Chemistry & Biology *13*, 399–407, April 2006 ©2006 Elsevier Ltd All rights reserved DOI 10.1016/j.chembiol.2006.02.004

Selective Kinase Inhibition by Exploiting Differential Pathway Sensitivity

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Summary

Protein kinase inhibitors are optimized to have high affinity for their intended target(s) to elicit the desired cellular effects. Here, we asked whether differences in inhibitory sensitivity between two kinase signaling pathways, controlled by the cyclin-dependent kinases Cdk1 and Pho85, can be sufficient to allow for selective targeting of one pathway over the other. We show the oxindole inhibitor GW297361 elicits a Pho85-selective response in cells despite having a 20-fold greater biochemical potency for Cdk1 in vitro. We provide evidence that partial inhibition of Pho85 is sufficient to activate Pho85-dependent signaling, but partial inhibition of Cdk1 is not sufficient to block Cdk1-dependent cell proliferation. Identification of highly sensitive kinases may provide a means to achieve selective perturbation of kinase signaling pathways complementary to efforts to achieve maximal differences between in vitro IC_{50} values.

Introduction

Protein kinases couple external or internal signals to specific outputs, such as gene transcription or activation of effector proteins. Inhibitors of protein kinases are a key resource for development of molecularly targeted therapeutics, as well as being important tools for studying the cellular functions of protein kinases [1–3]. The discovery of protein kinase inhibitors often begins with optimization of in vitro potency and selectivity by biochemical assays followed by cell-based assays. It is now widely appreciated that a number of factors within cells can greatly influence the cellular potency of kinase inhibitors, including competition with high cellular ATP concentrations, membrane permeability, the presence of cellular phosphatases, and the concentration of kinase substrates relative to different kinases [4]. The current generation of kinase inhibitors have been designed to bind their targets with low nanomolar binding affinities and to have suitable pharmacokinetic properties to efficiently penetrate cells, effectively mitigating the first two of these factors. However, the effect of the latter two factors, as well as a host of other cellular components, on kinase inhibitor efficacy is difficult to predict, as they require a detailed understanding of the network properties of signal transduction pathways. More specifically, these factors determine the degree of coupling between the activity of a given kinase and the activation or repression of downstream biological responses.

The complex relationship between the level of kinase activity and an elicited cellular response has parallels to well-established examples in receptor pharmacology, where substoichiometric receptor occupancy by an agonist can be sufficient to elicit full activation of the pathway [5, 6]. Thus, agonist *efficacy* is not solely dependent on the affinity of the agonist for a given receptor. Similarly, protein kinases often regulate cellular responses that are switch-like in nature. The level of kinase activity necessary to propagate a signal in a cell can be thought of as the intrinsic *sensitivity* of the pathway to perturbations in kinase activity from, for example, a small-molecule inhibitor. In principle, this pathway sensitivity may be a key factor in determining inhibitor efficacy, and differences in the sensitivity of pathways governed by different kinases may affect inhibitor target specificity.

Here, we asked whether differences in inhibitory sensitivity between kinase signaling pathways can be sufficient to allow for the selective targeting of one pathway over another within cells. By using a chemical screen of a small library of CDK inhibitors, we probed the inhibitory sensitivity of two kinases, Cdk1 and Pho85, in *Saccharomyces cerevisiae*. Cdk1 is an essential protein kinase that regulates each cell cycle transition in yeast [7]. Pho85, the most closely related kinase in the yeast kinome to Cdk1, is not essential for normal cell proliferation, but regulates nutrient sensing and stress responses to phosphate deprivation [8]. Given that it is impracticable to determine the precise degree of inhibition of a kinase within a cell, we reasoned that the identification of inhibitors that show little or no in vitro selectivity between these two kinases, yet elicit Cdk1- or Pho85-selective responses within cells, would provide powerful evidence of differences in pathway sensitivity between these two kinases.

We report the identification of GW297361 [9], **1** (Figure 1A), an oxindole CDK inhibitor that elicits a Pho85-selective response in cells despite having a 20-fold greater biochemical potency for Cdk1 in vitro. We explored the molecular basis for this selectivity, and provide evidence that the Pho85 signaling pathway is highly sensitive to perturbations in kinase activity. Thus, the ability of **1** to inhibit Pho85, but not Cdk1, in vivo reveals distinct sensitivities of these kinases to chemical inhibition. Systematic determination of the quantitative contribution of each kinase in a pathway to its biological output could reveal the optimal nodes to block cell signaling by small-molecule kinase inhibitors.

Results

In order to measure the cellular effects of Cdk1 and Pho85 inhibitors, we used a recently described strategy

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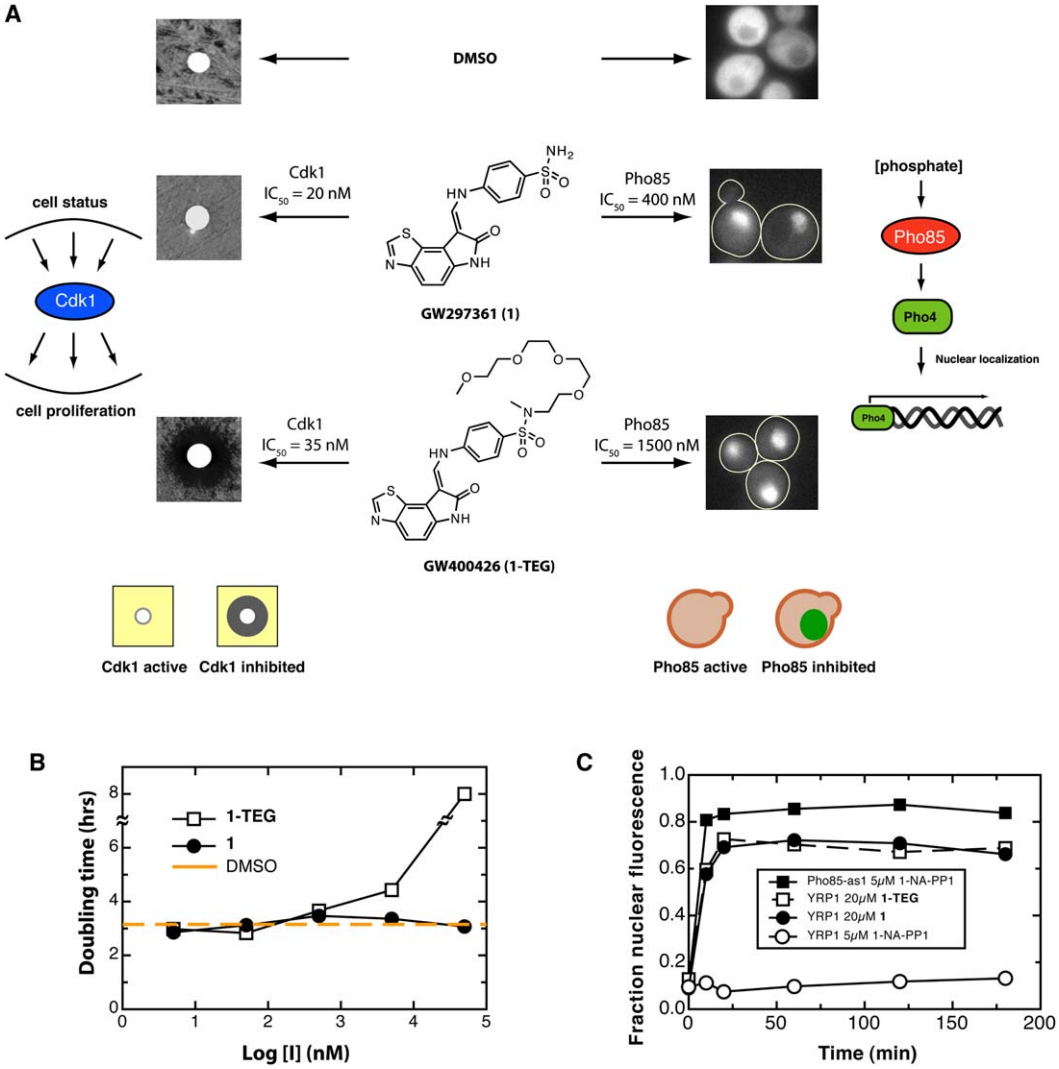

Figure 1. Functional Assays for Cdk1 and Pho85 Inhibition

(A) Oxindole CDK inhibitors were screened in functional assays for inhibition of Cdk1 and Pho85. (Left) Halo assays of GW400426 (**1-TEG**) or GW297361 (**1**) showing differential effects on cell proliferation of YRP1 yeast. A 10 μl aliquot of a 2 mM DMSO stock of each compound was added to the disc. (Right) **1-TEG** and **1** both induce nuclear localization of Pho4-GFP when added at 20 μM to YRP1 cells. Shown are fluorescence images overlaid with brightfield edge traces of the cells.

(B) **1-TEG** induces cell cycle arrest in YRP1 cells with an EC_{50} = 20 μM, while **1** has no effect at concentrations up to 50 μM.

(C) Time course of Pho4-GFP assay as depicted in (A). **1-TEG** and **1** are nearly indistinguishable at all time points.

for determining the molecular targets of protein kinase inhibitors [10]. In that work, we used a chemical genetic method of potently and monospecifically inhibiting an engineered kinase (an "analog-sensitive kinase"), along with microarray profiling and other cell-based assays, to identify molecular biomarkers diagnostic of inhibition of specific kinases. We show that the CDK inhibitor GW400426, **1-TEG** (TEG = tetraethyleneglycol; Figure 1B), inhibits both Cdk1 and Pho85 kinases in the YRP1 drug-sensitized yeast strain background. **1-TEG** inhibits yeast cell proliferation with an EC_{50} of roughly 20 μM—an activity that is accounted for by its potent inhibition of the essential kinase Cdk1 (IC_{50} = 35 nM). **1-TEG** treatment also induces the nuclear localization of the GFP-tagged transcription factor Pho4 by inhibiting its phosphorylation by Pho85, thus providing a sensitive fluorescence-based assay to monitor inhibition of Pho85.

We used these two cellular readouts to screen a small library of oxindole inhibitors (see Figure S1A in the Supplemental Data) originally developed as inhibitors of mammalian CDK2 [9]. We identified **1** as a molecule that showed activity in the Pho4-GFP localization assay that was nearly indistinguishable from **1-TEG** (Figure 1C). However, **1**, despite sharing a high degree of structural similarity to **1-TEG**, did not inhibit yeast cell proliferation as evaluated by halo assay (Figure 1B), suggesting that it was not inhibiting Cdk1 within cells. We confirmed these results by evaluating the ability of **1** to affect cellular proliferation in liquid culture. At concentrations up to 50 μM, **1** did not measurably affect cell proliferation (Figure 1C). Concentrations greater than

50 μM could not be evaluated due to solubility constraints.

Next, we assessed whether the observed cellular target specificity of **1** was reflected by its in vitro potency against these two kinases. Surprisingly, we found that **1** is actually a more potent inhibitor of Cdk1 than **1-TEG** ($IC_{50,1}$ = 20 nM versus $IC_{50,1\text{-}TEG}$ = 35 nM). Moreover, while **1** also inhibits Pho85 (IC_{50} = 400 nM) more potently than **1-TEG** does (IC_{50} = 1.5 μM), it does so 20-fold less potently than it inhibits Cdk1. Thus, the inhibitory activity of **1** against these two kinases within yeast cells is reversed from that predicted by in vitro biochemical measurements. **1-TEG** and **1** share very similar IC_{50} values against a wide variety of human kinases (Figure S1C), suggesting that the inverted cellular target specificity of **1** does not reflect an ability to bind kinases in a significantly different orientation.

While cell proliferation reflects the final outcome of Cdk1-mediated signal transduction within the cell, we sought to understand in greater detail the cellular effects elicited by **1**. The regulation of cell proliferation by Cdk1 is a complex process involving the phosphorylation of hundreds of substrates [11] that regulate the transcription of cell-cycle genes. We therefore examined both the global effects on gene transcription of **1** as well as its impact on the phosphorylation of Orc6, a direct substrate of Cdk1 [12].

We conducted whole-genome transcriptional profiling and examined the effects of **1** treatment on the transcription of a set of 213 genes whose expression is dependent on Cdk1 activity [10]. **1-TEG** strongly represses the transcription of these genes (Figure 2A, lanes 4 and 5), to a similar degree as from monospecific inhibition of Cdk1 (Cdk1-as1 strain, lane 6), while **1** has a much weaker repressive effect on these genes (lanes 2 and 3). Similar results were obtained when we evaluated the expression of several Cdk1-dependent genes (*CLB2*, *CDC20*, *HOF1*) by quantitative PCR (Figure 2E).

In log phase cells, Orc6 migrates as two distinct phosphoisoforms on SDS-PAGE that are present at approximately equal levels. Upon inhibition of Cdk1 by **1-TEG**, the majority of the Orc6 is converted to the faster-migrating isoform (lower:upper = 4:1) (Figure 2B). Upon treatment of YRP1 cells with **1**, an intermediate level of the faster-migrating phosphoisoform was obtained (lower:upper = 2:1). Thus, examination of both Cdk1-dependent gene transcription and substrate phosphorylation reveals that **1** partially inhibits Cdk1 within cells. The fact that **1**, unlike **1-TEG**, is unable to block cell proliferation suggests that cell cycle control by Cdk1 is buffered against small changes in Cdk1-mediated phosphorylation and transcription.

Next, we evaluated the effect of **1** on Pho85-mediated signaling by monitoring a set of Pho4-dependent genes involved in the response to low phosphate conditions that are induced upon inhibition of Pho85. We previously found that **1-TEG** treatment results in a modest induction in gene expression compared to monospecific inhibition of Pho85 in a Pho85-as1 strain, consistent with its observed IC_{50} value (1.5 μM) against Pho85 in vitro [10]. While **1** is a slightly better Pho85 inhibitor (IC_{50} = 400 nM) in vitro than **1-TEG**, it induces the transcription of these genes to a similar degree (Figure 2C). These results, along with the Pho4-GFP localization data, are consistent with **1** inhibiting Pho85 in cells to a similar or somewhat lesser degree compared to **1-TEG**. Furthermore, it is clear that inhibition of Pho85 by either **1-TEG** or **1** is incomplete, as comparison with a Pho85-as1 strain treated with 1-NA-PP1 results in both a stronger transcriptional and Pho4-localization response (Figures 2C and 1D). This is most likely the result of the relative impermeability of the oxindole scaffold to the yeast cell wall compared to the pyrazolo-pyrimidine scaffold of PP1 derivatives [10].

One factor that might begin to explain the inverted target specificity of **1** is the relative cellular concentrations of the two kinases within cells. If the concentration of Pho85 was much greater than that of Cdk1, it could compensate for the difference in biochemical affinity [4]. However, quantitative measurements of the concentrations of these two kinases under identical growth conditions to those in our experiments show that both Cdk1 and Pho85 are present at nearly equal levels [13]. Moreover, this explanation would not account for the inhibition of both Cdk1 and Pho85 by **1-TEG**, a molecule with roughly equivalent in vitro potency to that of **1**.

If the Pho85 signaling pathway was inherently more sensitive to changes in catalytic activity than the Cdk1 pathway, the data could be explained, if **1-TEG** was able to achieve greater intracellular concentrations than **1**. Under this scenario, **1** reaches a steady-state cellular concentration that is only high enough to activate Pho85 signaling, while being insufficient to fully inhibit Cdk1 and block cellular proliferation.

It is difficult to precisely evaluate the concentrations of small molecules within cells due to the complex factors (permeability, efflux, subcellular localization, etc.) involved [14]. Given the shared oxindole scaffold between **1-TEG** and **1**, we reasoned that the yeast generic environmental stress response (ESR) [15] to the presence of foreign molecules might make a reasonable surrogate marker to approximate the intracellular concentrations of these molecules. When we examined the expression of genes involved in the ESR upon treatment with **1-TEG** or **1**, we found that, at 20 μM, **1** had a slightly diminished response in both induction and repression of ESR genes (Figure 2D). At 40 μM, the responses of **1-TEG** and **1** were very similar. These data suggest that **1** may be less biovailable than **1-TEG**. However, the similarity in the ESR between **1-TEG** at both concentrations, **1** at 40 μM and 1-NA-PP1 at 5–20 μM (data not shown), suggests that the upper limit of the ESR has been reached, and these markers are not suitable to make conclusive judgments about the concentration of the molecules within the cell.

Any enhanced bioavailability of **1-TEG** could, in principle, be conferred due to a change in a physicochemical property, such as the superior solubility in DMSO and aqueous media of **1-TEG** compared to **1**. Alternatively, it could result from a discrete molecular recognition event mediated by the TEG moiety that results in enhanced bioactivity in yeast, such as inhibition of an efflux pump or recognition by an importer. There is some precedent for the latter scenario, as long-chain alkyl esters of adenosine monophosphate (AMP) were found to cause cytotoxic effects in yeast due to modulation of a signaling pathway that activates a plasma membrane proton pump [16]. The TEG group found on **1-TEG** is

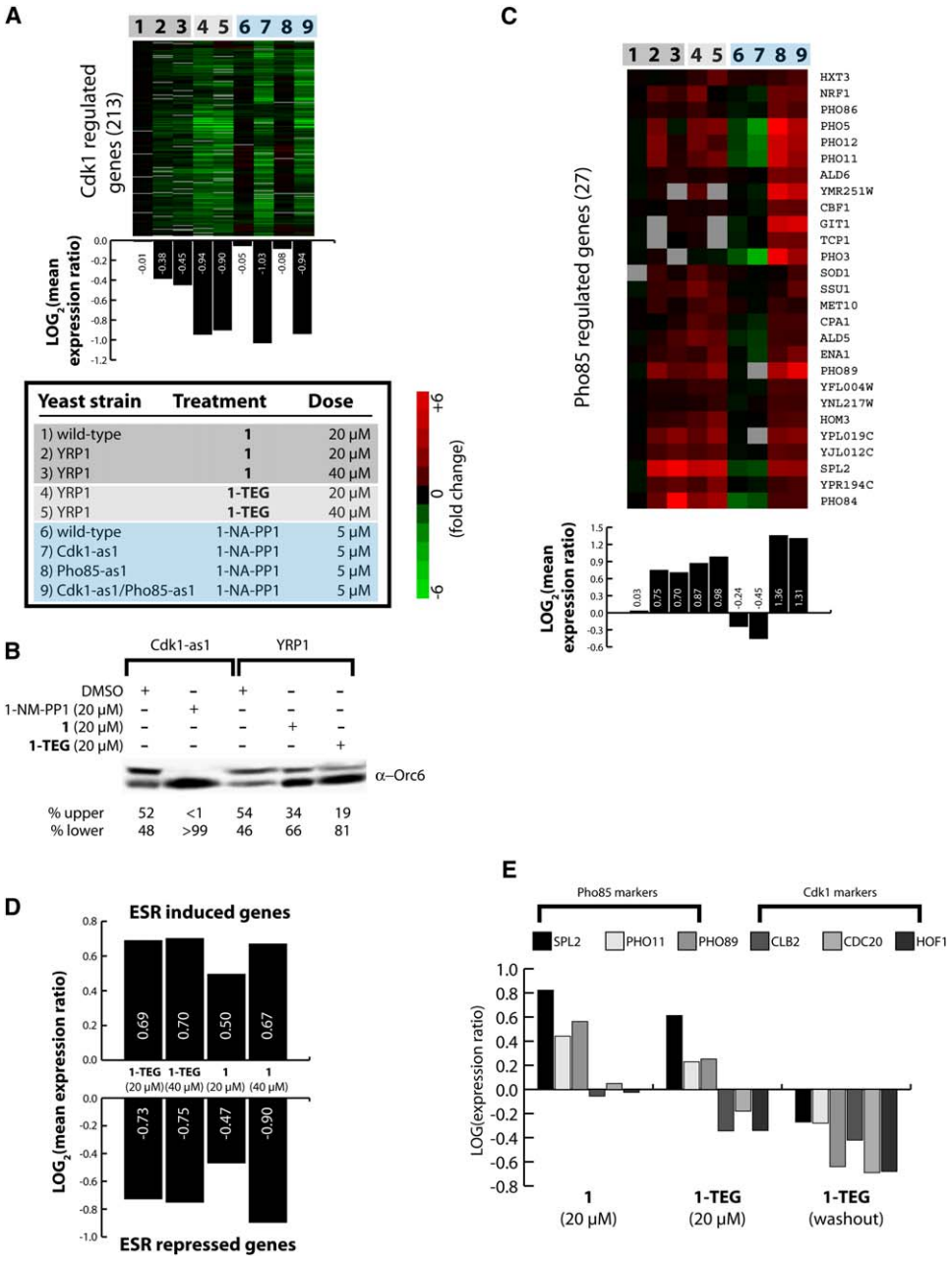

Figure 2. Validation of Target Specificity of **1** and **1-TEG**

(A) Cluster of 213 genes whose expression is repressed upon inhibition of Cdk1. Quantitation of mean log expression ratios is shown below each column. **1** exhibits partial inhibition of Cdk1-dependent gene expression compared to full inhibition achieved by **1-TEG** or in a Cdk1-as1 strain inhibited with 1-NA-PP1.

(B) Western blot quantitating Orc6 phosphoisoforms present in Cdk1-as1 or YRP1 cells following treatment for 15 min with DMSO, 1-NA-PP1, or **1-TEG** or **1**. The effect of **1** is consistent with partial inhibition of Cdk1 within cells.

(C) Cluster of 27 genes whose expression is induced upon inhibition of Pho85. Both **1** and **1-TEG** upregulate Pho4-dependent genes. Comparison to a Pho85-as1 strain inhibited with 1-NA-PP1 suggests partial inhibition of Pho85 by **1** and **1-TEG** within cells. The columns are the same as in (A).

(D) Effects of **1-TEG** and **1** on expression of genes involved in the yeast ESR. A 20 μM treatment with **1** results in a milder response than 40 μM **1** or treatment with **1-TEG**, providing indirect evidence that **1** has reduced bioavailability in YRP1 yeast.

(E) Quantitative PCR measurements of expression changes in three markers of Pho85 inhibition (*SPL2*, *PHO11*, *PHO89*) and Cdk1 inhibition (*CLB2*, *CDC20*, *HOF1*) upon treatment of YRP1 yeast for 10 min with **1-TEG** or **1**. Values represent the change in expression compared to DMSO treatment. For the washout experiment on the right, cells were treated with **1-TEG** for 10 min, pelleted, and resuspended in fresh media containing 1% DMSO for 10 min.

a distinct SAR element in this series of molecules, as we had found that all three molecules with antiproliferative activity in our screen contained a similar polyethylene glycol (PEG) moiety coupled to the sulfonamide moiety, while molecules with other substituents did not (Figure S1B). PEGylation has been used to improve the

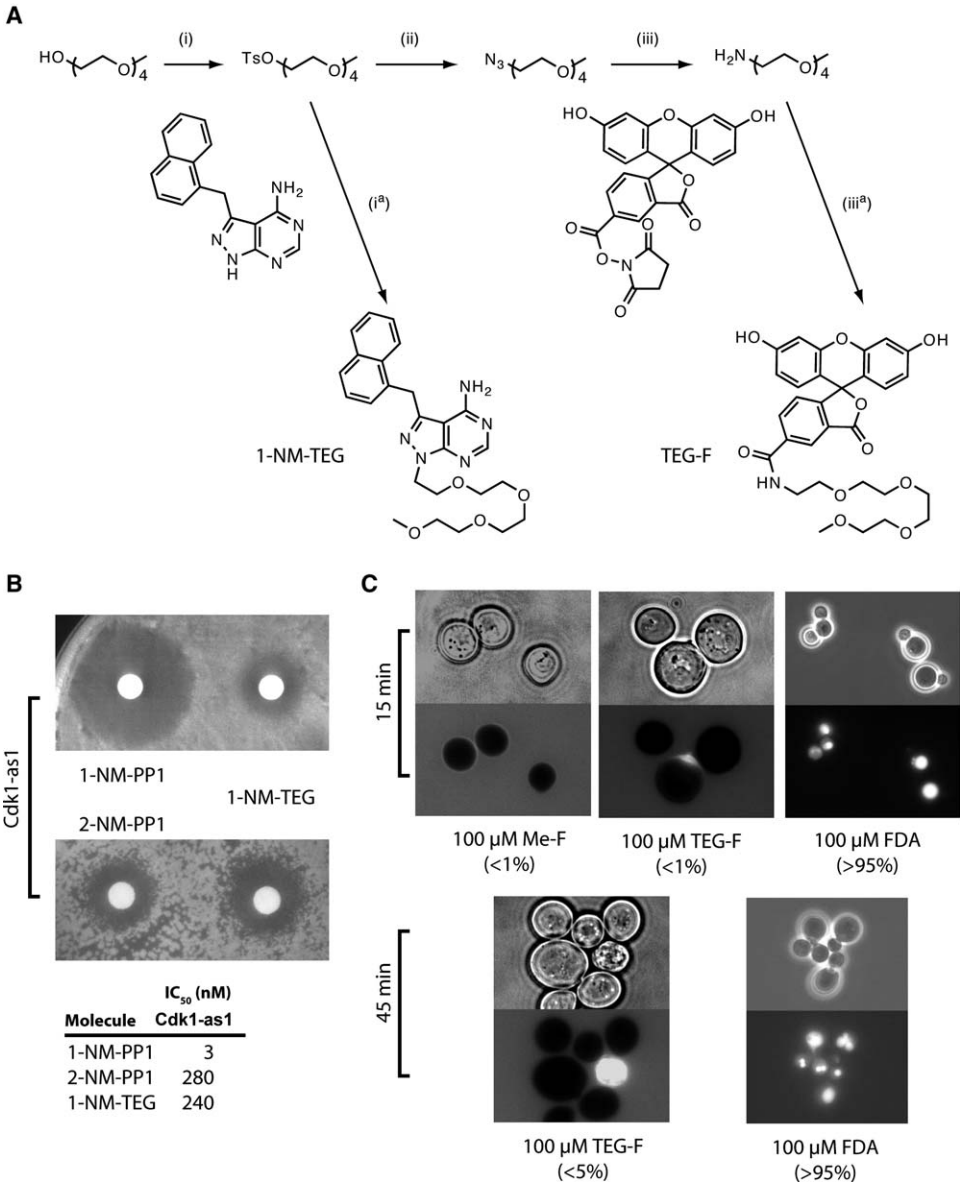

Figure 3. Probing the Molecular Basis for the Difference in Target Specificity between **1** and **1-TEG**

(A) (i) TsCl, TEA, −10°C; (i[a]) LiBr, $NaHCO_3$ then H-1-NM, K_2CO_3, DMF; (ii) NaN_3, TBAHS, DMF; (iii) H_2, Pd/C, MeOH; (iii[a]) FAM-SE, DMF. Further synthesis details in Experimental Procedures.

(B) Halo assays showing effects on cellular proliferation of 1-NM-PP1-, 2-NM-PP1-, and 1-NM-TEG-treated Cdk1-as1 cells. In this series of molecules, in vitro potency closely tracks the cellular efficacy.

(C) Neither TEG-Me nor TEG-F is able to penetrate the cell wall of YRP1 yeast. FDA treatments demonstrate compartmentalization of fluorescein molecules following hydrolysis of acetyl groups.

pharmacokinetic properties of both proteins [17] and small molecules [18].

In an attempt to distinguish between the two possibilities, we synthesized TEG-based "molecular chimeras" to see if the TEG moiety would confer enhanced bioactivity in yeast in a general fashion to small molecules. We first asked if TEG could increase the cellular concentrations of a small molecule. We synthesized 1-NM-TEG, a derivative of 1-NM-PP1 (Figure 3A). As 1-NM-PP1 is already able to cross the yeast cell wall, we were interested in seeing if attaching the TEG moiety would enhance its potency against analog-sensitive kinases.

Using a standard halo assay, we found that 1-NM-TEG failed to suppress yeast cell proliferation as potently as 1-NM-PP1 when applied at the same concentration (Figure 3B, top). However, we also found that 1-NM-TEG (IC_{50} = 240 nM) is a much weaker inhibitor than 1-NM-PP1 (IC_{50} = 3 nM) of Cdk1-as1 in vitro, consistent with known structure-activity relationships for PP1 analogs [19]. Therefore, we asked whether 1-NM-TEG demonstrated unusually high potency within cells given its weaker in vitro potency. We assessed the inhibitory properties of 2-NM-PP1, a molecule with an in vitro potency against Cdk1-as1 very similar to 1-NM-TEG

(IC_{50} = 280 nM). Treatment of YRP1 cells with 2-NM-PP1 resulted in a halo virtually identical to that associated with 1-NM-TEG treatment, demonstrating that in this series of molecules, in vitro inhibitory potency translated directly to in vivo efficacy (Figure 3B, bottom) irrespective of the TEG moiety. This result also rules out the possibility that the TEG moiety is generally toxic to yeast.

Next, we asked if TEG could by itself overcome the impermeability of a cell membrane to a small molecule by synthesizing a TEG-conjugated fluorescein fluorophore (Figure 3A, TEG-F). Fluorescein is unable to cross the cell wall of wild-type yeast or YRP1 yeast (data not shown). We asked whether TEG-F would be able to overcome this impermeability by treating YRP1 yeast with either TEG-F or a methyl amide fluorescein control compound (Me-F) at concentrations up to 100 μM and visualizing the cells with fluorescence microscopy. As a positive control to ensure we could detect cellular entry of fluorescein, we used a yeast-permeable form of fluorescein, fluorescein diacetate (FDA). FDA is taken up by yeast cells and hydrolyzed to the fluorescent species within 15 min by intracellular esterases (Figure 3C). In contrast, we found that there were few, if any, cells that took up either TEG-F or Me-F after 15 min (Figure 3C). After 45 min, the small (<5%) percentage of cells that demonstrated fluorescein uptake appeared to be dead stationary phase cells that presumably had compromised membrane permeability. Consistent with this notion, the fluorescence in these cells did not become localized to discrete cellular compartments, as seen in the FDA-treated cells. At all concentrations, the cell permeability of Me-F and TEG-F were indistinguishable. Collectively, these experiments suggest that there appears to be no TEG-specific molecular recognition event that confers enhanced bioactivity to **1-TEG** over **1**.

If the Pho85 signaling pathway was more sensitive to changes in Pho85 catalytic activity through chemical inhibition than was the Cdk1 pathway, it might also be expected to recover more quickly upon removal of the drug [20]. Therefore, we investigated the dynamic properties of the Pho85 and Cdk1 signaling pathways. We treated YRP1 cells with **1-TEG** for 10 min to induce both Pho85 and Cdk1-dependent responses, and then washed out the drug for 10 min. Quantitative PCR experiments monitoring the expression of Pho85 and Cdk1-dependent genes (*SPL2*, *PHO11*, *PHO89*, *CLB2*, *CDC20*, *HOF1*) confirmed that, upon wash out, the Pho85 transcriptional signature is quickly abrogated, whereas the Cdk1 signature is sustained (Figure 2E, right). These results demonstrate that the Pho85 signaling pathway is rapidly switchable between activated and repressed states.

Although there are other plausible explanations that we cannot rule out at this time, our results are consistent with a model in which the cellular target specificity of **1** in budding yeast is governed by its poor bioavailability due to its pharmacokinetic properties. Our data suggest that partial inhibition of Pho85 by **1** or **1-TEG** is sufficient to induce a significant biological response. Thus, **1** is able to activate the Pho85-mediated environmental sensing response, but is unable to attain the cellular concentrations necessary to achieve significant Cdk1 inhibition.

One prediction of this model would be that it should be possible to identify a dose at which **1-TEG** would have the same cellular effects as **1** (e.g., inhibition of Pho85 but not of Cdk1). We were unable to identify such a dose. Treatment of YRP1 cells with 5 μM **1-TEG** results in nearly no biological response as measured by microarray profiling [10]. This may reflect the underlying and often underappreciated complexity of cellular permeability to small molecules. Cellular phenotypes were only observed for the oxindole series of compounds in the drug-sensitized yeast strain YRP1, which contains disruptions of two genes encoding small-molecule efflux pumps (*SNQ2*Δ, *PDR5*Δ) and one gene (*ERG6*Δ) involved in the biosynthesis of ergosterol, a key component of the cell membrane. Treatment of wild-type strains (Figure S1D) and yeast strains harboring any two of the three gene disruptions in YRP1 (data not shown) failed to elicit any cellular response. Thus, even in YRP1 yeast, the concentration of oxindole-based inhibitors, such as **1-TEG** and **1**, within the cell may not correspond linearly to the concentration added to the media. Recent studies have demonstrated that estrogen, and perhaps other lipophilic hormones, rely on active transport, not passive diffusion, to enter mammalian cells. These revelations about such a well-studied molecule illustrate that the mechanisms underlying small-molecule bioavailability are complex and poorly understood [21].

Discussion

In this paper, we sought to address a fundamental question in understanding the molecular basis for the cellular effects of protein kinase inhibitors, the relationship between the level of kinase activity and the output of a signal transduction pathway. We asked whether we could selectively target a protein kinase based on the inhibitory sensitivity of its cellular signaling pathway to modulation of kinase activity rather than the relative binding affinities of a small molecule to the kinases within a cell. By comparison to the structurally similar inhibitor **1-TEG**, whose cellular targets were well defined based on previous studies by chemical genetic analysis in our lab, we have identified **1** as a CDK inhibitor that elicits specific Pho85-dependent responses within yeast cells, thus exhibiting a dramatically different cellular specificity profile than predicted based on its in vitro profile. While we can not rule out all possible explanations for the cellular activity of **1**, we believe that the inverted target specificity of **1** is strong evidence that kinase signaling pathways within cells are differentially sensitive to levels of kinase activity. To our knowledge, this is the first example of a kinase inhibitor to be shown to achieve its target specificity using such a mechanism.

In the field of receptor pharmacology, the identification of signaling pathways that achieve maximal response upon substoichiometric occupancy by agonists has led to the concept of "spare receptors," with other cellular components being the limiting factor in signal transduction [22]. For protein kinases, differences in pathway inhibitory sensitivity might be expected to lie in the nature of the signal integration that occurs to convert individual protein phosphorylation events into specific biological responses. For instance, in *S. cerevisiae*,

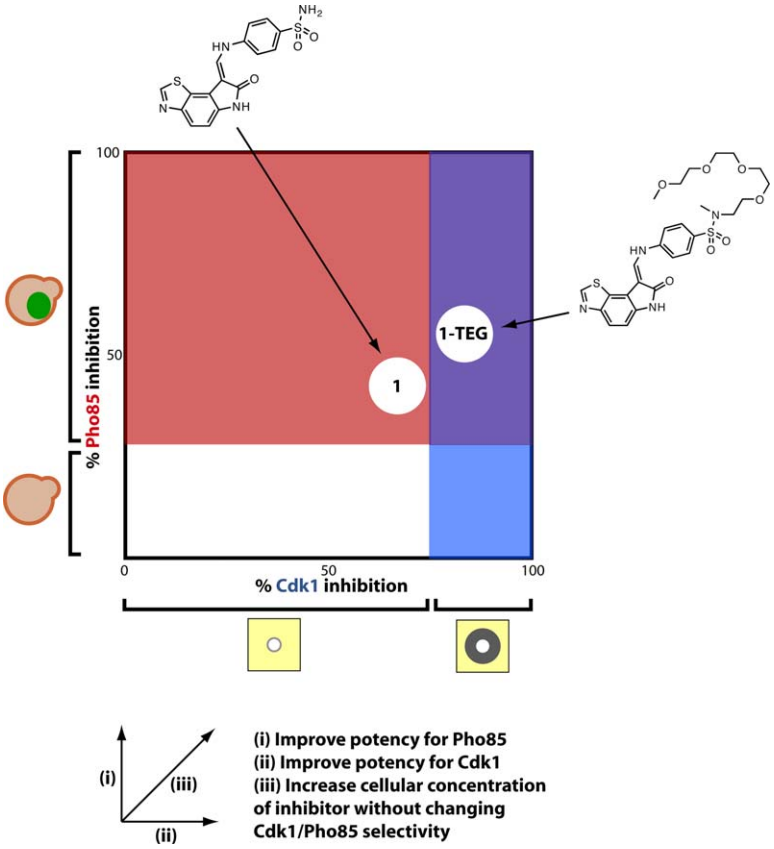

Figure 4. Inhibitor Selectivity via Differential Pathway Sensitivity

Model to explain cellular effects of **1-TEG** and **1** within the cell. The x and y axes show the percent inhibition of Cdk1 and Pho85, respectively. All values are for schematic purposes only. The level of inhibition of Pho85 necessary to induce Pho85 localization (~30%, shaded in red) is relatively low compared to that necessary to block cellular proliferation through inhibition of Cdk1 (~75%, shaded in blue). **1-TEG** is able to block both kinases within yeast cells and falls within the purple area. If an inhibitor is dosed at a concentration to achieve >30% inhibition of Pho85 and <75% inhibition of Cdk1, than it would be able to selectively inhibit Pho85, despite having no apparent selectivity by in vitro biochemical assays (as illustrated by **1**).

the phosphorylation of Sic1, an inhibitor of the Cdk1-Clb CDK-cyclin complex, by Cdk1-Cln on more than six phosphorylation sites has been shown to be a mechanism by which yeast cells impose a switch-like response at the G1-S transition [23]. In that study, degradation of Sic1 was achieved if six or more, but not five or fewer, of nine CDK phosphorylation sites were present, illustrating the precise tuning of biological responses to protein kinase activity. Similarly, the extent of phosphorylation of Pho4 on one to four phosphorylation sites by the Pho85-Pho80 CDK-cyclin complex controls the transcriptional efficiency of genes involved in the response to various environmental stresses [24].

The cellular circuitry in which each kinase is embedded is also likely to play an important role in pathway sensitivity. The cell cycle transitions orchestrated by Cdk1, through phosphorylation of hundreds of substrates, are irreversible processes in which a significant fraction of Cdk1 substrates are regulated by other mechanisms aside from an opposing phosphatase, such as proteolytic degradation [11]. In contrast, the rapid reversibility of the Pho85-Pho4 pathway is consistent with tight coupling to an as-yet unidentified cellular phosphatase(s) in a relatively simple regulatory circuit. We hypothesize that the precise cell cycle transitions governed by Cdk1-cyclin complexes might have evolved to be highly resistant to biological noise, and therefore to chemical inhibition as well. This point is illustrated by the inability of **1** to block cellular proliferation despite having a quantitatively significant effect on Cdk1-mediated gene transcription and substrate phosphorylation. Efficient adaptation to changes in the cellular environment might require that the Pho85 signaling pathway respond to even small changes in catalytic activity as a measure of fluctuations in extracellular phosphate concentration [8]. As seen schematically in Figure 4, differences in the level of inhibition necessary to achieve a biological response for Pho85 or Cdk1 inhibition would account for the cellular target specificity of **1** and **1-TEG**.

The identification of hypersensitive kinase pathways may play an important role in selecting targets for inhibitor design. These kinases may themselves be attractive targets, or they may need to be included in counterscreens in the process of lead optimization to minimize off-target side effects. Identification of hypersensitive pathways will require detailed quantitative analysis of the role of kinase activation in downstream signaling. Already, it is apparent that even closely related kinases may have significant differences in their network properties and, therefore, may respond differently to pharmacological inhibition. For instance, elegant studies in *Xenopus* oocytes have shown that activation of the MAP kinase JNK by progesterone stimulation occurs in a manner whereby, in a population of oocytes, each individual oocyte either has full JNK activation or none [25]. In contrast, a recent report demonstrates that mammalian ERK MAP kinase activity increases in a graded fashion in response to agonist, with subsequent switch-like behavior regulated at the level of gene transcription [26].

Our current efforts to elucidate the functions of protein kinases using chemical genetic analysis should aid in the process of systematically identifying and

dissecting the signaling dynamics of such pathways. Chemical tools, such as specific, covalent inhibitors of protein kinases [27], combined with chemical genomic profiling, may allow us to address these questions in a quantitative fashion.

Significance

The use of small-molecule protein kinase inhibitors to modulate signal transduction mediated by protein kinases has become a key focus of biomedical research. The ability to predict the cellular effects of kinase inhibitors is dependent on understanding the true cellular targets of a molecule. However, protein kinase inhibitors are usually optimized against their intended targets through in vitro biochemical assays. This paper addresses a complementary aspect of inhibitor target specificity; namely the intrinsic inhibitory sensitivity of the cellular pathways controlled by a given kinase. By using cellular readouts of the activity of the closely related CDKs Cdk1 and Pho85, we screened a library of oxindole CDK inhibitors and identified GW297361 as a molecule that appears to inhibit Pho85 selectively within cells despite a 20-fold preference for Cdk1 in vitro. This supports the hypothesis that the environmental sensing Pho85 signaling pathway is tuned to be highly responsive to changes in Pho85 activity levels, whereas Cdk1 signaling, whose function is to regulate the cell cycle, appears to be buffered against partial inhibition of Cdk1. The existence of "hypersensitive" kinases may offer a new strategy to target this class of signaling enzymes. The differential inhibitory sensitivity of kinase signaling pathways may account for a subset of situations where there is a significant discrepancy between a molecule's in vitro specificity and its observed cellular activities.

Experimental Procedures

Chemical Synthesis

1-TEG, **1**, 1-NA-PP1, 1-NM-PP1, and 4-(naphthalen-1-ylmethyl)-1H-pyrazolo[3,4-d]pyrimidine were synthesized as previously described [9, 28]. Methyl ether tetraethylene glycol amine was synthesized as previously described [29]. To synthesize TEG-F and Me-F, FAM-SE (1 eq) and methyl ether tetraethylene glycol amine (1.2 eq) or methylamine (1.2 eq) were dissolved in DMF, and the reaction was allowed to stir for 4 hr at room temperature. Following removal of DMF in vacuo, the reaction products were purified by HPLC and lyophilized. ESI/MS analysis confirmed formation of the expected product.

1-NM-TEG was synthesized by reacting methyl ether tetraetylene glycol tosylate with LiBr. The crude brominated product was coupled to H-1-NM in DMF with K_2CO_3. Products were purified by HPLC and lyophilized. ESI/MS analysis confirmed formation of the expected product.

Strains and Plasmids

YRP1 was a gift from Karl Kulcher (Vienna University, Vienna, Austria). Pho85-as1, Cdk1-as1, and dual Cdk1-as1/Pho85-as1 have been described previously [10, 30, 31].

Kinase IC_{50} Assays

Cdk1-Clb2 and Pho85-Pho80 kinase assays were performed as previously described to determine IC_{50} values for **1-TEG** and **1** [30].

Orc6 Phosphorylation

Exponentially growing Cdk1-as1 or YRP1 cells were treated with either DMSO, 1-NA-PP1, **1-TEG**, or **1** for 15 min. Cellular proteins were extracted into urea lysis buffer (20 mM Tris [ph 7.4], 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 50 mM NaF, 80 mM BGP, 1 mM Na_3VO_4, 1 mM PMSF), run out on SDS-PAGE, and blotted to nitrocellulose. The blot was probed with a monoclonal antibody against Orc6 (SB49, 1:1000) and visualized by ECL after probing with an HRP-conjugated goat anti-mouse antibody (1:1500; Pierce). Densitometry quantitation was done using ImageJ software (http://rsb.info.nih.gov/ij/).

Pho4-GFP

Pho85-as1 or YRP1 cells carrying the Pho4-GFP plasmid were grown under selection to an OD of 0.5 and treated with 5 μM 1-NA-PP1, 20 μM **1-TEG** (YRP1), or 20 μM **1** (YRP1). Samples were taken at each time point and analyzed with static microscopy. At least 50 cells were counted for each treatment.

Microarray Analysis

Microarray preparation and experimental procedures were performed, and clusters from Figure 2 were identified as previously described [10]. Briefly, yeast cells of the appropriate strain were grown to an OD of 0.7 and treated with either inhibitor or the equivalent volume of dimethyl sulfoxide for 10 min. Cells were collected by filtration and flash-frozen in liquid nitrogen. Yeast total RNA was isolated, followed by selection for polyadenylated mRNA from 1 mg of total RNA. First-strand cDNA synthesis was carried out in the presence of a dNTP/amino-allyl-dUTP mixture. The cDNA from paired samples was then labeled with either Cy3 or Cy5 dyes and hybridized to microarrays containing ~93% of yeast ORFs. Fluorescence ratios were obtained with an Axon 4000A scanner. Each experiment was done in replicate with Cy3 and Cy5 labeling reversed between inhibitor and DMSO treatments in the replicate experiments. "Dye-flipped" expression ratios were inverted and then averaged in log-space with their nonflipped counterparts. To identify functional clusters of genes as depicted in Figure 2A, expression ratios were converted to log-space and the data set was filtered to include genes whose expression changed by 1.6-fold or more in at least two experiments. Genes were clustered with Cluster 3.0 software (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/manual/) by average-linkage hierarchical clustering.

Quantitative PCR

Sequences for 5′-3′ and 3′-5′ primers were as follows: ACT1: TC ACTATTGGTAACGAAAGATTCAG, AAGCCAAGATAGAACCACCAA TCCA. SPL2: AGAGTGCAGGAGGCTCAATC, TCGTCAATGACACAG TCGTTC. PHO11: TCCAGTGTTCCAATGACACC, AGTTACTGACGC TGCTGACG. PHO89: TCGGTTCTCCAAGCCATTAC, AAGGCAACA CCACCATAAGC. CLB2: ATCAACAGCCGCACAAGAG, CATGCTGG ATTATCTCCTTCG. CDC20: TGTGGAATGCCACCACTG, AATGAA CCGATACGGACACC. HOF1: AACCAAGGCCGATTGTTG, GGCCA TTGAACTTACGGATG.

Yeast log-phase cultures were treated with DMSO, 20 μM **1-TEG**, or 20 μM **1** for 10 min, harvested by centrifugation, and frozen at −80ºC. RNA was isolated by hot phenol/chloroform extraction. A total of 1 μg of RNA was resuspended in a total volume of 10 μl with 250 nM of 3′-5′ primer and heated to 70ºC for 10 min. The reverse transcription was carried out with StrataScript enzyme (Stratagene), according to the manufacturer's instructions, at 50ºC for 45 min. Following ethanol precipitation, reaction products were amplified in a GeneMachines (MJ Research) thermal cycler in the presence of SYBR Green dye (Molecular Probes). Quantitation was carried out by first measuring cycle threshold values in duplicate from a dilution series spanning four orders of magnitude of a standard template. Cycle threshold values were measured in duplicate from drug-treated and DMSO-treated samples and verified to fall within the linear range of the standard series. Measured values were normalized to ACT1 levels, which were found by microarray analysis to be unchanged following drug or DMSO treatment.

Supplemental Data

Supplemental Data, including supplemental figures, are available online at http://www.chembiol.com/cgi/content/full/13/4/399/DC1/.

Acknowledgments

We thank Lee Kuyper and Scott Dickerson (GlaxoSmithKline) for providing the oxindole library, Bruce Stillman (Cold Spring Harbor Laboratory) for providing the Orc6 antibody, Erin O'Shea (Harvard University) for Pho85-related reagents, and Dave Morgan, Quincey Justman, and Jimmy Blair for helpful discussions. Funding was provided by AI grant NIHR01-AI44009.

Received: December 10, 2005
Revised: January 29, 2006
Accepted: February 6, 2006
Published: April 21, 2006

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