Probes for Cell Division

HR22C16: A Potent Small-Molecule Probe for the Dynamics of Cell Division**

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Proper cell division requires that each daughter cell receive a single and complete copy of DNA.[1] Errors in this process can lead to severe developmental defects and diseases in humans. To divide the genetic material, the cell assembles a multicomponent apparatus that converts chemical energy into mechanical energy for the transport of DNA. The entire process takes minutes, and several steps occur within seconds. Cell-permeable small molecules allow us to intervene in this process on this time scale, whereas genetic methods, including RNA interference, are too slow, effective on a time scale of hours to days.[2a] Although many small molecules, including taxol, disrupt cell division by binding to tubulin, only a few small molecules that target the numerous other proteins involved in cell division have been identified.[2b,c] A strategy referred to as "forward chemical genetics" is a particularly promising tool for the discovery of new small-molecule inhibitors.[3] In forward chemical genetics, small molecules are selected if they yield a desired cellular phenotype or perturbation. The identification of the target of this small molecule can then link a known or an unknown protein to the observed phenotype. The small molecule can then be used to study the function of the target protein in its cellular context.

Herein we report the discovery of HR22C16 (**1**, Figure 1), a new cell-permeable small-molecule inhibitor of cell division, identified by a high-throughput microscopy-based forward-chemical-genetic screen. We show that HR22C16 blocks cell division by targeting Eg5, a molecular-motor protein whose function is required for cell division ($IC_{50} = 800 \pm 10$ nM).[4a] Furthermore, we report an efficient, diastereoselective, traceless solid-phase synthesis of HR22C16 analogues.

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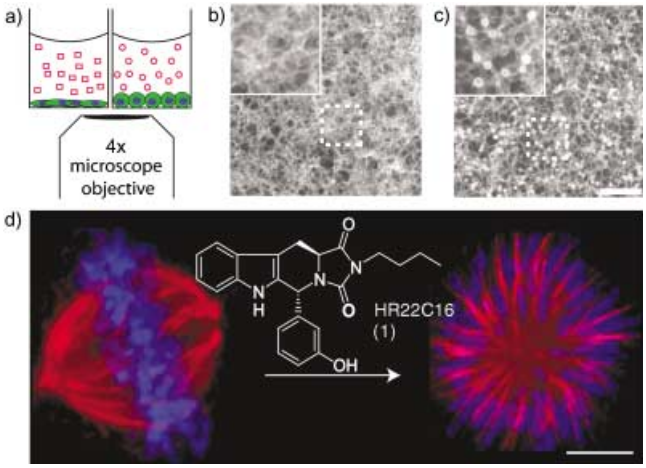


Figure 1. HR22C16 (**1**), a cell-division inhibitor discovered from a forward-chemical-genetic screen. a) Schematic representation of the assay. Vertebrate cells (BS-C-1) in a multiwell plate are stained for the actin cytoskeleton and imaged from below by using a 4× objective. Primary data from the screen show b) the effect of a compound that causes no morphological change (mostly flat cells with very few cells undergoing division) and c) the effect of HR22C16 with hundreds of cells blocked in mitosis as round cells (inset: 2.5×; scale bar: 250 μm). d) Configuration of microtubules (red: stained with antitubulin antibodies) and DNA (blue: stained with Hoechst 33342) in a control cell (left) and a HR22C16-treated cell (right; scale bar: 5 μm).

We have used these analogues in cellular and in vitro assays to clarify the structural basis of the activity of HR22C16 and to identify an Eg5 inhibitor that is approximately ninefold more potent than HR2216. Finally, guided by these studies, we have designed a photolabile protecting (photocaging) strategy for HR22C16 that allows fast temporal control over Eg5 function during cell division.

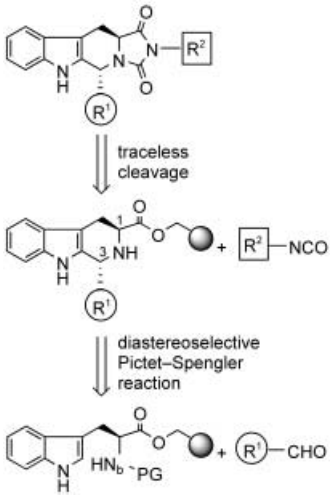
To identify small molecules that inhibit cell division we used the morphology of cells blocked in division as our readout, rather than limiting our search to one for an inhibitor of a particular protein. Cells are flat until they initiate cell division, when they adopt a round shape. Inhibition of cell division, then, increases the fraction of cells that exhibit a round morphology. Analysis by fluorescence microscopy (at only 4× magnification) provided a direct and simple readout for the inhibition of cell division. Based on this approach we examined the effects on mammalian cells of 16000 compounds from a collection of diverse small molecules.[5a,b] Figures 1b and 1c show primary data from this cell-based screen. Most compounds did not block cell division and therefore flat cells were mostly observed. In contrast, compounds that blocked cell division led to a higher proportion of round cells (i.e. dividing cells) and were easily detected by the eye or automated methods. HR22C16 (**1**) was discovered to be one of these compounds.

Subsequent examination of the microtubule cytoskeleton (red) and chromosomes (blue) in cells treated with HR22C16 revealed a monoaster phenotype (Figure 1d). Such a phenotype can arise in dividing cells by several mechanisms, including the loss of function of the ATPase Eg5, a motor protein that generates mechanical forces required for cell division.[4a] This phenotype is also obtained when cells are treated with the dihydropyrimidine monastrol, a known cell-permeable small-molecule Eg5 inhibitor ($IC_{50} = 14\ \mu M$).[4b] We used in vitro assays for Eg5 motor function and indeed found that HR22C16 inhibited Eg5 ($IC_{50} = 800 \pm 10$ nM).

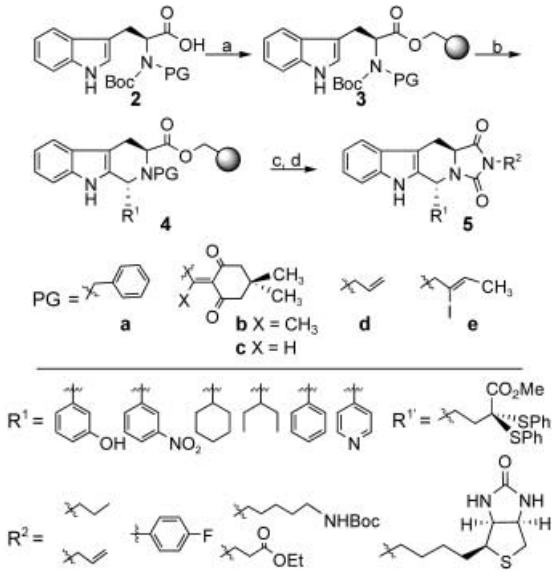
HR22C16 is a new and potent inhibitor of a key enzyme involved in cell division. To understand and characterize the structural basis for the activity of HR22C16, we sought to develop an efficient solid-phase synthesis of HR22C16 analogues with defined stereochemistry. We envisaged that the HR22C16 scaffold could be generated by an initial Pictet–Spengler cyclization of tryptophan and an aldehyde, followed by treatment with an isocyanate to form the terminal hydantoin ring (Scheme 1). We recognized that the diastereoselectivity of the Pictet–Spengler cyclization would have to be examined carefully as a diastereoselective version of this reaction had not yet been reported on a solid support.[6] However, if successful, this transformation would not only provide access to HR22C16 analogues, but would also be useful for the synthesis of natural products in the sarpagine and ajmaline class.[7a]

An additional key consideration was the cleavage of the products from the solid supports. We envisaged that the compounds could be attached to the solid support by the tryptophan carboxy group. After Pictet–Spengler cyclization, treatment with isocyanates would lead to formation of the hydantoin ring and concurrent cleavage of the ester bond, thus resulting in traceless release from the solid support.

Next, we required a protecting group for N_b that could be removed under homogeneous reaction conditions, as required for solid-phase synthesis, and that would allow diastereoselective Pictet–Spengler cyclization. In solution N_b-substituted tryptophan esters undergo the Pictet–Spengler cyclization to yield the 1,3-*trans* tetrahydro-β-carboline diastereoisomers preferentially.[7] We evaluated a number of protecting groups for N_b, including benzyl (**2a**), 4,4-dimethyl-2,6-dioxocyclohexylidene ethyl (**2b**), and 4,4-dimethyl-2,6-dioxocyclohexylidene methyl (**2c**; Scheme 2), but these protecting groups were either difficult to cleave under homogeneous conditions or did not afford any diastereoselectivity in the Pictet–



Scheme 1. Design of the traceless, diastereoselective solid-phase synthesis of diverse HR22C16 analogues. PG is a protecting group that induces transfer of asymmetry.



Scheme 2. Diastereoselective traceless solid-phase synthesis of HR22C16 analogues. a) NovaSyn TG-S-OH (0.25 equiv; for aldehyde $R^{1\prime}$: Wang resin), 1,1′-carbonyldiimidazole (1 equiv), CH_2Cl_2, room temperature, 15 h; b) R^1CHO (20 equiv), trifluoroacetic acid (5% in CH_2Cl_2), 50°C, 24 h; c) $[Pd(PPh_3)_4]$ (0.4 equiv), *N*,*N*′-dimethylbarbituric acid (6 equiv), CH_2Cl_2, 50°C, 6 h; d) R^2NCO (10 equiv), THF, 55°C, 36 h.

Spengler cyclization. However, when the two readily cleavable groups N_b-allyl (**2d**) and N_b-(*Z*)-2-iodo-2-butenyl (**2e**) were used, the solid-phase Pictet–Spengler reaction with 3-hydroxybenzaldehyde afforded the products in good yields (>90%) and the *trans* isomer was favored over the *cis* isomer by 9:1.[5a] Notably, the solid-phase diastereoselective Pictet–Spengler cyclization of **3e** with 5-oxo-2,2-bis(phenylthio)pentanoic acid methyl ester ($R^{1\prime}CHO$)[8] yielded a key intermediate in the synthesis of the naturally occurring indole alkaloids corynantheidol and geissoschizine.[9] We are currently pursuing the diversity-oriented synthesis of analogues of these bioactive small molecules.

We carried out the diversity-oriented synthesis of HR22C16 analogues by first coupling N_b-Boc-N_b-allyl-L-tryptophan (**2d**) to hydroxy-TentaGel resin with 1,1′-carbonyldiimidazole (Scheme 2). The resin-bound amino acid **3d** was subjected to Pictet–Spengler cyclization conditions in the presence of various aldehydes, including heterocyclic, substituted aromatic, and aliphatic aldehydes.[5a,c] A key advantage of solid-phase chemistry is that large excesses of soluble reactants can normally be used to drive intermolecular reactions to completion. However, this feature does not apply to intramolecular cyclizations. We therefore devised a strategy to remove any uncyclized material that remained on the solid support. Toward this end, the resins were treated with propyl isocyanate after the Pictet–Spengler cyclization. Uncyclized starting material (typically <5%) was cleaved as the hydantoin to leave behind the desired N_b-allyl-1,2,3,4-tetrahydro-β-carboline. After Pd-mediated deprotection of the N_b-allyl functionality, the resin was treated with diverse isocyanates (R^2NCO). The hydantoin-forming reaction provided traceless cleavage of the HR22C16 analogues **5** from the solid support.[5a] Excess isocyanate was then removed by incubation with an amine scavenger resin. The average overall yield of HR22C16 analogues was 46%, and the products were obtained in >90% purity as determined by HPLC and ^1H NMR spectroscopic analysis.

Although our strategy can readily yield large libraries of HR22C16 analogues, we initiated our studies by synthesizing a small library of 50 compounds. Cell-based assays revealed that minor changes in the R^1 substitution on HR22C16 resulted in a marked decrease in potency.[5a] However, several analogues with variations in R^2 were active. These compounds were tested in in vitro assays with recombinant Eg5. Compound **6** (Figure 2) inhibited Eg5 motility with an IC_{50} value of 90 ± 40 nM, thus making it the most potent inhibitor currently available for this key cell-division protein.

The physical separation of genetic cargo during cell division (i.e. anaphase) typically takes 10–15 minutes in vertebrate cells. However, it has been reported that antimitotic agents, including the Eg5-inhibitor monastrol, take 10–15 minutes to achieve inhibitory concentrations inside cells.[10] Thus, greater temporal control in perturbing this process is critical. Light can be controlled with outstanding temporal and spatial precision to localize photochemistry noninvasively in a biological context. Photolabile protecting groups have been widely used to "cage" bioactive compounds for regulated physiological release.[11] When a caged compound is

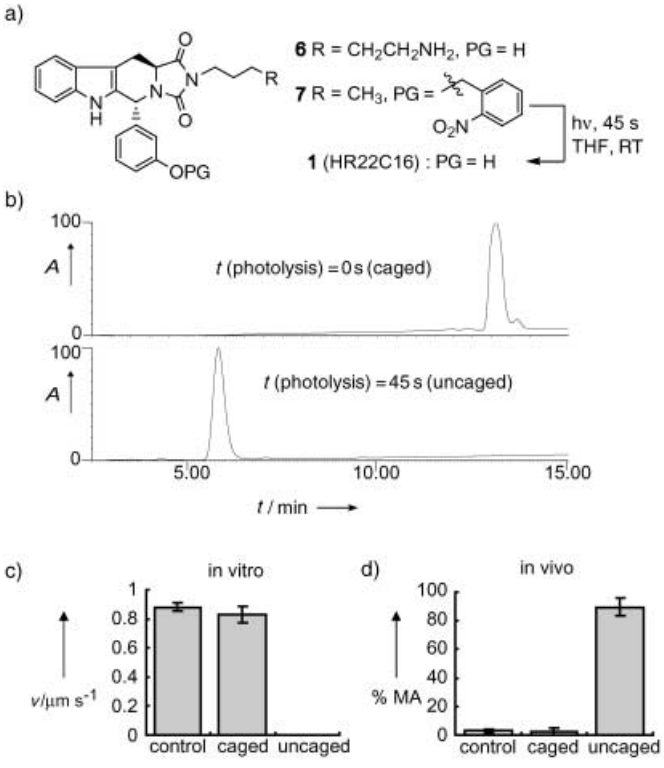


Figure 2. A strategy for the rapid photolytic release of "caged" HR22C16. a) Structures of HR22C16 analogues. b) HPLC analysis of photolysis. A 10 mM solution of **7** was completely uncaged upon exposure for 45 s to a He–Cd laser. Caged HR22C16 **7** does not c) inhibit Eg5 in in vitro motor assays (2.5 μM) or d) arrest cell division in human tumor cells (HeLa; 5.0 μM; control = dimethyl sulfoxide alone; %MA = percent monoastral spindles in mitotic cells). Uncaged HR22C16 blocks Eg5 function in both assays.

used, cells are incubated to a point of equilibration with a caged inhibitor that does not block the function of its target. Then at the desired stage photolysis releases the active inhibitor to perturb the function of the target.

We envisaged that photocaging of HR22C16 would allow Eg5 to be inhibited with increased temporal precision. Based on our studies with HR22C16 analogues, we predicted that substitution at the phenolic moiety of HR22C16 would result in inactivation. Thus, we synthesized[5a] the *ortho*-nitrobenzyl ether **7** (Figure 2). Indeed, this "caged" HR22C16 analogue does not inhibit Eg5 in vitro or block cell division in a human tumor cell line. Compound **7** can be completely "uncaged" through the use of a He–Cd laser in under 45 seconds, with the release of active HR22C16. We anticipate that this strategy, combined with the use of more physiologically benign photolabile protecting groups such as brominated 7-hydroxycoumarin-4-ylmethyl, will provide the degree of temporal control needed to examine the complex dynamics of cell division. Furthermore, localized uncaging of HR22C16 analogues may allow spatial control over the inhibition of cell division, whereby a subset of dividing cells in an organelle (or tumor) in a living organism may be targeted.

In summary, we report the discovery of a novel antimitotic, HR22C16, identified by a forward-chemical-genetic screen. Our efficient solid-phase traceless synthesis of HR22C16 analogues has provided access to new tools for studying Eg5, including one molecule that is about 155-fold more potent than other available Eg5 inhibitors. Current efforts involve the use of "caged" HR22C16 to examine Eg5 function at high temporal resolution during cell division. The results of these studies will be reported in due course.

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