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A small molecule inhibitor selective for a variant ATP-binding site of the chaperonin GroEL

Eli Chapman [a,*], George W. Farr [b], Krystyna Furtak [b], Arthur L. Horwich [a,b,c]

[a] The Scripps Research Institute, Molecular Biology, 10550 North Torrey Pines Road, Mb46, La Jolla, CA 92037, USA
[b] Department of Genetics, Yale University School of Medicine, New Haven, CT 06510, USA
[c] Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06510, USA

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ABSTRACT

The chaperonin GroEL is a megadalton-sized molecular machine that plays an essential role in the bacterial cell assisting protein folding to the native state through actions requiring ATP binding and hydrolysis. A combination of medicinal chemistry and genetics has been employed to generate an orthogonal pair, a small molecule that selectively inhibits ATPase activity of a GroEL ATP-binding pocket variant. An initial screen of kinase-directed inhibitors identified an active pyrazolo-pyrimidine scaffold that was iteratively modified and screened against a collective of GroEL nucleotide pocket variants to identify a cyclopentyl carboxamide derivative, EC3016, that specifically inhibits ATPase activity and protein folding by the GroEL mutant, I493C, involving a side chain positioned near the base of ATP. This orthogonal pair will enable in vitro studies of the action of ATP in triggering activation of GroEL-mediated protein folding and might enable further studies of GroEL action in vivo. The approach originated for studying kinases by Shokat and his colleagues may thus also be used to study large macromolecular machines.

Chaperonins are megadalton-sized double ring protein complexes, found in a variety of cellular compartments, that provide essential kinetic assistance to protein folding through the consumption of ATP. The bacterial chaperonin, GroEL, provides such assistance through a reaction cycle involving two major states. In one state, an open ring binds non-native polypeptide in its central cavity via contacts with its hydrophobic wall, forestalling misfolding and aggregation. In the second state, after GroEL cooperatively binds ATP in the 7 subunits of the ring, the cochaperonin 'lid' protein GroES is recruited, and the non-native polypeptide is released into the encapsulated cavity, the 'Anfinsen cage', where it attempts to properly fold in an isolated, now hydrophilic environment where aggregation cannot occur. After what is the longest step of the reaction cycle (~10 s), ATP hydrolyzes in the GroES-bound ring, gating ATP binding in the (unoccupied) opposite ring, the latter step allosterically ejecting GroES and polypeptide into the bulk solution. ATP binding and hydrolysis thus play a crucial role in driving the GroEL/GroES reaction cycle.[1] While a mutant, D398K, that blocks ATP hydrolysis has been identified, arresting the machine in a folding-active state,[2] to date there have been no mutants isolated that block the step of ATP binding. Such inhibition would allow analyses of the step of folding activation, for example addressing how many subunits need to bind ATP in order to bind GroES or trigger folding of polypeptide. Inhibition of ATP binding would seem to be readily accomplished by small molecule occupation of the ATP-binding site. To this end, the chemical biological tools developed by Shokat and coworkers[3] were adapted to generate a small molecule inhibitor tuned specifically to a GroEL variant with an amino acid substitution in the ATP-binding pocket.

First, residues in the ATP-binding pocket were selected for substitution, employing the X-ray crystallographic model of an asymmetric GroEL/GroES/ADP·AlF_3 complex (PDB 1svt).[4] Eleven residues were selected, each was altered to cysteine, and several to glycine and alanine as well. Mutation of bulky residues, for example, I493, N479, or M488, was particularly attractive, because it would create a 'hole' in the binding pocket that could specifically accommodate a compound that would not be recognized by the wild-type binding site.[5] Substitution with cysteine comprised an attempt to generate a covalent link with an electrophile-bearing small molecule. Function of the variants was tested in vivo by transforming a GroEL-deficient strain of *Escherichia coli* with the respective mutant *groE* operons and inspecting for rescue.[6] With the exception of double mutants and the I493G variant, all of the substitutions were tolerated (see Supplemental Table 1). The rescuing variants were then over-produced and purified for biochemical study.

An initial collection of ~120 molecules,[7] based on histidine and pyrazolo-pyrimidine scaffolds, was screened for inhibition of wild-type and variant GroEL ATPase activity, as measured using a malachite green assay in 96-well plate format. From this, a number of inhibitors obtained, all from the pyrazolo-pyrimidine scaffold.

* Corresponding author. Tel.: +1 858 784 7386; fax: +1 858 784 7388.
E-mail address: echapman@scripps.edu (E. Chapman).

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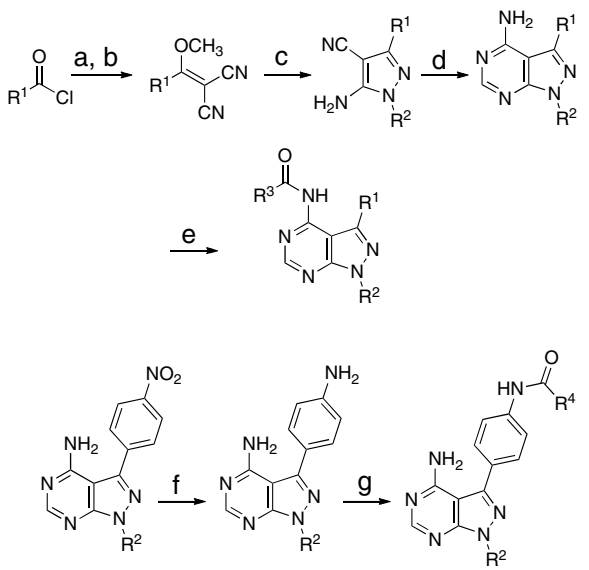
Because only modest variant selectivity and inhibition were observed, a small collection of derivatives of this scaffold was synthesized in an effort to increase selectivity and potency (Scheme 1).[8]

Briefly, acid chlorides bearing R^1 were added to the anion of malononitrile and after extractive workup, the resulting product was refluxed with dimethylsulfate (DMS) in 9:1 dioxane:H_2O to yield the vinyl ether derivatives after chromatographic purification. These two steps could also be combined into one step by adding DMS directly to the first reaction and heating to reflux followed by extractive workup and chromatographic purification. Formation of the pyrazolol ring was carried out by refluxing the vinyl ethers with a hydrazine bearing R^2 in ethanol followed by workup and chromatography. Finally heating to 180 °C in formamide yielded the pyrazolol-pyrimidine. Position R^3 was introduced by reaction with the corresponding acid chloride in pyridine. In the cases in which R^1 was a nitro containing aryl group, the molecule could be further elaborated by catalytic hydrogenation of the nitro group to yield the aniline followed by filtration and condensation with an acid chloride containing R^4 using three equivalents of pyridine in chloroform.

Positions R^1–R^4 (Fig. 1a) were varied giving a total of ~100 compounds to be tested against chaperonin ATPase activities. It was discovered that substitutions at position R^3 were not tolerated. Position R^2 tolerated a methyl, ethyl, isopropyl or *tert*-butyl group, but no aryl substitutions were accepted and the *tert*-butyl group showed the best activity. At position R^1, only aryl substitutions were tolerated. While screening compounds bearing electrophiles at positions R^1 and R^4, it was noted that despite only modest covalent bond formation with one of the mutants (G32C, data not shown), the incorporation of an acrylamide group or a chloroacetamide at R^4 yielded increased potency versus the unsubstituted amine. This guided the synthesis of a small collective of aryl and alkyl substitutions at the R^4 position, with larger alkyl substituents showing greater potency up to a cyclohexyl group which showed diminished efficacy.

The ATPase activity of wtGroEL and each of the mutants was measured in the presence and absence of each of the pyrazolo-pyrimidine derivatives in a single point, 96-well plate format.[9]



Scheme 1. General synthesis of pyrazolo-pyrimidines used in the present study. (a) NaH, malononitrile, THF, 0–23 °C; (b) $(CH_3)_2SO_4$, $NaHCO_3$, dioxane/H_2O, reflux; (c) R^2NHNH_2, EtOH, reflux; (d) formamide, 180 °C; (e) R^3COCl, pyridine, 23 °C; (f) H_2, Pd/C, EtOH, 23 °C; (g) R^4COCl, pyridine, $CHCl_3$, 23 °C.

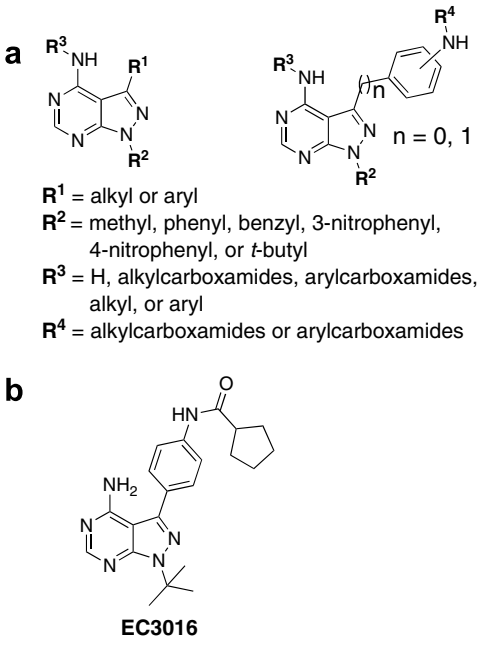


Figure 1. The variable positions used to generate the panel of small molecules used in the present study is indicated in (a). A small molecule with excellent selectivity for the I493C GroEL variant is shown in (b).

Inhibition of activity was seen for several of the mutant/molecule pairs, but the most sensitive discovered was I493C, in combination with EC3016 (Fig. 1b). This pair was further assessed in a collective of standard GroEL assays, ATP hydrolysis (Fig. 2a) and the refolding of two GroEL substrate proteins, bovine rhodanese and pig heart malate dehydrogenase (MDH; Fig. 2b).

The combined chemical and genetic approach taken here to generating a small molecule ligand that targets a specific genetically modified protein to effect selective activation or inhibition of activity has been employed in a number of previous studies. An early example was the use of FK1012 and FKBP to generate chemical control of protein dimerization.[10] The current work directly borrows from an approach originally taken with kinases, where uniquely for a selected kinase, a specific ATP analogue was identified that could fit into the genetically engineered ATP pocket of the kinase to enable labeling of its specific substrates.[11] Subsequently, inhibition of individual kinases was programmed using similar approaches with genetically engineered kinase ATP pockets and small molecule pyrazolol-pyrimidine inhibitors like those employed here.[12] Such combined chemical genetic approaches have been adapted to the molecular motors, kinesin[13] and myosin,[14] employing modified ATP pockets and N^6-substituted ATP or ADP analogues, enabling action of individual isoforms of these machines to be recognized.[15] The present study of the GroEL chaperonin takes the same general approach as these earlier studies, that is, modifying the nucleotide pocket at the position adjacent to the N^6 of ATP and using as a ligand a small molecule pyrazolol-pyrimidine compound related to those employed by Shokat and coworkers,[12] to inhibit ATP binding by the GroEL machine. Here, instead of a single or, as in the case of some of the motors, two targeted sites, there are seven sites within a ring. Beyond this initial study, the use of varying numbers of targetable variant sites versus non-recognized wild-type sites, by virtue of forming rings with mixed subunit composition, has already enabled investigation of the ATP requirements for activating the machine at the level of a ring.[16] The experiments presented here and in the recent mixed ring study have been carried out in vitro, but they establish the feasibility of using the combination of a small molecule ligand

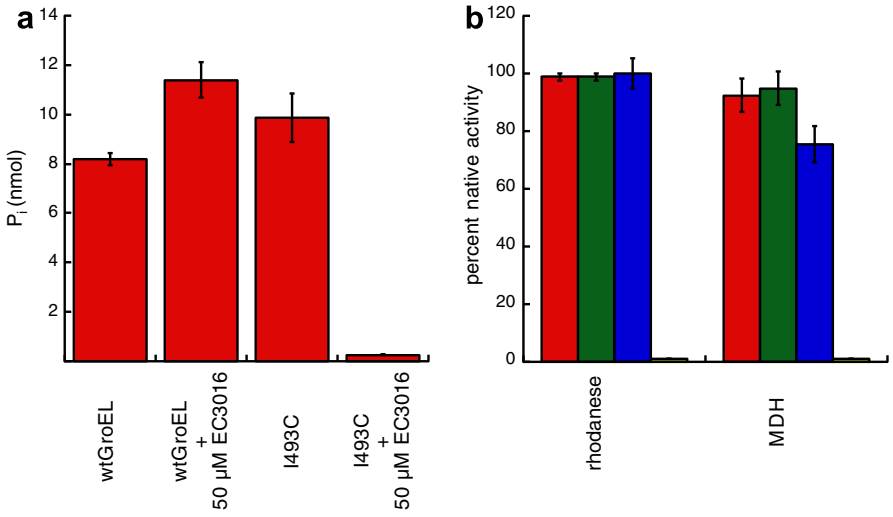


Figure 2. Biochemical properties of wtGroEL and I493C measured in the presence or absence of EC3016. (a) The amount of inorganic phosphate released 10 min after addition of ATP as measured by malachite green complex formation. (b) The maximum amount of rhodanese, MDH, or DHFR activity recovered. Red bars are wtGroEL, green bars are wt GroEL plus 50 μM EC3016, blue bars are I493C, and yellow bars are I493C plus 50 μM EC3016. For both panels, each point was repeated in triplicate and averaged and the error bars indicate one standard deviation from the mean.

and a cell containing homooligomeric variant GroEL to address the action of GroEL in vivo. For example, addition of a cell permeable small molecule ligand that rapidly and specifically inactivates variant GroEL by binding to its modified nucleotide pocket, blocking ATP access during cell growth, should potentially be able to address which proteins are the immediate substrates of the chaperonin by their selective misfolding and aggregation. General physiological defects of GroEL-deficient cells may also be further defined by such an experiment.

In conclusion, a small molecule inhibitor that acts selectively on a genetically modified GroEL ATP-binding pocket has been generated through use of a combination of synthetic chemistry and genetics.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.12.015.

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