NECA derivatives exploit the paralog-specific properties of the site 3 side pocket of Grp94, the endoplasmic reticulum Hsp90

The hsp90 chaperones govern the function of essential client proteins critical for normal cell function as well as cancer initiation and progression. Hsp90 activity is driven by ATP, which binds to the N-terminal domain and induces large conformational changes that are required for client maturation. Inhibitors targeting the ATP-binding pocket of the N-terminal domain have anticancer effects, but most bind with similar affinity to cytosolic Hsp90α and Hsp90β, endoplasmic reticulum Grp94, and mitochondrial Trap1, the four cellular hsp90 paralogs. Paralog-specific inhibitors may lead to drugs with fewer side effects. The ATP-binding pockets of the four paralogs are flanked by three side pockets, termed sites 1, 2, and 3, which differ between the paralogs in their accessibility to inhibitors. Previous insights into the principles governing access to sites 1 and 2 have resulted in development of paralog-selective inhibitors targeting these sites, but the rules for selective targeting of site 3 are less clear. Earlier studies identified 5′-N-ethylcarboxamido adenosine (NECA) as a Grp94-selective ligand. Here we use NECA and its derivatives to probe the properties of site 3. We found that derivatives that lengthen the 5′ moiety of NECA improve selectivity for Grp94 over Hsp90α. Crystal structures reveal that the derivatives extend further into site 3 of Grp94 compared with their parent compound and that selectivity is due to paralog-specific differences in ligand pose and ligand-induced conformational strain in the protein. These studies provide a structural basis for Grp94-selective inhibition using site 3.

Hsp90 chaperones are required for the conformational maturation and late-stage activation of hundreds of client proteins, many of which are essential for cell viability (1–3). Grp94, the endoplasmic reticulum Hsp90 paralog, is required for localization of cell surface receptors, including Toll-like receptors, Lrp6, Her2, and integrins (4–7), as well as secreted proteins, such as insulin-like growth factors (8). Overexpression of Grp94 occurs in various cancers, including multiple myeloma and Her2+ breast cancer, and correlates with a poor prognosis (9, 10). Recent studies have shown that blocking Grp94 activity in these cancer cells leads to client degradation and reduces cancer cell viability (4, 11).

Over a dozen Hsp90 inhibitors that target the ATP-binding pocket in the N-terminal domain (NTD) have been tested in clinical trials, but a lack of biomarkers for patient selection, compound- or target-specific toxicities, and, under some conditions, up-regulation of compensatory chaperone systems have hindered their progress to approval (12–17). In addition to Grp94, humans possess two Hsp90 paralogs in the cytosol (Hsp90α and Hsp90β) and one in the mitochondria (Trap1). Because Hsp90 paralogs have distinct sets of client proteins with widely diverse cellular functions, selective inhibition (targeting just one of the four paralogs) may mitigate the limitations of some of the pan-hsp90 inhibitors and improve drug efficacy in cancer treatment (18).

The residues that comprise the ATP-binding pocket of the NTDs are almost universally conserved, making hsp90 paralog selective drug discovery a challenge. Three distinct side pockets, however, adjoin the central ATP-binding cavity of hsp90s (Fig. 1), and paralog selectivity can be achieved by accessing these side pockets in one paralog but not another. Although the residues that line the side pockets are also conserved between paralogs, selective access is governed by intrinsic conformational differences between the NTDs of each paralog as well as by ligand-driven rearrangements (4, 19–23). In one of the best-studied examples, two of these side pockets, site 1 and site 2, have been shown to distinguish between cytosolic Hsp90 and Grp94. Crystal structures of each paralog bound to PU-H54, a purine-based (PU) Grp94-selective inhibitor, revealed that the flexibility of the Grp94 lid (helices 4 and 5), compared with its Hsp90 counterpart, permits conditional access to site 2 upon inhibitor binding. In Grp94:PU-H54, rearrangement of the lid causes Phe-199 to swing toward site 1, exposing the hydrophobic cleft of site 2. This movement allows the nonpolar 8-aryl derivatives to bind to site 1 and site 2.
moiety of PU-H54 to enter site 2 in an energetically favorable conformation. On the other hand, the structure of Hsp90:PU-H54 showed that the lid is unaffected by inhibitor binding and that Hsp90 Phe-138, the equivalent of Grp94 Phe-199, blocks site 2, forcing the 8-aryl moiety of PU-H54 to remain in the energetically less favorable site 1.

While differential access to site 1 and site 2 offers selectivity within the PU inhibitor scaffold, less is known about how other regions of the ATP-binding pocket could be exploited for paralog selectivity. 5′N-ethylcarboxamido adenosine (NECA) is an ATP mimic that selectively binds Grp94 in pulldown experiments (24, 25) and shows higher affinity for Grp94 than for Hsp90 (26). The structure of Grp94:NECA revealed that the 5′N-ethylcarboxamido moiety occupies the side pocket called site 3, which sits adjacent to the termini of the helix 4/5 lid (23). Modeling the NECA binding pose into the structure of unliganded yeast Hsp90 showed that access to site 3 is disfavored by a predicted clash between the terminal methyl group of the 5′ moiety and the backbone carbonyl oxygen of Hsp90 Gly-121. In Grp94:NECA, the equivalent Gly-196 is displaced by 3.6 Å backbone carbonyl oxygen of Hsp90 Gly-121. In Grp94:NECA revealed that the 5′N-ethylcarboxamido moiety is formed by Met-85, Asn-162, Leu-163, Thr-165, Ala-167, Thr-171, Gly-196, Val-197, Phe-199, and Tyr-200, sits adjacent to the central adenine-binding cavity of the N-terminal domain. We designed 3 ligands, N-propylcarboxamido adenosine (NPCA), N-hydroxyethylcarboxamido adenosine (NEoCA), and N-aminoethylcarboxamido adenosine (NEaCA), which lengthen the 5′ moiety of NECA by an additional methyl, hydroxyl, or amino group, respectively (Fig. 2). We assessed the binding properties of these ligands to Grp94 and Hsp90 using isothermal titration calorimetry (ITC). As seen in Fig. 3 and Table 1, NECA, NPCA, and NEoCA bind to Grp94 with 5-fold greater affinity to Grp94 than to Hsp90, whereas NPCA, NEoCA, and NEaCA exhibit 9.1-, 7.4-, and 5.4-fold higher affinity for Grp94 than for Hsp90. In this series, the order of -fold selectivity of compounds for Grp94 over Hsp90 is NPCA > NEoCA > NEaCA ~ NECA.

NECA and its 5′-modified derivatives bind selectively to Grp94 over Hsp90

Analysis of the structure of Grp94 in complex with NECA (23) showed that the 5′N-ethylcarboxamido moiety does not completely fill site 3 of the ATP-binding pocket. Site 3, which is formed by Met-85, Asn-162, Leu-163, Thr-165, Ala-167, Thr-171, Gly-196, Val-197, Phe-199, and Tyr-200, sits adjacent to the central adenine-binding cavity of the N-terminal domain. We designed 3 ligands, N-propylcarboxamido adenosine (NPCA), N-hydroxyethylcarboxamido adenosine (NEoCA), and N-aminoethylcarboxamido adenosine (NEaCA), which lengthen the 5′ moiety of NECA by an additional methyl, hydroxyl, or amino group, respectively (Fig. 2). We assessed the binding properties of these ligands to Grp94 and Hsp90 using isothermal titration calorimetry (ITC). As seen in Fig. 3 and Table 1, NECA, NPCA, and NEoCA bind to Grp94 with $K_d$ values of 2.8 μM, 6.3 μM, and 9.1 μM, respectively. The measured affinity of NECA was considerably worse, with a $K_d$ value of 130 μM.

NECA and its derivatives exhibit preferential binding to Grp94 over Hsp90. As seen in Fig. 3 and Table 1, NECA, NPCA, NEoCA, and NEaCA bind to human Hsp90α with $K_d$ values of 14, 58, 68, and 708 μM, respectively. When comparing the ratio of $K_d$ values for Grp94 and Hsp90, it is apparent that NECA binds with 5-fold greater affinity to Grp94 than to Hsp90, whereas NPCA, NEoCA, and NEaCA exhibit 9.1-, 7.4-, and 5.4-fold higher affinity for Grp94 than for Hsp90. In this series, the order of -fold selectivity of compounds for Grp94 over Hsp90 is NPCA > NEoCA > NEaCA ~ NECA.

We compared the $K_d$ values determined by ITC with $K_i$ values calculated from a fluorescence polarization (FP) competition displacement assay using geldanamycin-Cy3b as the tracer (27, 28). As seen in Fig. 4 and Table 2, the $K_i$ values for NECA, NPCA, NEoCA, and NEaCA, calculated from the $IC_{50}$ measurements according to the method of Nikolovska-Coleska et al. (29) yielded values of 1.0, 2.0, 3.9, and >20 μM for Grp94 and 7.9, 32.8, >40, and >40 μM for Hsp90. The $K_i$
Characterizing a Grp94-selective side pocket

Table 1
Thermodynamic parameters of ligand binding

<table>
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<tr>
<th>Ligand</th>
<th>Protein</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G$ (kcal/mol)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$T\Delta S$ (kcal/mol)</th>
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<tr>
<td>NECA</td>
<td>Grp94N</td>
<td>2.8 ± 0.1</td>
<td>-7.545 ± 0.030</td>
<td>-18.8 ± 0.6</td>
<td>-11.26 ± 0.54</td>
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<tr>
<td>NPCA</td>
<td>Grp94N</td>
<td>14.0 ± 2.8</td>
<td>-6.601 ± 0.120</td>
<td>-11.4 ± 0.0</td>
<td>-4.78 ± 0.15</td>
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<tr>
<td>NEoCA</td>
<td>Grp94N</td>
<td>6.3 ± 0.1</td>
<td>-7.071 ± 0.006</td>
<td>-16.3 ± 0.6</td>
<td>-9.23 ± 0.57</td>
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<tr>
<td>NEaCA</td>
<td>Grp94N</td>
<td>57.5 ± 2.1</td>
<td>-5.762 ± 0.022</td>
<td>-8.3 ± 0.0</td>
<td>-2.54 ± 0.02</td>
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<tr>
<td>NPCA</td>
<td>Hsp90N</td>
<td>9.1 ± 0.3</td>
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<td>-13.25 ± 0.30</td>
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<td>NEoCA</td>
<td>Hsp90N</td>
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<td>Hsp90N</td>
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<td>-8.43 ± 3.08</td>
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Structures of Grp94 and Hsp90 bound to NECA derivatives

To understand the structural basis of the observed differences in affinity between NECA and its derivatives for Grp94 and Hsp90, we determined the crystal structures of the N-terminal domains of Grp94 and Hsp90 in complex with these compounds. Structures of the Grp94 N-terminal domain with (Grp94N) and without (Grp94NΔ41) the charged linker in complex with NECA have been reported previously (PDB codes 1U2O, 1YSZ, and 6D28) (23, 30). Crystals of ligand complexes containing Grp94NΔ41 were determined for NPCA, NEoCA, and NEaCA (PDB codes 2GQP, 2HG1, and 2HCH). Data collection and refinement statistics are presented in Table 3.

The Grp94NΔ41:ligand complexes crystallize in a different space group than the Grp94N:ligand complexes, and in the Grp94NΔ41:ligand complexes, the residues corresponding to...
We also determined the structures of human Hsp90α in complex with NECA, NPCA, NeoCA, and NeCA. Crystals of apoHsp90N were soaked with the ligand prior to freezing and data collection. Crystals of Hsp90:NECA and Hsp90:NPCA diffracted to a resolution of 1.6 Å and 1.65 Å, respectively, whereas crystals of Hsp90:NeoCA and Hsp90:NeCA each diffracted to a resolution of 1.5 Å. All structures were solved by molecular replacement. Data collection and refinement statistics are presented in Table 3. The position of the bound ligands is supported by strong electron density (Fig. S1A).

In the Hsp90:NECA complex, the NECA ligand occupies the ATP-binding pocket. Comparison of the structures of Hsp90:NECA and Grp94:NECA shows that the adenine and ribose moieties of NECA make similar interactions within the ATP-binding pocket but differ significantly in the poses of the 5′ moieties of the bound NECA. As seen in Fig. 6A, compared with Grp94-bound NECA (NECAGrp), the dihedral angle of the amido-carbon bond of NECAHsp undergoes a 190° rotation, resulting in the ε methyl group pointing toward the back face of Hsp90 site 3. In contrast, the pose of NECAGrp places the ε methyl closer to the front face of site 3. The entrance to site 3 in Hsp90 is constricted compared with the entrance to site 3 in Grp94. This constriction is due to differences in the position of the carbonyl oxygen of Gly-135 compared with the equivalent Gly-196 in Grp94. As described previously for yeast Hsp90 (23), modeling the NECAGrp ligand into the structure of human Hsp90:NECA shows that the NECAGrp would clash with the carbonyl oxygen of Gly-135 (Fig. 6B). The rearranged pose of NECAHsp thus reflects the need to avoid steric clashes with Gly-135 in the Hsp90 pocket.

NPCA binds to Hsp90 with a 3.5-fold lower affinity than NECA. Examination of the structure of the Hsp90:NPCA complex shows that, although the adenine and ribose again occupy their expected positions, the 5′ moiety fits deeper into site 3 of Hsp90 compared with NECA and packs closely against the side chain of Tyr-139 (Fig. 6C). In the high-resolution structure of Hsp90:NPCA, the electron density for Tyr-139 is consistent with two side-chain rotamers. In the more favored “distal” rotamer, the side chain of Tyr-139 is 3.5 Å away from the episol methyl of NPCA. In the “proximal” rotamer, on the other hand, the side chain of Tyr-139 is rotated 19.6° away from the distal position. This decreases the distance from the ε methyl of NPCA to Tyr-139 to 2.4 Å, a distance that is too close to be energetically favorable (Fig. 6C). The distal rotamer conformation appears to be a consequence of the longer 5′ moiety of NPCA because comparison of the high-resolution Hsp90:NECA and Hsp90:NPCA structures shows that, in Hsp90:NECA, Tyr-139 occupies only the “normal” proximal rotamer conformation.

The distal rotamer conformation of Tyr-139 enlarges site 3 in Hsp90:NPCA and prevents a van der Waals clash with the longer 5′ moiety of NPCA. The distal rotamer of Tyr-139 seen in Hsp90:NPCA, however, pushes the side chain of the residue deeper into the hydrophobic core of Hsp90. Although the energetic consequences of this movement are unknown, an analysis of the 194 human Hsp90α N-terminal domain structures in the PDB shows that only 10 other structures exhibit this Tyr-139 conformation (Fig. S2). This set includes eight related com-
Characterizing a Grp94-selective side pocket

Table 2
Comparison of FP IC₅₀, FP Kᵢ, ITC Kᵢₙ, and ATPase IC₅₀ values

Selectivity = Hsp90/Grp94. ND, not determined.

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<th>Ligand</th>
<th>Protein</th>
<th>FP IC₅₀</th>
<th>FP Kᵢ</th>
<th>ITC Kᵢₙ</th>
<th>ATPase IC₅₀</th>
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<td>NECA</td>
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<td>Grp94</td>
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<td>&gt;20</td>
<td>130 ± 11</td>
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Table 3
Data collection and refinement statistics

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Refinement

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<td>Completeness (%)</td>
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Data of Hsp90 bound to tricyclic imidazo pyridines (31) and two structures of complexes with benzimidazol-substituted pyrrolo pyridine carboxamides. These ligands insert into site 1 of the Hsp90-binding pocket and make direct hydrogen bond interactions between the Tyr-139 OH and nitrogen substituents of the ligand. In contrast, in Hsp90:NPCA, Tyr-139 makes no stabilizing ligand interactions and instead moves only to accommodate the impinging 5’ ligand moiety from site 3. Compared with Hsp90:NECA, this movement likely accounts for the energetic penalty incurred when Hsp90 binds NPCA.

Compared with Hsp90:NPCA, the structures of Hsp90:NEoCA and Hsp90:NεaCA reveal altered positioning of the terminal hydroxyl and amine groups. In Hsp90:NEoCA, the ε OH of NEoCA points away from the back wall of site 3 and...
makes hydrogen bond interactions with the carbonyl oxygen of Gly-135 and with a water molecule (Fig. 6D). NEaCA, on the other hand, contains a charged terminal amino group that adopts two conformations of equal occupancy. In both cases, the ε amino group makes extensive hydrogen-bonding interactions with the carbonyl oxygens of Asn-106, Thr-109, and either the carbonyl oxygen of Ile-110 or the OH of Tyr-139, depending on the conformation of the terminal amino group. The $K_d$ of NEaCA binding to both Grp94 and Hsp90 is substantially worse than for NECA, NPCA, or NEoCA. This cannot be ascribed to unfavorable steric clashes because the poses of NEoCA and NEaCA are essentially identical when bound to Grp94, and the conformation of both Grp94 and Hsp90 is unaltered in the protein:NEaCA complexes. Rather, the reduced binding affinity likely reflects the energetic costs of burying a fully charged amino group deep in a protein cavity, perhaps displacing stabilizing water molecules in the process.

**Discussion**

Of the three side pockets identified near the ATP-binding cavity of hsp90 chaperones, sites 1 and 2 have been exploited previously for the design of paralog-selective inhibitors (4, 19–22, 32). Site 3, in contrast, has been relatively neglected, in part because site 3 is further away from the central ATP-binding cavity, requiring longer linkers between the central scaffold and its attached substituents and making targeting more difficult. The structural differences between site 3 in Grp94 and Hsp90 are also subtle, and access to site 3 is not governed by structural rearrangements to the N-terminal domain as they are for sites 1 and 2.

Here we have shown that inhibitors based on the NECA scaffold all target site 3 and bind with a 5- to 9-fold preference for Grp94 over Hsp90. The origins of the preferential binding of these compounds to Grp94 over Hsp90. The origins of the preferential binding of these compounds to Grp94 appear to be due to the effect of Gly-135 on the trajectory of the NECA binding pocket. Initial modeling studies (23) indicated that NECA would not be able to access site 3 in yeast Hsp90 in the pose adopted when it bound to Grp94. The structural data presented here indicate that, although the adenine and ribose of NECA bind to the central pocket of Hsp90 in the same manner as Grp94, Hsp90-bound NECA redirects its carbamido moiety toward the rear of site 3 by altering the amido dihedral angle to avoid a clash with the carbonyl oxygen of Gly-135. Although the energetic strain imposed by the adoption of this alternate ligand binding pose correlates with the modestly lower binding affinity of NECA to Hsp90 compared with Grp94.
Characterizing a Grp94-selective side pocket

In an attempt to improve ligand affinity and selectivity for Grp94, we hypothesized that compounds that insert deeper into site 3 would incur a binding advantage over NECA because of increased interactions between the ligand and the pocket. However, the binding data reported here show that NPCA, NEoCA, and NEaCA all bind to Grp94 and Hsp90 with weaker affinity than the parent compound, NECA. In the case of Grp94, examination of the structures of the Grp94:NPCA, Grp94:NEoCA, and Grp94:NEaCA complexes shows that the 5’ moieties all adopt similar poses that direct the 5’ moiety further toward the rear of site 3. This results in a gauche conformation at the terminal end of the 5’ substituents, which incurs a modest energetic penalty compared with the anti pose. In the case of NPCA and NEoCA, the energetic penalty for this conformation compared with NECA reduces the binding affinity by only 2- to 3-fold. Incorporation of the amino substituent, however, leads to a nearly 50-fold decrease in binding affinity, indicating that burial of a charged group in such a constrained pocket is strongly disfavored.

A similar trend in the binding affinities for NPCA, NEoCA, and NEaCA is also observed for Hsp90. Incorporation of the additional methyl or hydroxyl group results in a 4- to 5-fold loss of binding affinity, whereas addition of the charged amino group lowers the affinity by 50-fold compared with the parent compound, NECA. Interestingly, the maximal selectivity between Grp94 and Hsp90 in this series is found with NPCA, which gains an additional factor of 2 in selectivity for Grp94 over Hsp90 compared with NECA. This can be rationalized by the structure of Hsp90:NPCA, which shows that the nonpolar ε methyl group is positioned facing the rear of the binding site, where it impinges on the position of Tyr-139, in turn displacing Tyr-139 deeper into the hydrophobic core of the Hsp90 N-terminal domain. The observed repositioning of Tyr-139 seen in Hsp90:NPCA is rare and has only been observed in Hsp90:ligand complexes where the ligand forms hydrogen-bonding interactions with the hydroxyl of the tyrosine side chain, which presumably stabilizes the altered rotamer. No such ligand-mediated stabilization is observed between NPCA and Tyr-139, leading to the conclusion that the distal rotamer is energetically disfavored.

Because of the large sample requirements and low-throughput nature of ITC, most ligand binding assays for Hsp90 chaperones have made use of a fluorescence polarization competition binding assay (27,28). We compared binding of NECA and its derivatives to Grp94 and Hsp90 using both ITC and FP and showed that the IC₅₀ measurements resulting from the FP assay underestimate the binding affinity for Hsp90, leading to overestimation of the selectivity of Grp94-selective inhibitors. The difference between the two assays can be traced to the different affinities for Hsp90 and Grp94 of the fluorescent tracer ligand. When IC₅₀ measurements are converted to Kᵣ values, the FP and ITC measurements are in good agreement. This suggests that future studies of paralog-selective ligands may benefit from comparing Kᵣ values instead of IC₅₀ values.

The binding data presented here agree with earlier studies showing that NECA binds selectively to Grp94 (24–26). A recent report from Liu and Street (33), however, suggests that NECA binds preferentially to Hsp90, not Grp94. The discrepancy between these conclusions likely reflects the assays used to measure selectivity. Liu and Street (33) monitored ligand selectivity by interference with ATP hydrolysis, which relies on competition between NECA and ATP for the ATP-binding site on the chaperone. It has been established previously that, although the affinity of ATP for Grp94 is on par with that reported for NECA here, the affinity of ATP for human Hsp90 is nearly two orders of magnitude weaker than for Grp94 (28, 34). Competition between ATP and NECA for Grp94 and Hsp90 that does not account for the difference between the ATP affinities of the two paralogs would significantly underestimate the intrinsic selectivity of NECA in favor of Hsp90.

Recently, several inhibitors have been reported that were designed to target site 3 in Grp94 (35, 36). These compounds, which contain bisphenyl (PDB Chemical Component (CC) ID VC1, VC5) or furan-imidazole (CC ID 6C0) substituents off the core resorcylic scaffold exhibit submicromolar affinity for Grp94 and have FP IC₅₀ selectivities over Hsp90 of up to 70-fold. The evidence that site 3 is targeted by the resorcylic substituents of these inhibitors comes from crystal structures (PDB codes 5IN9, 6AOL, and 6AOM) of complexes formed by soaking the compounds into preformed crystals of apoGrp94NΔ41. Although the structures are modeled with the furan imidazole and bisphenyl substituents inserted into site 3, the electron density for these pendant moieties is discontinuous and weak. This observation is inconsistent with the submicromolar IC₅₀ values reported for these compounds and prompts reconsideration of their possible mode of binding. Notably, all of the compounds designed to target site 3 bear a strong resemblance to the resorcylic benzyl imidazole compound BnIm (CC ID 9QY) (37). A recent structure of BnIm bound to Grp94NΔ41 that was generated by cocrystallization of the ligand with the protein (PDB code 5WMT) showed that the benzyl imidazole moiety, whose placement is supported by strong electron density, occupies site 1 in the ATP pocket, not site 3. In addition, in Grp94NΔ41:BnIm, the resorcylic scaffold is flipped about its pseudosymmetric resorcylic-imidazole axis to insert the methyl ester moiety into an exposed site 2 (22). Unlike site 3, sites 1 and 2 are inaccessible in unliganded Grp94, and the structure of Grp94NΔ41:BnIm showed that significant conformational changes to the protein were required to open sites 1 and 2 for ligand occupancy. These conformational changes are not possible in apo Grp94N or Grp94NΔ41 crystals without disrupting the crystalline lattice. Thus, soaking site 1 or site 2–binding ligands into preformed apoGrp94 crystals limits the occupancy possibilities for the resorcylic substituents to either site 3 or the open mouth of the ATP-binding cavity, which is what is observed in the soaks. Taken together, the similarities between BnIm and VC1, VC5, and 6C0 and the lack of strong crystallographic support for the modeled conformations suggest that the later compounds target sites 1 and 2 of Grp94 but not site 3. This, in turn, suggests that, to date, the only compounds that are bona fide site 3 binders are Radamide and the NECA series described here.

The rigidity of the NECA scaffold effectively directs the carboxamido substituent into site 3 of Grp94, and modest selectiv-
ity over Hsp90 suggests that site 3 has potential as a paralog-selective side pocket. However, functional and therapeutic exploitation of the NECA scaffold has been slowed by concerns about cross-reactivity with adenosine A2 receptors as well as by the modest affinity of NECA for Grp94. With regard to the later concern, NECA and the derivatives tested here exhibit high solubility in aqueous solutions (>200 mM). It is possible that next-generation NECA derivatives, designed with higher lipophilicity, might effectively drive the equilibrium in favor of Grp94 binding and improve the potency of these compounds.

**Experimental procedures**

**Protein expression and purification**

The N-terminal domain of Grp94 (Grp94Δ41) (residues 69–337; Δ41 refers to deletion of the charged linker, residues 287–327, which are replaced by four glycine residues) was overexpressed in *Escherichia coli* strain BL21 Star DE3 as GST fusions. Expression, purification, and removal of GST by thrombin proteolysis were done as described previously (23). Near full-length Grp94 (residues 73–754 Δ41) was expressed in *E. coli* and purified as described previously (38). The N-terminal domain of human Hsp90α (residues 1–236) was expressed as an N-terminal His tag fusion and purified as described previously (39).

**Reagents**

NECA was purchased from Sigma. NPCA, NEoCA, and NEαCA were provided by E. Toone (Duke University) and synthesized in a two-step process starting from 2',3',3'-isopropylidenadenosine and propylamine, isopropenyl chloroformate, or ethylenediamine (all from Aldrich) according to published protocols (40).

**Analytical HPLC-MS**

Mass spectra of NECA and its derivatives were obtained on an Advion Expression LCMS instrument with electrospray ionization. Analytical HPLC was performed on an Agilent Technologies 1260 Infinity Quaternary LC system. The purity and identity of each compound were verified by HPLC-MS using the following method: 12-min gradient of increasing concentrations of acetonitrile in water (5% → 100%) containing 0.1% formic acid with a flow rate of 0.4 ml/min and UV detection at λ = 218 and 260 nm on an Agilent Poroshell 120 EC-C18, 3.0 mm × 450 mm, 2.7-μm column. Compounds had a purity of 95% or more.

**Isothermal titration calorimetry**

Proteins were equilibrated into DTT-free assay buffer by repeated concentration and dilution using an ultrafiltration spin filter (Millipore). Concentrated ligand solutions in DMSO, typically 100 mM, were diluted into assay buffer. The standard ITC assay buffer contained 40 mM Hepes (pH 7.5), 100 mM NaCl, and 1% DMSO. Titrations were carried out at 25 °C using a VP-ITC calorimeter (Microcal, Inc.) with the ligand solution (500–1500 μM) loaded into the titration syringe and the protein (30–150 μM) into the cell. ITC titration against PU-H71, a well-characterized Hsp90 inhibitor, was used as a benchmark for both instrument and protein quality control.

ITC assays consisted of 29 injections of 10 μl (2 μl for the first injection) each, with 5-min intervals between injections, and the stirring speed was set to 310 rpm. The first injection was discarded in all titrations. Reference power was set at 10 or 15 μCal s⁻¹. Data were fit to a one-site model using Origin 7 Software. Two replicate titrations were carried out, and the reported parameters represent the average of the two replicates.

The fluorescence polarization competition binding assay was carried out as described previously (28). The protein concentration was 10 nM, and the geldanamycin-Cy3b concentration was 6 nM. Competition reactions were incubated for 24 h at 4 °C prior to analysis.

**Kᵢ calculation**

*Kᵢ* values for competition binding experiments were calculated from the FP IC₅₀ values using the method of Nikolovska-Coleska et al. (29) using equations as described previously (41) and implemented in a spreadsheet calculator (29). Input data for the calculation were total protein concentration (10 nM), tracer ligand concentration (6 nM), tracer ligand *Kᵢ* for the protein, and measured IC₅₀ values. Tracer ligand *Kᵢ* values for Grp94 (2.5 nM) and Hsp90α (0.7 nM) were taken from a previous report (28).

**NECA inhibitor IC₅₀ measurements**

ATP hydrolysis rates were measured using the PiPer Phosphate assay kit (Thermo Fisher Scientific) in 96-well fluorescent assay plates (Corning). Nearly full-length Grp94 (73–754Δ287–327) was buffer-exchanged into 1× ATPase buffer (40 mM HEPES-KOH (pH 7.4), 150 mM KCl, and 5 mM MgCl₂), concentrated to 50 μM, and diluted prior to the experiment. Experimental setup included a 50 μl:50 μl mixture of PiPer reagent (100 μM Ampex Red reagent containing 4 units/ml maltose phosphorylase, 0.4 mM maltose, 2 units/ml glucose oxidase, and 0.4 units/ml horseradish peroxidase) and the ATPase reaction (5 μl of ATP solution, 5 μl of inhibitor solution, and 40 μl of protein or 40 μl of 1× ATPase buffer for ATP-only wells). Inhibitors were prepared from stocks in DMSO and serially diluted in 1× ATPase buffer before addition to the plate. The effects of NECA, NPCA, and NEαCA were tested in the range of 0.781–100 μM. NEαCA was tested in the range of 2.91–372 μM. The final concentration of protein in each well was between 2.2–2.7 μM depending on the plate, and the final ATP concentration was 15 μM. Plates were incubated at 37 °C for 4 h, and the reactions were quenched on ice.

Fluorescence was measured at 544 nm/590 nm (excitation/emission) on a SpectraMax Gemini XS plate reader (Molecular Devices) with 30 readings/well. Data were background-corrected by subtracting the average of wells containing ATP only. Percent activity was calculated by dividing the measured fluorescence of inhibitor-treated wells by the average of wells containing protein, ATP, and DMSO (i.e. no inhibitor). DMSO had no effect on ATPase activity. Experiments are averages of six independent measurements. The data were plotted using Prism, and inhibitor concentration was transformed to logarithmic scale. The data were then fit using the dose–response inhibition equation: log(inhibitor) versus response (percent
activity compared with DMSO). IC_{50} was determined by measuring the concentration of inhibitor required to inhibit 50% activity.

### Crystallization and structure solution

Complexes between Grp94NA41 and NPCA, NEoCA, and NEaCA were formed by mixing concentrated protein at 30 mg/ml with 1–2.5 μl of concentrated ligand in DMSO to a final concentration of 5 mM and incubating the mixture on ice for 15 min. Crystals were formed by hanging drop vapor diffusion at 18 °C by mixing 2 μl of Grp94NA41:ligand with an equal volume of reservoir solution consisting of 100 mM Tris (pH 7.6), 25 mM MgCl₂, and 30%–35% PEG 400. Crystals formed in 1–3 days. Crystals of apoHsp90N were formed by hanging drop vapor diffusion at 4 °C by mixing 2 μl of protein at 25 mg/ml with an equal volume of cold reservoir solution consisting of 100 mM BisTris propane (pH 6.4), 10–50 mM MgCl₂, and 10–20% PEG 3350. Crystals formed in 1–3 days. Soaks were performed by adding 1 μl of a 9 mM ligand solution in reservoir solution to the crystal-containing drops. Crystals were harvested after 3–4 h of soaking.

Grp94NA41 crystals were harvested directly from crystallization drops without further stabilization and flash-frozen in liquid nitrogen. Crystals of Hsp90:ligand soaks were removed from the mother liquor in a loop, placed briefly in a drop containing reservoir solution, followed by transfer to a cryostabilization solution of 100 mM BisTris propane (pH 6.4), 25 mM MgCl₂, and 30% PEG 3350. Crystals were removed after a few seconds and flash-frozen in liquid nitrogen.

Structure solution was carried out by molecular replacement using PDB code 1Q5Y as the search model for Grp94 and 1YER as the search model for Hsp90. Refinement was carried out in Phenix.


### Acknowledgments—We thank Dr. E. Toone (Duke University) for chemical synthesis of the NECA derivatives, and W. J. Aw and C. G. C. and D. T. G. project administration. G. C. and D. T. G. methodology; G. C. and D. T. G. writing—original draft; G. C. and D. T. G. supervision; G. C. and D. T. G. data curation; G. C. and D. T. G. writing—original draft; G. C. and D. T. G. project administration.

### References

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