poorly, if at all [3, 6]. In other cases where ligand binding is kinetically slow compared to the rate of transcription (e.g., for the FMN [12] and purine [11] riboswitches), pausing allows the aptamer domain sufficient time to bind ligand if the metabolite's intracellular concentration is sufficiently high. This binding interaction allows the RNA to proceed from the F to F* state (Figure 1B). For the *pbuE* adenine riboswitch [5], acquisition of the F* state determines that transcription will be aborted and thus turns off gene expression.

In the absence of ligand, the 3′ side of the P1 helix dissociates from the aptamer domain and is used to form a stem-loop structure in the expression platform that dictates the second genetic outcome, upregulation of transcription (Figure 1B). A second pause site, usually found in the middle of the expression platform, enables riboswitches to perform the intrinsically slow secondary structural rearrangement (F → F‡ in Figure 1B) that leads to active transcription of the downstream coding sequence [12].

Previous studies have noted the necessity for the loop-loop interaction in ligand recognition, but they were unable to provide a plausible connecting mechanism. This coupling is an important issue, as three-way junctions are a ubiquitous structural motif for higher-order organization of RNA, involved in forming protein binding and catalytic active sites [13]. Characterization of the natural ("fast") hammerhead ribozyme revealed that a loop-loop interaction facilitates organization of the catalytic site located in a three-way junction, allowing it to cleave at greatly accelerated rates in physiologically relevant magnesium concentrations [14, 15]. Strikingly, this RNA has strong architectural similarities to the adenine riboswitch, illustrating that organization of functional RNA junctions is often facilitated by other elements of tertiary structure [16]. These recent studies underscore that we are still just beginning to understand how the ability of RNA to fold into intricate three-dimensional structures allows them to execute their diverse cellular functions.

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Discovering New MAP Kinase Inhibitors

The current study by Kim et al. [1] (in this issue of *Chemistry & Biology*) uses a genetic approach with the yeast *Schizosaccharomyces pombe* to identify a highly specific inhibitor of Spc1 MAP kinase that competes with protein substrates for Spc1 interactions, but not with ATP binding.

Mitogen activated protein (MAP) kinases are key signaling proteins that mediate cellular responses to extracellular signals. Overactivation of the MAP kinase signaling pathways, primarily through genetic mutations of upstream regulatory proteins, is thought to play a prominent role in inflammatory diseases and tumor cell proliferation [2]. Thus, there is wide interest in the development of specific and effective MAP kinase inhibitors. In this issue of *Chemistry & Biology* [1], Kim et al. describe the identification of a highly specific, small molecular weight isoquinolinium compound that appears to inhibit only a specific MAP kinase isoform found in the yeast *Schizosaccharomyces pombe* (*S. pombe*) but has little effect on the activity of other yeast MAP kinases, MAP kinase activators, or homologous MAP kinases from mammalian species. These findings may help set the stage for the development of highly specific and hopefully more clinically effective MAP kinase inhibitors.

The identification of the inhibitory isoquinolinium compound, which the authors termed HWY 5069, utilized a genetic approach involving the yeast *S. pombe*. This "model organism" approach, which has also been exploited in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* [3], involves the genetic manipulation of proteins to create mutant strains of *S. pombe* that contain deletions of specific signaling proteins found within the various MAP kinase pathways. Subsequently, the proliferation of the various mutant strains along with the wild-type *S. pombe* was examined in the presence or absence of test compounds; in this study a proprietary collection of 80 isoquinolinium derivatives was examined [1]. It is quite interesting that all of the *S. pombe* deletion mutants are viable and appear to have similar proliferation rates. The premise for this model is that inhibition of *S. pombe* proliferation will only occur if both the inhibitory test compound and its target protein are present. In contrast, proliferation of the mutant strain lacking the protein target of the inhibitory test compound would not be affected by compound treatment. As such, *S. pombe* strains with the intact Spc1 MAP kinase protein were sensitive to HWY 5069, whereas mutant strains lacking Spc1 were resistant to the test compound, implicating this MAP kinase as the HWY 5069 target. There is precedent for this type of model. For example, the antimicrobial and immunosuppressant compounds rapamycin and FK506 are ineffective inhibitors of proliferation in yeast strains that lack the rapamycin and FK506 target protein FKBP12 [4]. It is the rapamycin or FK506 molecule in complex with FKBP12 that acts as the functional inhibitor of other proteins involved in inflammation and cell proliferation. In the current paper [1], the authors propose a somewhat similar model where HWY 5069 must first form a complex with Spc1, inducing a conformational change in the protein that allows the recruitment of another protein(s) that inhibits Spc1 activity and prevents cell proliferation. Future studies will be needed to identify any recruited proteins and to establish firmly whether this model reflects the mechanism of action for HWY 5069 inhibition of Spc1.

However, Kim et al. have begun to shed some light on how this molecule works. For example, the authors address the question of whether HWY 5069 inhibits Spc1 activity by competing with ATP binding or with substrate protein interactions. To test this, the authors preincubated purified Spc1 with varying amounts of ATP or myelin basic protein (MBP) as Spc1 substrates [1]. Whereas preincubation with ATP had no affect on HWY 5069 inhibition of Spc1 kinase activity, preincubation with increasing concentrations of MBP reversed HWY 5069's ability to inhibit Spc1 activity in vitro. These findings provide evidence to support the conclusion that HWY 5069 functions in an ATP-independent manner and involves competition with substrate proteins that are phosphorylated by Spc1. This is an important finding, as kinase inhibitors that compete with ATP binding are notorious for their nonspecificity due to the generally conserved nature of ATP binding domains on protein kinases [5]. While MBP is a commonly used MAP kinase substrate in activity assays, it is also phosphorylated by many other types of kinases. Thus, it will be interesting to determine whether HWY 5096 can also compete with and

prevent the phosphorylation of more physiological Spc1 substrates such as the transcription factor Atf1 [6].

The authors further demonstrate that HWY 5069 does not require functionally active Spc1 protein to be effective. In these experiments, *S. pombe* cell lines that expressed either the wild-type or a kinase-inactive mutant of Spc1 were treated with HWY 5069 [1]. Interestingly, the proliferation of both cell lines was equally sensitive to the inhibitory effects of HWY 5069, indicating that Spc1 activity was irrelevant to the proposed HWY 5069-induced conformational changes in Spc1. This model will likely be tested by identifying the domain(s) on Spc1 involved in HWY 5069 interactions. The characterization of the HWY 5069 binding site on Spc1 will be important not only for generating appropriate chemical modifications to improve the efficacy of this compound but also for the development of novel inhibitors against other MAP kinases that are relevant in human diseases.

The authors suggest that HWY 5069 interactions with Spc1 are relatively weak and probably noncovalent. This was shown by preincubating the Spc1 kinase with increasing concentrations of NaCl in vitro [1]. The premise of this experiment is that the more avid the interactions between HWY 5069 and Spc1, the higher the NaCl concentration required to disrupt these interactions and to prevent HWY 5069 from inhibiting Spc1. However, as the authors show, as little as 40 mM NaCl prevented HWY 5069 from inhibiting Spc1, indicating weak associations between the test compound and its putative target. However, definitive details as to the mechanism of action for HWY 5069 will require further structural information using methods such as X-ray crystallography to characterize binding interactions of HWY 5069 with Spc1. Structural analysis is not a trivial undertaking and will depend upon the ability of HWY 5069 to cocrystallize with Spc1.

Extensive structural information is available for p38α MAP kinase, which is the closest mammalian homolog of Spc1 [7]. As a result, several docking domains on p38α MAP kinase have been shown to facilitate specific interactions with protein substrates [8]. It will be interesting to see if the 3D structure of these docking domains on p38α and Spc1 MAP kinases is different given that HWY 5069 has no effect on p38α activity [1]. A structural difference could explain HWY 5069 specificity, as the p38α MAP kinase common docking (CD: Asp313, Asp315, Asp316) and ED (Glu160, Asp161) domains differ by a single amino acid in the corresponding regions of Spc1 (CD: Asp304, Thr306, Asp307; ED: Glu151, Asn152).

The study by Song and coworkers describes a powerful genetic-based method for the identification of a specific inhibitor of a yeast MAP kinase. Because of the high conservation of MAP kinases from yeast to humans, this approach may be tested with other chemical libraries for identifying novel inhibitors of other family members. While the ultimate utility of HWY 5069 or derivatives of this molecule for use as clinical agents is yet to be determined, the findings presented suggest that this compound may show promise for development into an antifungal agent. To further build on these data, additional studies incorporating structural and cell biology techniques will be essential to discern the mode of action for HWY 5069. This information will help move forward the development of this molecule and may lead

to the discovery of novel inhibitors that are selective against other MAP kinases involved in human diseases.

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