitor chemotypes [27]. The second is the crystal structures of RSK1 NTKD that was determined in complex with purvalanol A and staurosporine [28]. The two RSK structures have similar topology. As shown in Fig. (3A) for the RSK2 NTKD model, each contains two lobes. The smaller N-terminal lobe contains mostly sheet structures, and the larger C-terminal lobe is comprised of mostly helical structures. The ATP-binding site occupies the cleft between the two lobes. A hinge region defines the interior of the ATP-binding site and links the two lobes. In RSK2, the polypeptide backbone of Asp148 and Leu150 form the hydrogen bond interaction sites in this hinge region.

Because RSK2 is the most closely linked with human cancers among the different isoforms, this review focuses on small molecule RSK2 inhibitors. However, where applicable, the activity of

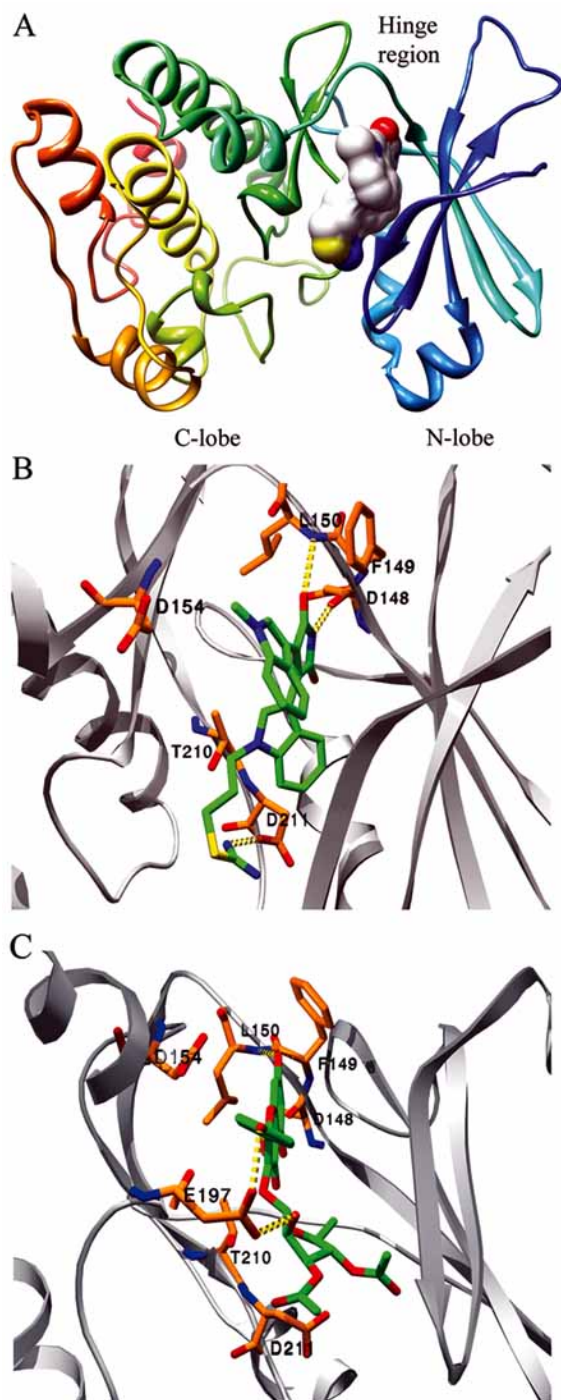


Fig. (3A). Modeled RSK2 NTKD structure. RSK2 is rendered in ribbon. The secondary structures on the amino-lobe (N-lobe) are colored blue and cyan, and those in the carboxy-lobe (C-lobe) are colored green, yellow, orange and red. The hinge region that connects the two lobes is labeled. To depict the ATP-binding site in the RSK2 NTKD structure, Ro31-8220 is drawn bound at the cleft between the two lobes based on the reported binding model [27]. Ro31-8220 is rendered in surface with carbon atoms colored grey, nitrogen atoms blue, oxygen atoms red and sulfur atoms yellow. Hydrogen atoms are not shown for clarity. **(B)** RSK2 NTKD-Ro31-8220 binding model. RSK2 is rendered in grey ribbon with Asp148, Phe149, Leu150, Thr210, and Asp211 also drawn in stick. Ro31-8220 is shown in stick. Nitrogen atoms are colored blue, oxygen red, and sulfur atoms yellow. The carbon atoms of RSK2 are colored orange, and those of Ro31-8220 are colored green. Hydrogen bonds are depicted as dashed yellow lines. **(C)** RSK2 NTKD-SL0101 binding model. The figure is rendered in the same fashion as Fig. 3B.

these compounds against the other RSK isoforms is presented. Described in this review are inhibitors that are characterized by IC_{50} 's of less than $1 \mu\text{M}$ that are either nonspecific kinase inhibitors, or RSK specific inhibitors, and inhibitors with undetermined kinase specificity. Not expectedly, RSK specific inhibitors are of tremendous interest due to their utility in dissecting the RSK signaling cascade, and their therapeutic potential.

STAUROSPORINE-LIKE COMPOUNDS

The two bisindoylmaleimide compounds GF109203X and Ro31-8220 (Fig. (4)) were both shown to be potent but nonspecific RSK2 inhibitors [29]. In *in vitro* kinase assays, GF109203X and Ro31-8220 inhibited RSK2 with IC_{50} 's of 310 nM and 36 nM, respectively. GF109203X and Ro31-8220 had similar activities for RSK1 and RSK3, with Ro31-8220 demonstrating slightly greater potency for the RSK isoforms relative to GF109203X. GF109203X is characterized by IC_{50} 's of 610 nM and 120 nM for RSK1 and RSK3, respectively. Ro31-8220 inhibited RSK1 and RSK3 with IC_{50} 's of 200 nM and 5 nM, respectively [30]. Both compounds showed significant inhibition of RSK activity in intact adult rat ventricular myocytes (ARVM) at concentrations of $\geq 3 \mu\text{M}$ and $\geq 1 \mu\text{M}$, respectively [30].

Both GF109203X and Ro31-8220 are classic PKC inhibitors, and each inhibited PKC with slightly greater potency than RSK. GF109203X and Ro31-8220 inactivated the PKC isoforms with IC_{50} 's of 8 – 12 nM and of 4 – 8 nM, respectively. In addition, it is not surprising that the two compounds were shown to bind to a number of cellular targets in a proteomic screen [31]. Because of the structural similarity between PKC and the RSK2 NTKD, it is presumed that GF109203X and Ro31-8220 derived their RSK2 activity by binding at the NTKD ATP pocket.

Molecular modeling was used to determine the binding modes of GF109203X and Ro31-8220 in complex with RSK2 NTKD [27]. As predicted in the model, the two compounds have similar orientations in the adenine recognition site of the ATP pocket. The maleimide groups of GF109203X and Ro31-8220 are hydrogen bonded to the backbone atoms of Asp148 and Leu150. Additionally, the isothiourea of Ro31-8220 may hydrogen bond to the carboxylate side chain of Glu197. Fig. (3B) shows the modeled binding mode of Ro31-8220 in RSK2 NTKD [27].

Maleimide SB216763 is also a potent but nonspecific RSK2 inhibitor with an IC_{50} of $\sim 0.1 \mu\text{M}$ [32]. In addition to its RSK2 activity, SB216763 is a well-known GSK-3 inhibitor [33]. At present, it has not been experimentally established whether SB216763 binds at the ATP-binding site of the NTKD, CTKD, or both. Because SB216763 is a maleimide, its hydrogen bonding interactions with RSK may be similar to that of the other maleimide inhibitors, GF109203X and Ro31-8220. In the instance of the RSK2 NTKD, the maleimide motif of SB216763 may be hydrogen bonded to the polypeptide of Asp148 and Leu150 in the hinge region of the adenine recognition site.

Staurosporine itself is a potent but nonspecific RSK inhibitor. The IC_{50} of staurosporine against RSK1 is 0.3 – 1 nM, which makes it among the most potent RSK inhibitor. However, staurosporine has activities in the nanomolar range against a wide range of protein kinases. The crystal structure of staurosporine in complex with RSK1 NTKD has been determined [28] and shows the lactam moiety of staurosporine is hydrogen bonded to the backbone atoms of Asp142 and Leu144 of the hinge region, which is similar to the RSK2-Ro31-8220 modeled complex. These two hydrogen bonds are complemented by an additional hydrogen bond between the N-methyl amino group of staurosporine and the backbone carboxyl of Glu191. The orientation of staurosporine in the ATP-binding site of RSK1 is similar to its pose in the ATP-binding site of other kinases such as CDK [34]. In addition to the RSK1-staurosporine crystal structure [28], Ikuta *et al* also reported the crystal structure of purvalanol A in complex with RSK1 NTKD. RSK1-bound purvalanol

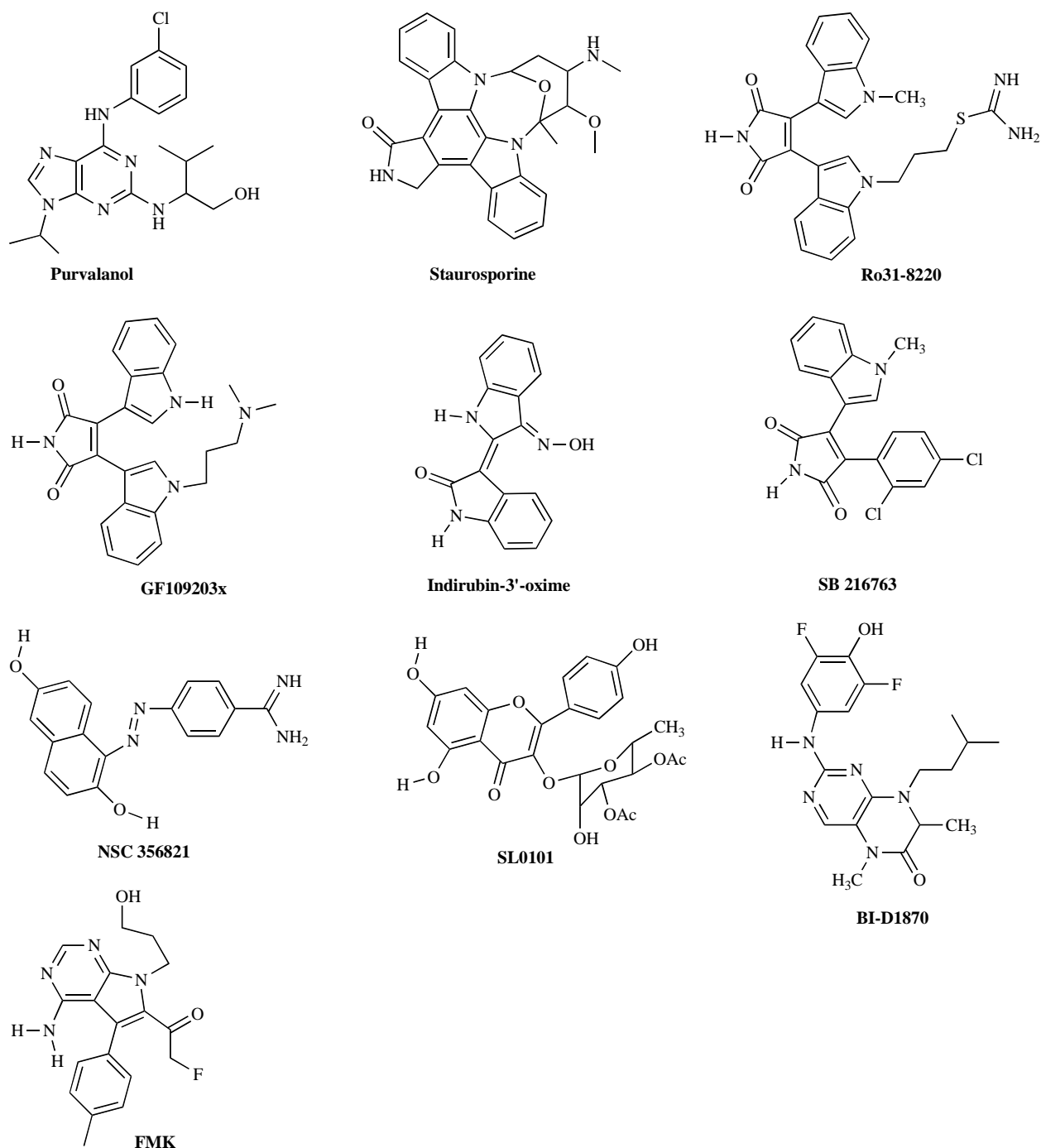


Fig. (4). Chemical structures of RSK inhibitors.

A forms a pair hydrogen bonds to the hinge region in the ATP-binding site. The imidazole N7 nitrogen atom of purvalanol is hydrogen bonded to backbone NH of Leu144, and its amino nitrogen is hydrogen bonded to the backbone carbonyl of Leu144.

INDIRUBIN-3-OXIME

Indirubin-3'-oxime is a potent but nonspecific RSK2 inhibitor with an IC_{50} of $\sim 0.1 \mu M$ against RSK2 [32]. However, the activity of indirubin-3-oxime is not limited to RSK. Indirubin-3-oxime has been reported to be a potent inhibitor of both the CDK isoforms with IC_{50} 's of 0.1 – 3.3 μM [35], and GSK-3 β with an IC_{50} of 22 nM [36]. CDK and GSK-3 are of intensive medicinal interest due to their role in cancer. The binding mode of indirubin-3-oxime in

complex with both CDK5 [37] and GSK-3 β [38] has been determined. In both instances, the compound binds at the ATP-binding site with its amide motif hydrogen bonded to the adenine recognition loop of the protein kinases. It is reasonable to assume that indirubin-3-oxime would bind in a similar fashion in the ATP-binding site of RSK NTKD. However, at present, it has not been established if indirubin-3-oxime binds in the ATP pocket of the NTKD, CTKD, or both.

DIHYDROXYLNAPHTHALENE NSC356821

The dihydroxynaphthalene compound NSC356821 was reported to be RSK2 inhibitor with an IC_{50} of 1 μM for RSK2 in an *in vitro* assay [27]. The compound was selected from the several hundred

thousand compounds in the NCI chemical repository *via* a virtual screen using the RSK2 NTKD model was a template [27]. Because the activity of NSC356821 against other kinases has not been investigated, its kinase specificity is unknown. However, a binding mode for NSC356821 in the RSK2 NTKD ATP-binding site has been proposed [27]. In this model, NSC356821 forms three hydrogen bonds to RSK2. The first is between the 6-hydroxyl of NSC356821 and the amide backbone of Leu150, which form the hinge region of the adenine recognition site. The second is between the 2-hydroxyl and the side chain hydroxyl of Thr210. The third is between the amidine functionality and the side chain carboxylate of Glu197.

KAEMPERFOL-GLYCOSIDE SL0101

The kaempferol-glycoside SL0101 is a natural product obtained from the tropical plant *Fosteronia refracta* and was the first identified RSK specific inhibitor. SL0101 was reported to inhibit RSK2 with an IC_{50} of 90 nM at an ATP concentration of 10 μ M. Furthermore, SL0101 is characterized by a K_i of 1 μ M and an EC_{50} was 50 μ M in intact cells [39]. These results indicate that the activity of SL0101 in intact cells is significantly weaker than *in vitro*. Because of its RSK2 activity, the synthesis of SL0101 was recently achieved.

SL0101 is ATP competitive and binds at the RSK NTKD ATP-binding site [39]. An active truncated RSK2 mutant which lacks the C-terminal domain was effectively inhibited by SL0101, suggesting the efficacy of SL0101 is linked to its interaction with the NTKD. Furthermore, a second active RSK2 mutant, in which the adenosine-interacting loop (AIL) in the NTKD ATP-binding site was replaced with p70 S6K (RSK2-AIL), was effectively inhibited by Ro-318220, but was much less affected by SL0101. Accordingly, this suggests that SL0101 forms key binding interactions to the AIL in the NTKD. Because the mechanism of action of SL0101 centers of its binding to the ATP pocket in the NTKD, which is responsible for phosphorylating exogenous substrates, SL0101 is an excellent chemical probe for delineating the biochemistry of the downstream targets.

In addition, SL0101 demonstrated remarkable *in vivo* potency and specificity. In studies of human breast cancer cell line MCF-7, Smith *et al* reported that SL0101 did not affect either the phosphorylation of RSK2, or the activation of MAPK, an indication that SL0101 does not significantly interfere with the upstream kinases necessary for RSK activation [39]. However, preincubation of the intact cells with 100 μ M SL0101 did abrogate PDB-induced p140 phosphorylation. Since p140 is a RSK substrate, this demonstrates the efficacy of SL0101 on downstream targets. Additionally, since SL0101 inhibits the proliferation of MCF-7 cells by blocking the cell cycle in the G1 phase, but had little effect on the growth of normal breast cell line MCF-10A, this suggests that SL0101 preferentially inhibits the growth of breast cancer cells relative to normal breast cells. The efficacy of SL0101 in these *in vivo* experiments highlights its cell-permeability. In addition, SL0101 was shown to be nontoxic. After removal of the inhibitor, SL0101-treated MCF-7 cells exhibited growth [39].

The kaempferol and the rhamnose moieties of SL0101 contribute differently to its binding affinity. The kaempferol motif was shown to inhibit RSK with an IC_{50} of 15 μ M, suggesting that the rhamnose moiety of SL0101 is responsible for the >150-fold increase in RSK affinity. Further examination of the rhamnose moiety revealed the varying contributions of its molecular constituents. SL0101 contains two acetyl groups substituted at the 3'' and 4'' positions of its rhamnose moiety, and it was recently shown that the addition of a third acetyl group to the 2'' position on the rhamnose group gave a derivative termed 3Ac-SL0101 that had similar RSK activity and specificity as SL0101 in intact cells, and additionally, was more potent in inhibiting MCF-7 cell proliferation and cell growth [40]. As for the kaempferol moiety, the three hydroxyl groups at the 5, 7 and 4' positions make different contributions to

SL0101 activity. This is demonstrated by the RSK2 activity of three SL0101 congeners: the 5-deoxy-SL0101, 7-deoxy-SL0101, and the 4',7-dideoxy-SL0101 derivatives which had IC_{50} 's of , 24.6 μ M, 1.56 μ M and 13.2 μ M, respectively [41]. Because its elimination resulted in a >65-fold reduction in activity, the 5-hydroxyl plays an important role in SL0101 activity. On the other hand, the 7-hydroxyl has less influence on SL0101 activity since its elimination resulted in only a 4-fold reduction in activity. Meanwhile based on the activity of the 7-deoxy-SL0101 and 4',7-dideoxy-SL0101 derivatives, it can be deduced that the 4-hydroxyl has a significant influence on activity, but less than that of the 5-hydroxyl.

These results delineate the structural basis for SL0101 activity. A binding model of SL0101 in complex with RSK2 NTKD has been reported [27]. As shown in Fig. (3C), the model depicts hydrogen bonds between the 5,7-dihydroxyl moiety of SL0101 and the backbone of residues Asp148 and Leu150 in the hinge region. Additional hydrogen bonds can potentially be formed between the keto oxygen atom and rhamnose ring oxygen atom of SL0101 and the Thr210 hydroxyl group, and between the 4'-hydroxyl and rhamnose hydroxyl groups of SL0101 and the Glu197 side chain carboxylate. In the same report, a binding model for kaempferol was also presented [27]. The kaempferol model shows a different hydrogen bond pattern. In this instance, the kaempferol is oriented nearly 90° relative to the same moiety in SL0101, and has its keto oxygen and 5-hydroxyl oxygen hydrogen bonded to the peptide backbone of Asp148 and Leu150.

DIHYDROPTERIDINONE BI-D1870

Dihydropteridinone BI-D1870 is a potent and RSK specific inhibitor. BI-D1870 was synthesized and assayed as a racemate. It was shown that BI-D1870 inhibited the four RSK isoforms with *in vitro* IC_{50} 's of 15-30 nM at an ATP concentration of 100 μ M [42]. While there is a two-fold differential in BI-D1870 activity for RSK4 relative to RSK1, each of the RSK isoforms is significantly inhibited. More importantly, BI-D1870 was shown to be remarkably selective for RSK relative to other kinases, even those from the same AGC subfamily as RSK.

The activity of BI-D1870 against a large panel of kinases was determined [42]. At 0.1 μ M, BI-D1870 inhibited RSK2 by >98%. In contrast, most of the other kinases were not significantly affected. The most affected kinase was PLK1, which had its activity reduced by 83% and was found to have an IC_{50} of 100 nM, which is 3- to 10-fold higher than RSK. Following PLK1 in activity were Aurora B, DYRK1a, CDK2-A, Lck, CK1, and GSK3 β , which had IC_{50} values of 340 nM to 2.2 μ M, representing a 10- to 100-fold difference in activity relative to RSK. The remarkable RSK specificity of BI-D1870 is further demonstrated by the fact that its RSK activity was >500-fold higher than the other AGC kinases tested in this panel: MSK1, PKB α , PKB β , S6K1, SGK1, PKC α , PDK1, PKA, PRK2, and ROCKII. In fact, MSK1, which is the most closely related kinase to RSK, was not significantly inhibited by 0.1 μ M BI-D1870 and its activity was only reduced by 13%.

Besides its *in vitro* potency and specificity, BI-D1870 was also shown to be cell-permeable. BI-D1870 was incubated in HEK-293 cells and the effect on RSK-catalyzed phosphorylation of its known substrate was determined. PMA was used as an agonist to activate ERK1/ERK2 and RSK isoforms. BI-D1870 significantly inhibited the PMA-induced phosphorylation of GSK3 α and GSK3 β , which are RSK substrates, while it had minimal effect on PMA-induced activation of ERK1/ERK2, which is catalyzed by Raf and MKK1, or the phosphorylation of CREB, which is catalyzed by MSK1 and MSK2. In addition, BI-D1870 did not significantly affect the phosphorylation of ribosomal S6 protein by S6K.

The effect of BI-D1870 on the *in vivo* activation of RSK was tested in the HEK-293 cells. PMA was used to stimulate the cells in the absence and presence of BI-D1870. RSK was immunoprecipitated with an antibody and assayed for activity in the absence of BI-

D1870. The BI-D1870 treatment of the HEK-293 cells did not apparently affect PMA-induced activation of RSK. However, similar experiments performed in Rat-2 cells indicated that the presence of BI-D1870 did increase ERK activity. This is in contrast to the results from the HEK-293 cell, but may be explained by a feedback inhibition from RSK to upstream constituents of the ERK cascade in this particular type of cell. The results of the *in vivo* studies indicate that BI-D1870 does not interfere with the activation of RSK or the upstream ERK signaling, but significantly affects the phosphorylation of its substrates. This characteristic makes BI-D1870 a powerful tool for understanding kinase activity in the ERK signaling pathway.

BI-D1870 binds at the NTKD ATP-binding site. BI-D1870 inhibited RSK1 and RSK2 with IC_{50} 's of 10 nM and 20 nM, respectively, at 100 μ M ATP but its activity was reduced to 5 nM and 10 nM for RSK1 and RSK2, respectively, at 10 μ M ATP [42]. Secondly, an active RSK2 mutant that lacked the C-terminal domain was inhibited by BI-D1870 with an IC_{50} of ~30 nM, which is similar to the 24 nM observed for the intact RSK2 protein, suggesting that BI-D1870's activity is primarily due to its interaction with the NTKD [42]. Additionally, given the remarkable potency and specificity of BI-D1870, the determination of its binding mode in RSK is critical to elucidating the key differences among kinases. However, at present, the binding mode of BI-D1870 in RSK has not been determined. Similar to SL0101, BI-D1870 is a useful chemical probe for elucidating the biochemistry of the RSK signaling cascade.

PYRROLOPYRIMIDINE FMK

The pyrrolopyrimidine fmk is a potent and RSK-specific inhibitor. Fmk was shown to inhibit RSK2 with an *in vitro* IC_{50} of 15 nM and an EC_{50} of 200 nM [43]. Fmk contains a reactive electrophile in its fluoromethylketone motif, and in this instance, derives its potent kinase activity from the covalent addition of its chloromethylketone functionality to the thiol group of Cys436 located in the ATP pocket of the RSK2 CTKD. When the pyrrolopyrimidine scaffold of fmk that lacked the fluoromethylketone group was assayed against RSK2, the scaffold had an IC_{50} of 1.2 μ M, which represents an 80-fold lower inhibitory activity for the scaffold relative to RSK2-fmk. Additionally, when fmk was assayed against a C436V mutant of RSK2, its IC_{50} value was >10 μ M. This represents a >600-fold decrease in the activity of fmk against the RSK2 mutant relative to RSK2 WT.

Despite having a mechanism of action involving covalent addition to the molecular target, fmk was shown to be remarkably RSK specific. In human epithelial cell lysate containing thousands of proteins, biotin-labeled fmk was found to react with only RSK1 and RSK2, which were identified using quantitative immunodepletion with specific antibodies [43]. Fmk was reported to also be highly selective for RSK1 and RSK2 in cells growing in culture. The effect of fmk on cellular function was examined. Fmk had little effect on ERK1/2 phosphorylation *in vitro* or in cell. Fmk treatment blocked phosphorylation of H3 histone, but had no effect on MSK1-mediated phosphorylation of H3 histone. The cell permeability of fmk is signified by its activity in the cell.

The remarkable RSK specificity of fmk is due to its stereoelectronic fit to the CTKD ATP-binding site. Using bioinformatic analysis of human protein kinases, Cohen *et al* [43] pointed out that the ATP-binding site of the RSK CTKD contains two ligand filters that make it unique among the human protein kinases. The first is a cysteine residue in the hydrophobic ATP pocket. In the case of RSK2, this is Cys436. The second filter is a threonine gatekeeper positioned at the ATP binding site. In the case of RSK2, this gatekeeper residue is Thr493. While other kinases such as MSK1 and PLK1 possess cysteine residues in their ATP pockets, these kinases do not possess the appropriate second selectivity filter. Even among the RSK isoforms, there exist differences in the selectivity filter.

While all four RSK isoforms contain the cysteine filter, RSK1 and RSK4 have equivalent threonine gatekeepers as RSK2, while RSK3 possesses a methionine gatekeeper.

While fmk is blocked from entering the ATP-binding site of many other kinases due to the presence of bulky gatekeeper such as methionine, leucine, or isoleucine, the compact size of the threonine gatekeeper in RSK2 allows the bulky aromatic groups of fmk to enter the ATP site. Mutagenesis studies confirmed the role of the Thr493 gatekeeper in the RSK specificity of fmk. A T493M mutant of RSK2 was >200-fold less affected by fmk than RSK2 WT.

Derivatives of fmk such as cmk, which has the same chemical structure as fmk with the exception that the fluorine atom is replaced by a chlorine atom [43], and fmk-pa, which is generated by replacing the primary hydroxyl of fmk with a propargylamine [44,45], were also found to be active against RSK *in vitro* and in cells.

While fmk is RSK specific, its mechanism of action is different from the two other RSK specific inhibitors SL0101 and BI-D1870. Whereas SL0101 and BI-D1870 are reversible inhibitors, fmk is an irreversible inhibitor. In addition, whereas SL0101 and BI-D1870 bind in the NTKD ATP-binding site, and therefore directly inhibit phosphorylation of downstream targets, fmk binds in the CTKD ATP-binding site and inhibits RSK autophosphorylation at Ser386 (Fig. (2)), which is essential for maximal enzyme activity. Despite its unconventional mechanism of action, the therapeutic potential of fmk was established by data showing that fmk induces significant apoptosis in human FGFR3-expressing, t(4;14)-positive multiple myeloma cells [46].

CONCLUSION

With the emergence of RSK as an anticancer target, the identification and optimization of small molecule RSK inhibitors has become a major focus. Because of the conserved structural and functional features found among serine/threonine kinases, it is not surprising that a number of well-known, nonspecific protein kinase inhibitors such as Ro31-8220, GF109203X, indirubin-3-oxime, and SB216763 have potent activity against RSK. More importantly, three RSK specific inhibitors have been identified in the literature: SL0101, BI-D1870, and fmk. Due to their specificity, each of these three inhibitors can be used as chemical probes for dissecting the complex signaling events associated with RSK, and are additionally therapeutic candidates.

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