Resorcinol-Based Grp94-Selective Inhibitors

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S Supporting Information

ABSTRACT: Glucose regulated protein 94 (Grp94) is the endoplasmic reticulum resident of the 90 kDa heat shock protein (Hsp90) family and represents a promising therapeutic target for the treatment of several diseases. Grp94 is the most unique member of the 90 kDa heat shock protein family due to a five amino acid insertion into its primary sequence, which creates hydrophobic subpockets exclusive to Grp94 that can be utilized for selective inhibition. The first resorcinol-based Grp94-selective inhibitor to take advantage of the hydrophobic S2 subpocket has been developed and shown to manifest low nanomolar affinity and ~10-fold selectivity for Grp94. Furthermore, these Grp94-selective inhibitors manifest low micromolar GI₅₀ values against multiple myeloma cells, supporting Grp94 as an emerging target for the treatment of this disease.

KEYWORDS: Heat shock protein 90, Grp94, multiple myeloma, cancer

The 90 kDa heat shock proteins (Hsp90) are responsible for the maturation of more than 300 nascent polypeptides into their biologically active conformations, as well as the rematuration of denatured proteins.^{1–3} Many substrates dependent upon Hsp90 are associated with signaling pathways that are commonly hijacked during transformation, including Her2, Akt, and Raf. In fact, Hsp90-dependent client proteins are represented in all ten hallmarks of cancer.^{4,5} Consequently, Hsp90 inhibition provides a combinatorial attack against cancer via a single molecular target. Hsp90 is a homodimeric protein and each monomer contains an N-terminal ATP-binding motif, a middle domain that is important for protein–protein interactions, and a C-terminal dimerization domain.⁶ Binding and hydrolysis of ATP at the N-terminus provides the requisite source of energy for client protein folding, and inhibition or disruption of this process results in ubiquitinylation of the substrate and its degradation via the proteasome.

Seventeen small molecule ATP-competitive N-terminal inhibitors have been identified and introduced into clinical trials for the treatment of cancer.^{7–9} Unfortunately, these trials have resulted in cardiotoxicity, hepatotoxicity, ocular toxicity, and/or hypoglycemia in many cases.¹⁰ In addition, all of these inhibitors induce the pro-survival heat shock response in which the heat shock proteins (Hsp90, Hsp70, Hsp27, etc.) are upregulated at the same concentration that is required for client protein degradation, requiring patients to receive increasingly higher doses that push the patient toward the maximum tolerated dose and toxicity. These detriments have produced concerns about Hsp90 as a therapeutic target and support the development of

alternative strategies for Hsp90 inhibition for the treatment of cancer.

The Hsp90 family of chaperones comprises four isoforms: Hsp90α and Hsp90β reside in the cytosol, Trap1 is localized to the mitochondria, and Grp94 is found in the endoplasmic reticulum. All of the investigational Hsp90 drugs that have undergone clinical evaluation are *pan*-Hsp90 inhibitors and manifest similar affinity against all four Hsp90 isoforms. Since each isoform plays a distinct role in cancer progression, targeting a specific isoform may induce the degradation of a smaller subset of client proteins and therefore reduce the liabilities associated with *pan*-Hsp90 inhibition. Unfortunately, the development of Hsp90 isoform-selective inhibitors is hindered by the fact that all four isoforms share >85% identity within their N-terminal ATP-binding site.^{11–13} The most unique isoform is Grp94, which contains a five amino acid insertion into its primary sequence that leads to unique secondary binding pockets within the ATP-binding site.

Grp94 is the ER resident isoform of Hsp90 and is responsible for the maturation and trafficking of proteins associated with cell signaling and cellular adhesion.¹⁴ Grp94 has been shown to play a key role in the maturation and intracellular trafficking of integrins and the maintenance of cell polarity.¹⁵ Other Grp94-dependent client proteins include Her2, LRP6, IGF-I and -II, toll-like receptors, and mutant myocilin.^{16–19} Inhibition of Grp94 has

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been implicated in various diseases, including hepatocellular carcinoma, multiple myeloma, rheumatoid arthritis, and glaucoma.^{20–22} Since Grp94 is only essential during embryonic development, Grp94 represents a nontoxic target that can be inhibited to treat these diseases. Albeit, some diseases exhibit an increased dependence upon a functional ER and the ER chaperone system for survival due to increased metabolic rate (hepatocellular carcinoma) or the unfolded protein response due to ER stress (multiple myeloma), which renders Grp94 as an ideal target for the treatment of these cancers.^{20,21} Due to the dependency of integrins on Grp94 for their maturation and trafficking to the cell surface, Grp94-selective inhibitors also represent a nontoxic method to inhibit cancer metastasis.^{23–25}

As a consequence of the five amino acid insertion into the primary sequence, the Grp94 ATP-binding pocket contains two distinct subpockets that are not present in other isoforms. Subpocket 1 (S1) is located near the solvent-exposed region of the binding pocket but is hydrophobic and surrounded by Ile166, Ala167, Phe195, Val197, and Tyr200. The corresponding residues in cytosolic Hsp90s (Hsp90 α and Hsp90 β) are similar, but access to this region is blocked by Asn92 and Lys90. Subpocket S1 has been utilized previously for the development of Grp94-selective inhibitors as evidenced by the rational design of BnIm and its subsequent analogue, KUNG38 (Figure 1a).

Figure 1. (a) Structure of KUNG38 and its interaction with the S1 subpocket of Grp94. (b) Structure of PU-H54 and its interaction with the S2 subpocket of Grp94. (c) Overlay of KUNG38 (green, PDBID: SIN9) and PU-H54 (blue, PDBID: 3O2F) co-crystal structures demonstrating the different binding modes between two Grp94-selective scaffolds. Red arrows indicate helix reorganization upon ligand binding resulting in Phe199 moving to allow access to S2 subpocket.

These resorcinol-based inhibitors orient the benzyl side chain into the S1 subpocket to impart selectivity for Grp94 (Figure 1c). Recently, a small library of purine-based inhibitors was screened for Grp94-inhibition and ultimately led to identification of PU-H54 and PU-WS13 as Grp94-selective inhibitors (Figure 1b).^{17,28} Co-crystal structures with these inhibitors identified a second Grp94-exclusive subpocket (S2) near the adenine binding region of the N-terminal ATP-binding pocket (Figure 1c). Subpocket S2 results from rotation of Phe199 in Grp94 (red arrow, Figure 1c) as helices 1, 4, and 5 reorganize upon ligand

binding (red arrow, Figure 1c). Surprisingly, access to S2 is obstructed in the crystal structure of KUNG38 bound to Grp94. To date, no resorcinol-based inhibitor has been shown to bind the S2 subpocket. Therefore, resorcinol-based inhibitors were designed to selectively interact with the S2 subpocket in an effort to produce Grp94-selective inhibitors and to identify the optimal subpocket for development of Grp94-selective inhibitors.

The resorcinol containing isoindoline **1** was chosen as a starting point to probe the S2 subpocket of Grp94 (Figure 2a), as

Figure 2. (a) Structure of **1**. (b) Proposed binding model of **1** in Hsp90 α (green) and Grp94 (blue). PDBIDs: 3O2F (Grp94) and 3O2I (Hsp90 α). Br = light blue. (c) Key hydrophobic residues comprising the S2 subpocket of Grp94. (d) Key hydrophobic residues comprising Site-1 of Hsp90 α . Isoindoline ring omitted for clarity.

it contains the resorcinolic pharmacophore present in the *pan*-Hsp90 inhibitor, AT13387, which remains under clinical investigation.^{29,30} As shown in Figure 2b, an overlay of **1** docked to both Grp94 and Hsp90 α provides insight into the development of Grp94-selective inhibitors. The 5-position of the resorcinol ring projects directly toward the hydrophobic S2 subpocket that is surrounded by Leu104, Phe199, Ala202, Val211, Trp223, and Leu249 (Figure 2c). The Hsp90 α binding site possesses a pocket that extends toward the solvent exposed region of the ATP-binding pocket (Site-1, Figure 2d), which has been utilized previously to develop *pan*-Hsp90 inhibitors. The S2 subpocket extends deeper into the protein and away from the solvent exposed region. In order to transform **1** into a Grp94-selective inhibitor, substitutions at the 5-position must exhibit a preference for binding the S2 subpocket versus Site-1 in Hsp90 α (Figure 2b). Molecular modeling studies suggested that attachment of an aryl group to the 5-position of the resorcinol ring (**3**) would produce detrimental steric interactions with the S2 subpocket. However, it appeared that a single atom linker could alleviate this steric clash and, instead, produce compounds that selectively interact with the S2 subpocket to manifest Grp94 selectivity. Molecules that connect a phenyl (proposed to bind to S2) and resorcinol ring via a sulfur (**4**), nitrogen (**5**), or methylene (**6**) were proposed (Scheme 1). In addition, a ring-constrained nitrogen-linked analogue (**7**) was also sought to limit rotation about the linker while projecting the aromatic ring into the S2 subpocket for selective inhibition of Grp94. In addition to

Scheme 1. Synthesis of Compounds 3–9^a

^aConditions: (a) BBr₃; (b) 3a, Pd(dppf)Cl₂; (c) 4a, Pd₂(dba)₃, NaO^tBu, XPhos; (d) 5a, Pd₂(dba)₃, RuPhos, NaO^tBu; (e) 6a, Pd(OAc)₂, RuPhos, K₃PO₄; (f) CuI, 7a; (g) Pd(TFA)₂, 8a, dppp; (h) MeOH, DBU.

these aryl-substituted analogues, small alkyl esters (8 and 9) were evaluated at the 5-position, which could also limit steric interactions within the S2 subpocket of Grp94.

Synthesis of the proposed analogues began via the preparation of 2, following literature procedures.³¹ Subsequent treatment of 2 with boron tribromide afforded resorcinol 1. Compounds 3–8 were prepared via a transition metal-catalyzed cross-coupling reaction between 2 and the corresponding coupling partners (3a–8a). Resorcinol 3 was synthesized via a Suzuki cross-coupling reaction with phenyl boronic acid (3a), followed by cleavage of the benzyl ethers upon exposure to boron tribromide. Coupling of 2 with thiophenol (4a) was achieved via the enlistment of tris(dibenzylideneacetone)dipalladium(0), sodium *tert*-butoxide, and XPhos to provide the corresponding thioether, which was exposed to boron tribromide to ultimately afford 4. Similarly, *N*-methyl aniline (5a) was coupled with 2 via tris(dibenzylideneacetone)dipalladium(0) in the presence of RuPhos and sodium *tert*-butoxide to give 5 after cleavage of the benzyl ethers. Coupling of potassium benzyltrifluoroborate (6a) with 2 was performed using palladium(II) acetate, RuPhos, and potassium phosphate tribasic followed by cleavage of the benzyl ethers to afford 6. Coupling of indoline (7a) with resorcinol ring 2 was achieved via a copper-catalyzed Ullmann reaction followed by cleavage of the benzyl ethers to yield 7. A palladium-catalyzed decarboxylative coupling reaction between 2 and ethyl potassium oxalate (8a) was performed prior to cleavage of the benzyl ethers to provide 8. Base-catalyzed transesterification of 8 in methanol provided methyl ester 9 in good yield.

Upon their preparation, compounds 3–9 were evaluated in a fluorescence polarization assay to measure their affinity for both Grp94 and Hsp90α.³² The results from such studies are summarized in Table 1. As hypothesized, direct attachment of an aryl appendage to the resorcinol ring was detrimental as evidenced by 3, which manifests an apparent *K*_d of >100 μM for both Grp94 and Hsp90α. However, incorporation of a linker (4–6) proved that the S2 subpocket could be exploited with the resorcinol scaffold to gain Grp94 selectivity with these analogues, resulting in 2–4-fold selectivity for Grp94. Thioether 4, *N*-methylaniline analogue 5, and methylene analogue 6 manifest excellent affinities of 16, 17, and 15 nM for Grp94, respectively. However, the low selectivity exhibited by these analogues suggest that rotation about the linker results in similar affinity for Hsp90α. Therefore, reduction of the rotational freedom about the linker atom was pursued via the incorporation of an indoline ring (7), which produced 5-fold selectivity. However, this was

Table 1. Evaluation of Compounds 3–9 via Fluorescence Polarization against Grp94 and Hsp90α^a

entry	Grp94 (μM)	Hsp90α(μM)	fold Grp94-selective
3	>100	>100	n/a
4	0.013 ± 0.002	0.026 ± 0.002	2
5	0.017 ± 0.003	0.034 ± 0.008	2
6	0.015 ± 0.003	0.066 ± 0.006	4
7	9.89 ± 1.1	>50	5
8	0.77 ± 0.1	3.52 ± 2.3	5
9	>10	>10	n/a

^aData are the average of at least two independent experiments ± SEM. n/a = nonselective.

accompanied by a significant loss of affinity for Grp94 with an apparent *K*_d of 9.9 μM.

The ethyl and methyl ester containing analogues, 8 and 9, also manifest decreased affinity for Grp94 (0.77 and >10 μM, respectively); however, ethyl ester 8 manifests improved selectivity (~5-fold) for Grp94. Compounds containing sulfur, nitrogen, and methylene linkers (4–6) also manifest low selectivity for Grp94 but maintain good affinity. Lack of selectivity for these analogues was not too surprising, as rotation about the linker atoms could project the phenyl ring into either the S2 subpocket of Grp94 or Site-1 of Hsp90α (Figure 3a,b).

Figure 3. Proposed binding mode of 4 in (a) Grp94 (blue) and (b) Hsp90α (green).

Similar rotational freedom has been observed for the thioether linked purine-based Grp94-selective inhibitors that target the S2 subpocket of Grp94. In an effort to improve the selectivity profile for these inhibitors, substitutions on the aryl side chain of 4 and 6 were explored.

Molecular modeling studies indicated that both Site-1 of Hsp90α and the S2 subpocket of Grp94 comprise similar amino acid residues, but these residues orient differently around the aryl side chain (Figure 3). Furthermore, the S2 subpocket of Grp94 is sterically less demanding as compared to Site-1 of Hsp90α. Therefore, it was proposed that these subtle differences could be exploited to improve Grp94 selectivity by the introduction of substituents onto the phenyl side chain. Selection of substituents was guided by docking studies and prior structure–activity relationship studies observed for the purine-based Grp94-selective inhibitors, due to the potential for overlapping binding modes.^{28,33} Compounds 10–12, which contain electron donating groups at the 2′-, 3′-, and 4′-positions, were considered (Scheme 2) as well as dimethyl (13–15) and dichloro (16–17) substituents on the phenyl side chain as mechanisms to reduce affinity for the smaller Site-1 binding region. The 2′-chloro group was hypothesized to produce detrimental steric interactions with the smaller Site-1 of Hsp90α and produce favorable interactions

Scheme 2. Synthesis of Sulfide-Containing Analogues 10–17^a

^aConditions: (a) Pd₂(dba)₃, XPhos, 10a–16a, NaO^tBu; (b) BBr₃; (c) Pd(OAc)₂, 17a, JosiPhos, NaO^tBu.

with the larger and more hydrophobic residues present in the S2 subpocket of Grp94. Compounds 10–16 were prepared via the synthetic route described for 4, but instead utilized coupling partners 10a–16a, followed by cleavage of the benzyl ethers with boron tribromide. Coupling of 2,4-dichlorothiophenol (17a) was accomplished with palladium(II) acetate and JosiPhos followed by treatment with boron tribromide to unmask the free phenols and provide 17.

Once synthesized, 10–17 were evaluated for affinity and selectivity via a fluorescence polarization assay, which provided information regarding the binding modes for these analogues (Table 2). While substitutions provided compounds with

Table 2. Evaluation of 10–17 in a Fluorescence Polarization Assay against Grp94 and Hsp90α^a

entry	Grp94 (μM)	Hsp90α (μM)	fold Grp94-selective
10	0.029 ± 0.004	0.065 ± 0.01	2
11	0.034 ± 0.002	0.037 ± 0.002	n/a
12	0.07 ± 0.002	0.052 ± 0.007	n/a
13	0.03 ± 0.002	0.07 ± 0.002	2
14	0.021 ± 0.003	0.066 ± 0.006	3
15	0.029 ± 0.001	0.031 ± 0.002	n/a
16	0.031 ± 0.003	0.029 ± 0.010	n/a
17	0.059 ± 0.006	0.460 ± 0.006	8

^aData are the average of at least two independent experiments ± SEM. n/a = nonselective.

excellent affinity for Grp94 (K_d s < 70 nM), many of these substitutions did not possess selectivity for Grp94 (e.g., 11, 12, 15, and 16). Lack of selectivity is likely due to Site-1 of Hsp90α possessing a higher degree of flexibility than originally suspected and observed via molecular modeling. Alteration of the electronic nature of the phenyl ring did not improve selectivity, as electron donating (11), neutral (15), and withdrawing (16) substitutions manifested no enhancement in selectivity. Substitutions at the 2'-position were detrimental for Hsp90α and resulted in improved selectivity for Grp94, as 17 manifested an apparent K_d of 59 nM against Grp94 and ~8-fold selectivity over Hsp90α. Additionally, the low selectivity manifested by analogues 13 and 16 (2-fold and nonselective, respectively) suggests the aryl side chain of these resorcinol-based Grp94-selective inhibitors orients differently than the side chain of purine-based Grp94-selective inhibitors, thus resulting in new structure–activity relationships.

Due to poor selectivity manifested by the majority of analogues containing a sulfide linker (10–16), attention turned toward modification of 6 to improve selectivity for Grp94. The 8-

fold selectivity manifested by 17 suggests that substitutions at the 2'- or 4'-positions were needed to improve selectivity. Therefore, 18 and 19 were sought to elucidate substituents that could further enhance Grp94 selectivity versus other Hsp90 isoforms. These analogues were synthesized via a palladium-catalyzed cross-coupling reaction between boronic ester 2a and 2-chloro- or 4-chlorobenzyl bromide (18a and 19a, respectively), followed by treatment with boron tribromide to ultimately produce 18 and 19 (Scheme 3).

Scheme 3. Synthesis of Methylene-Linked Analogues 18–21^a

^aConditions: (a) (BPin)₂, Pd₂(dba)₃, PCy₃, KOAc; (b) 18a–21a, Pd(PPh₃)₄, 2 M K₂CO₃; (c) BBr₃.

Evaluation of these analogues via a fluorescence polarization assay revealed 19 to manifest 5-fold selectivity for Grp94 and improved affinity as compared to sulfide analogues 10–17. Compound 19 exhibited lower Grp94 selectivity compared to 17. Due to the difference in size between the sulfur atom and the methylene group, substitutions at the 4'-position did not exhibit similar activities. Consequently, substitutions at the 4'-position were explored, which ultimately led to 20 and 21. It was determined that increasing the size of the substituent from chloro (19) to methyl (20) resulted in loss of selectivity and a 3-fold decrease in affinity, as compared to 19. In contrast, decreasing the size to a fluorine (21), led to ~10-fold selectivity for Grp94 and <10 nM affinity, which warranted further evaluation in cellular models of Grp94-dependent processes (Table 3).

Table 3. Evaluation of 18–21 in a Fluorescence Polarization Assay against Grp94 and Hsp90α^a

entry	Grp94 (μM)	Hsp90α (μM)	fold Grp94-selective
18	0.06 ± 0.01	0.20 ± 0.01	3
19	0.013 ± 0.004	0.06 ± 0.01	5
20	0.04 ± 0.001	0.04 ± 0.002	n/a
21	0.008 ± 0.001	0.077 ± 0.004	10

^aData are the average of at least two independent experiments ± SEM. n/a = nonselective.

ER chaperones are upregulated in multiple myeloma as a consequence of the unfolded protein response that leads to plasma cell differentiation and pathogenesis.³³ Grp94 has been shown to be critical for the development of multiple myeloma, and its genetic deletion led to inhibition of cell growth.²¹ Li and co-workers demonstrated this phenomenon to occur through loss of the canonical Wnt signaling pathway. LRP6, a Frizzled coreceptor, was also identified as a Grp94-specific client, and KD of Grp94 led to a loss of LRP6 and inhibition of Wnt signaling, ultimately leading to cell death via activation of caspase 9. Consequently, we sought to evaluate these Grp94-selective

inhibitors for their ability to decrease the proliferation of multiple myeloma cells. Grp94-selective inhibitors (6, 19, 21), non-selective inhibitors (11, 20), and inactive controls (3, 9) were evaluated for antiproliferative activity against RPMI8226 cells as summarized in Table 4. In general, compounds that demon-

Table 4. Evaluation of Inhibitors against RPMI8226 Cell Line^a

compound	GI ₅₀ (μM) RPMI8226
geldanamycin	0.003 ± 0.0006
BnIm	10.2 ± 0.5
3	>25
6	3.7 ± 0.8
9	13.8 ± 0.1
11	1.9 ± 0.2
19	3.0 ± 0.4
20	2.7 ± 0.4
21	1.4 ± 0.1

^aData are the average of at least two independent experiments ± SEM.

strated affinity via fluorescence polarization exhibited low micromolar GI₅₀ values against these cells. Compound 21 manifested a GI₅₀ value of 1.4 μM and was selected for further evaluation via Western blot analysis of RPMI8226 cell lysates. Treatment with 21 demonstrated reduction of LRP6 levels (Figure 4), as predicted via Grp94 KD studies, at concentrations

Figure 4. Western blot analysis of RPMI8226 cell lysates after treatment with 21, G (geldanamycin, a natural product, *pan*-Hsp90 inhibitor, 0.5 μM), or D (DMSO, 0.1% final concentration).

that mirrored cell viability, linking Grp94 inhibition to cell viability. Analysis of downstream Wnt signaling demonstrated that survivin, an antiapoptotic protein, was degraded in a dose-dependent manner, which suggested that 21 activated apoptosis through decreased Wnt signaling. Degradation of Akt, a cytosolic Hsp90-dependent client protein, was not observed until the highest concentration. Similarly, induction of Hsp70, a hallmark of *pan*-Hsp90 inhibition, was not observed until the highest concentration. Together, these data suggest that 21 manifests Grp94-selective inhibition in cells and represents a new scaffold for the development of Grp94-selective inhibitors.

Recently, Grp94-selective inhibition has emerged as a therapeutic target for the treatment of glaucoma, multiple myeloma, and metastatic cancer. Therefore, the development of new Grp94-selective chemotypes is highly desirable. In this study, a resorcinol-based and nonselective scaffold was modified through a rational drug design approach to probe an exclusive binding region present in Grp94 (S2 subpocket), which had not

been previously accessed with the resorcinol class of Hsp90 inhibitors. Structure–activity relationship studies resulted in the Grp94-selective inhibitors, 17 and 21, which manifest low nanomolar affinity and ~10-fold selectivity for Grp94. This work demonstrates that the S2 subpocket of Grp94 can be utilized to develop inhibitors that are selective for Grp94. Based on these studies, modifications to other resorcinol-based *pan*-Hsp90 inhibitors could alter their selectivity profiles and transform *pan*-Hsp90 inhibitors into Grp94-selective inhibitors. The data presented in this Letter demonstrates that Grp94-selective inhibitors can manifest low micromolar GI₅₀ values against multiple myeloma cells and that pharmacological inhibition of Grp94 recapitulates the effects of genetic knockdown studies, providing evidence that Grp94 is a therapeutic target for these cancers. Ongoing efforts are focused on the co-crystallization of 21 with Grp94 and Hsp90 in an effort to further develop this scaffold for Grp94-selective inhibition.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.7b00193.

Synthesis, characterization, and biological protocols (PDF)

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Author Contributions

^{||}These authors contributed equally to this work. All authors conceived the project and analyzed data. A.K. and V.C. performed synthesis. V.C. performed biological assays. All authors wrote and approved the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Grp94, glucose regulated protein 94; Hsp90, heat shock protein 90; LRP6, low density lipoprotein receptor-related family; IGF, insulin-like growth factor; ER, endoplasmic reticulum; XPhos, 2-dicyclohexylphosphino-2',4',6'-triisopropylbiphenyl; RuPhos, 2-dicyclohexylphosphino-2',6'-diisopropoxybiphenyl; JosiPhos,

(2R)-1-[(1R)-1-(dicyclohexylphosphino)ethyl]-2-(diphenylphosphino)ferrocene; KD, knockdown

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