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# The identification, synthesis, protein crystal structure and in vitro biochemical evaluation of a new 3,4-diarylpyrazole class of Hsp90 inhibitors

Kwai-Ming J. Cheung,[a,†] Thomas P. Matthews,[a,†] Karen James,[a] Martin G. Rowlands,[a] Katherine J. Boxall,[a] Swee Y. Sharp,[a] Alison Maloney,[a] S. Mark Roe,[b] Chrisostomos Prodromou,[b] Laurence H. Pearl,[b] G. Wynne Aherne,[a] Edward McDonald[a,*] and Paul Workman[a]

[a]*Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, Cancer Research UK and Haddow Laboratories, 15 Cotswold Road, Sutton, Surrey SM2 5NG, UK*
[b]*Section of Structural Biology, The Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, UK*

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**Abstract**—High-throughput screening identified the 3,4-diarylpyrazole CCT018159 as a novel and potent (7.1 μM) inhibitor of Hsp90 ATPase activity. Here, we describe the synthesis of CCT018159 and a number of close analogues together with data on their biochemical properties. Some initial structure–activity relationships are discussed, as well as the crystal structure of CCT018159 bound to Hsp90.


Heat-shock protein 90 (Hsp90) is a molecular chaperone responsible for ATP-dependent folding, stability and function of many 'client' proteins that are involved in the development and progression of cancer.[1–] These client proteins include ErbB2, c-Raf, Cdk4, mutant p53, hTERT, Hif1-α and the oestrogen/androgen receptors. The natural products geldanamycin **1a** and radicicol **2** (see Fig. 1) were known to be active in various biological assays[5] and were subsequently shown to inhibit Hsp90,[6,7] leading to degradation of client proteins by the ubiquitin–proteasome pathway.[8] Treatment of cells with Hsp90 inhibitors results in cell cycle arrest, differentiation and apoptosis.[9,10] Since cancers usually have multiple molecular oncogenic defects, the potential for simultaneous combinatorial disruption of multiple cancer-associated signalling proteins and pathways, and also for modulation of all the hallmark features of malignancy, makes Hsp90 a particularly attractive cancer drug target.[1,–] Hsp90 inhibitors should exhibit broad spectrum antitumour activity and a lower risk of inducing drug resistance. Good activity and an acceptable therapeutic index have been observed with the geldanamycin analogue 17-allylamino, 17-demethoxy-geldanamycin (17AAG) **1b** in human tumour xenograft models.[14] Hypotheses to explain the therapeutic selectivity for tumour versus normal cells include a greater dependence of cancer cells on oncogenic client proteins (oncogene addiction); the presence in malignant cells of overexpressed and mutated oncoproteins that necessitate high chaperone capacity; the stress conditions of cancer cells leading to a stronger need for molecular chaperone activity; the removal of the buffering effect of Hsp90 in tumour cells causing synthetic lethal effects; and the preferential accumulation of Hsp90 in cancer versus normal cells.[1,15,16] Furthermore, it has been reported that Hsp90 is present in an activated superchaperone complex that binds 17AAG much more tightly than the latent form in normal cells.[15,17]

Phase 1 clinical trials of 17AAG **1b** showed evidence of biological and clinical activities, including prolonged stable disease in two patients with melanoma.[18]

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* Corresponding author. Tel.: +44 208 722 4294; e-mail: ted.mcdonald@icr.ac.uk
† These authors contributed equally to this work.

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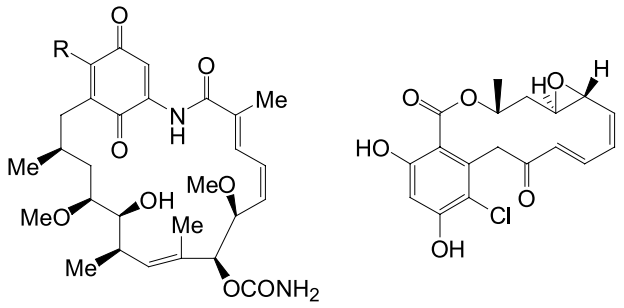



**Figure 1.** Structures of natural product based Hsp90 inhibitors.

However, a second generation of Hsp90 inhibitors is being sought to overcome some of the undesirable features of 17AAG, such as limited oral bioavailability, potential toxicity and poor aqueous solubility.[19,20]

Natural product inhibitors of Hsp90, such as geldanamycin **1a** and radicicol **2**, inhibit Hsp90 by binding to the ATP site located in the N-terminal domain, disrupting the ATPase activity that is essential for its chaperone function.[7,21,22] Derivatives of geldanamycin continue to be evaluated, including the more soluble 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17DMAG) **1c**, which has entered clinical trial.[23] Radicicol oximes have shown activity in animal models.[24] The first synthetic small molecule inhibitors of Hsp90 were based on the purine scaffold, for example, PU3 and PU24FCl.[12,25] Amongst other compounds,[19] novobiocin and cisplatin have been reported to inhibit Hsp90 in these cases by binding at a C-terminal site.[26]

High-throughput screening[27] of a library of 50,000 compounds, assayed at a final nominal concentration of 40 μM in 0.4% (v/v) DMSO, identified CCT018159 (Fig. 2, **3a**) as the most potent inhibitor of yeast Hsp90 ATPase activity (79% inhibition, $IC_{50}$ = 8.9 μM). Further interrogation of the data from this malachite green assay furnished a number of less potent analogues (Table 1), which provided some early structure–activity relationships (SAR). All the compounds share a similar structure and the more active ones possess the resorcinol ring, pyrazole and benzodioxan core. Methylation of the hydroxyl *para* to the pyrazole **4**–**8** appears to result in a significant fall in potency. The lipophilic (alkyl) group at the 5-position of the resorcinol seems to be important, as when the ethyl group in **3a** is replaced by a hydrogen **10** or propyl chain **9** we observe an 8- and 5-fold increase in $IC_{50}$, respectively.

A synthetic chemistry programme was undertaken to devise an efficient route to synthesise sufficient quantities of **3a** and a number of close analogues to confirm and further investigate the pharmacological properties of this class of compounds.

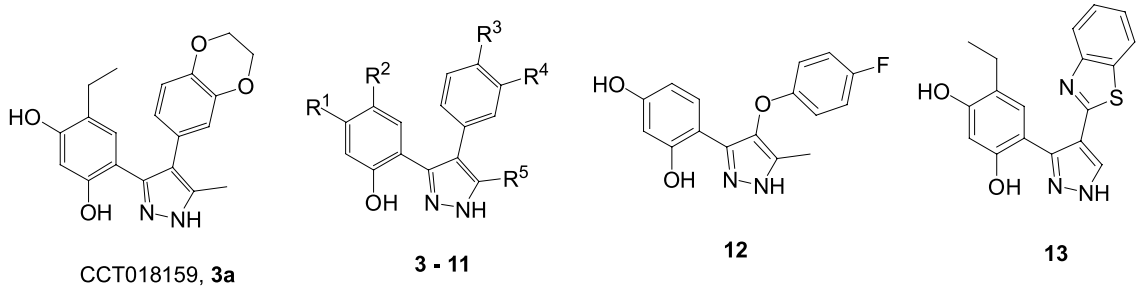



**Figure 2.** Molecular structures of CCT018159 **3a** and close analogues in the screening series.

**Table 1.** Inhibitory activity of hits identified in the high-throughput screen against yeast Hsp90 ATPase

| Compound | $R^1$ | $R^2$ | $R^3$ $R^4$ | $R^5$ | % Inhibition in screen at 40 μM | $IC_{50}$ (μM) ± SD |
|---|---|---|---|---|---|---|
| **3a** CCT018159 | OH | Et | –$OCH_2CH_2O$– | Me | 79 | 8.9 ± 0.72 |
| **4** | OMe | Et | –$OCH_2O$– | H | 20 | |
| **5** | OMe | H | –$O(CH_2)_3O$– | Me | 25 | |
| **6** | OMe | Pr | –$OCH_2CH_2O$– | H | 0 | |
| **7** | OMe | Pr | H H | H | 10 | |
| **8** | OMe | Et | OMe H | H | 13 | |
| **9** | OH | Pr | –$OCH_2CH_2O$– | Me | 25 | 46.1 ± 2.8 |
| **10** | OH | H | –$OCH_2CH_2O$– | Me | 26 | 60.8 ± 6.7 |
| **11** | OH | Pr | –$OCH_2O$– | H | 64 | 22.3 ± 2.5 |
| **12** | (See Fig. 2) | | | | 0 | |
| **13** | (See Fig. 2) | | | | 15 | |
| **1a** Geldanamycin[a] | (See Fig. 1) | | | | 90 | 4.8 ± 0.8 |
| **1b** 17AAG[a] | (See Fig. 1) | | | | 80 | 8.7 ± 2.3 |

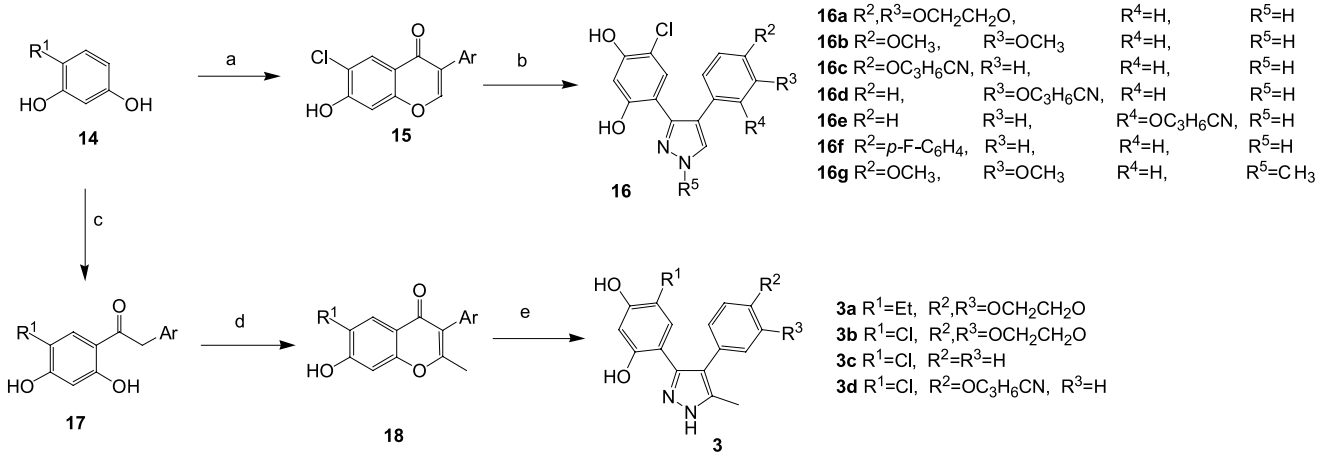
[a] Hsp90 inhibitor standards.

We prepared the hit **3a**[28] and analogues **3b–d** and **16a–g** based on a previously published procedure for the preparation of 3,4-diaryl pyrazoles by refluxing chromen-4-one intermediates[29] in ethanol with hydrazine hydrate or methyl hydrazine in the case of **16g**. The chromen-4-ones **15** and **18** were synthesised from phenylacetic acids by one of two methods[30] depending on whether the substituent at the 5-position of the pyrazole was hydrogen **16a–g** or a methyl group **3a–d** (Scheme 1).

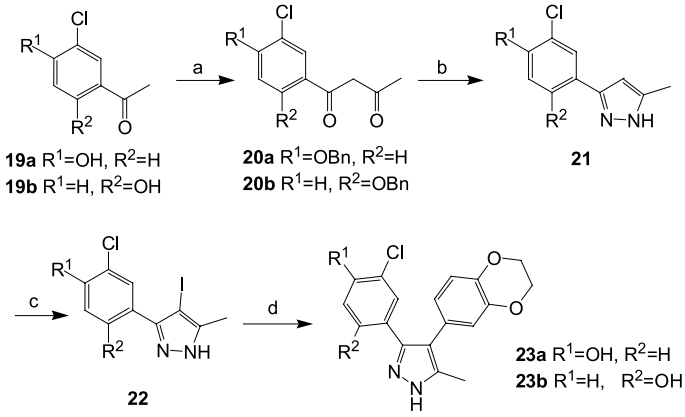
The phenylacetic acids required for **3c**, **16b** and **16g** are commercially available, whilst the biaryl phenylacetic acid used in the synthesis of **16f** was prepared by a literature procedure.[31] The required acids for **3a**, **3b**, **16a** and **16c–e** were synthesised by alkylating the corresponding mono- or dihydroxyphenylacetic acids with bromobutyronitrile and 1,2-dibromoethane, respectively.

Two compounds containing only one of the two resorcinol hydroxyls were synthesised by a different procedure detailed in Scheme 2. Whilst **19b** is commercially available, the phenolic starting material **19a** was prepared from a rearrangement of acetic acid 2-chlorophenyl ester with aluminium chloride.[32] From the resulting 5:1 mixture of isomers, the major *para* rearranged product **19a** was isolated by recrystallisation (NMR assignment was unambiguous). Next, a 1,3-diketone chain was built up on the benzyl protected phenols, followed by the ring-forming condensation with hydrazine hydrate to form the pyrazole. Iodination, followed by a Suzuki–Miyaura coupling and benzyl ether deprotection, gave the desired analogues **23a** and **23b**.

The 13 compounds synthesised were assayed for activity against the yeast Hsp90 ATPase using a more sensitive malachite green assay,[33] which gave an $IC_{50}$ of 7.1 μM ($n = 2$) for **3a**. This compound also inhibited human Hsp90 in the presence of the co-chaperone Aha-1[34] with a closely similar $IC_{50}$ of 3.1 μM ($n = 2$). In this assay, geldanamycin gave an $IC_{50}$ of 4.2 μM against the human enzyme and 17AAG gave a value of 3.5 μM. A sulforhodamine B (SRB) assay[35] was used to determine the $GI_{50}$ against HCT116 colon cancer cells (Table 2). This cell line was used as it is relatively sensitive to Hsp90 inhibitors and because we previously characterised in detail both its molecular response, in terms of client protein depletion and Hsp70 induction, and also its cellular response, with respect to cell cycle arrest and apopto-




**Scheme 1.** Reagents and conditions: (a) One pot (i) $ArCH_2CO_2H$, $BF_3 \cdot OEt_2$, (ii) $PCl_5$, DMF; (b) hydrazine hydrate (or methyl hydrazine **16g**), EtOH, reflux; (c) $ArCH_2CO_2H$, $BF_3 \cdot OEt_2$, 80 °C, 90 min; (d) $K_2CO_3$, acetic anhydride, DMF, reflux, 6 h; (e) hydrazine hydrate, EtOH, reflux.




**Scheme 2.** Reagents and conditions: (a) (i) BnBr, $K_2CO_3$, acetone, reflux, (ii) Na, EtOAc; (b) hydrazine hydrate, ethanol, reflux; (c) NaI, $I_2$, $Na_2CO_3$, water/THF, microwave, 120 °C; (d) (i) $ArB(OH)_2$, $Pd(PPh_3)_4$, $Cs_2CO_3$, LiCl, 1-propanol/water, microwave, 120 °C, (ii) NaI, $BF_3$, MeCN, rt, 18 h.

**Table 2.** Inhibitory potency of **3a** and close analogues against yeast Hsp90 ATPase and proliferation of HCT116 human colon cancer cells

| Compound | $IC_{50}$ (μM) $n = 2$ | $GI_{50}$ (μM) |
|---|---|---|
| **3a** CCT018159 | 7.1 | 4.1 ± 0.4 |
| **3b** CCT072440 | <1[a] | 4.0 ± 0.6 |
| **3c** CCT072457 | 1.65 | 6.4 |
| **3d** CCT073657 | <1[a] | 3.6 |
| **16a** CCT072442 | 1.6 | 23 |
| **16b** CCT069965 | 2.4 | 13 |
| **16c** CCT072453 | <1[a] | 6.8 ± 0.7 |
| **16d** CCT074897 | 2.4 | 30 |
| **16e** CCT074898 | 12.85 | 31 |
| **16f** CCT072454 | 1.55 | 11 |
| **16g** CCT075450 | >100 | — |
| **23a** CCT073667 | >100 | — |
| **23b** CCT072439 | 25.5 | — |
| **1a** Geldanamycin | 4.8 | 0.067 |
| **1b** 17AAG | 6.6 | 0.021 |

Where multiple assays were conducted, the results are shown as ±SE.
[a] $IC_{50}$ of compounds **3b**, **3d** and **16c** were close to the detectable limit of the assay. Percentage inhibition values for yeast Hsp90 at 1 μM with these compounds were 61.5%, 77.8% and 64.7%, respectively.

sis.[10,36] The SRB assay showed that the hit compound **3a** effectively reduced cellular proliferation with a $GI_{50}$ of 4.1 μM.

Gene expression microarray studies (data not shown) and Western blotting[37] have helped us to define the molecular signatures that are characteristic of Hsp90 inhibition, which include the downregulation of c-Raf and Cdk4, and the induction of Hsp70 proteins.[36] Figure 3 shows that after treatment with **3a** in HCT116 cells, depletion of c-Raf and Cdk4 and elevation of Hsp70 were observed, confirming the action of **3a** as an Hsp90 inhibitor.

Protein X-ray crystallography (1.6 Å resolution) confirmed that **3a** binds to the ATPase pocket within the N-terminal domain of yeast Hsp90 (Fig. 4). Details of the expression and purification of His-tagged Hsp90 constructs and co-crystallisation of the N-terminal domain of Hsp90 with inhibitors are described in Panaretou et al.[38] and Roe et al.,[7] and the amino acid numbering refers to yeast Hsp90. In the complex, the two phenolic hydroxyls and the adjacent pyrazole N-atom of **3a** form a network of hydrogen bonds with amino acids together with associated water molecules lying

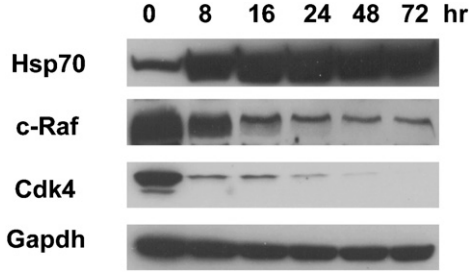



**Figure 3.** Western blot showing that in HCT116 cells **3a** (5 × $GI_{50}$) downregulates Hsp90 client proteins (c-Raf and Cdk4) and elevates levels of Hsp70. GAPDH was used as loading control.

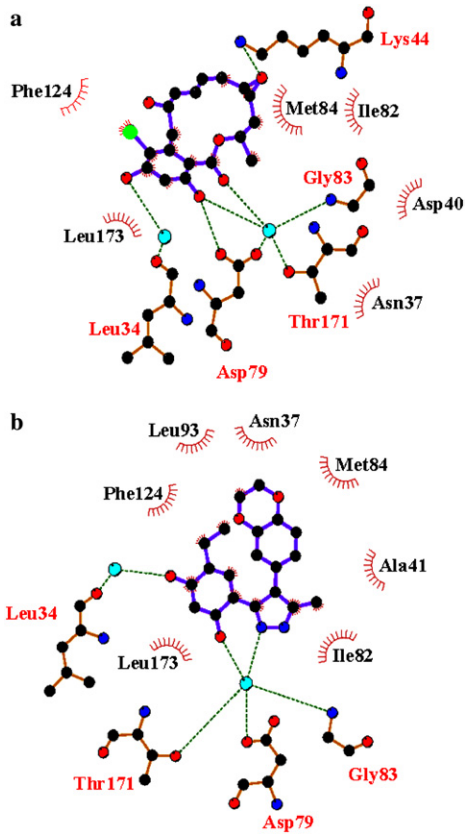



**Figure 4.** Ligplot diagrams showing the binding interactions of (a) **2** and (b) **3a** with the yeast Hsp90. Hydrogen-bonding interactions are shown as broken lines. Amino acids shown in red are involved in hydrogen bonding. Atoms in van der Waals contact have 'spokes' directed at each other. Blue spheres represent water molecules.

at the base of the pocket that binds radicicol **2** (Fig. 4) and geldanamycin **1a**.[7] In fact, interactions that **2** and **3a** make with Hsp90 are strikingly similar. Most importantly, interactions with inhibitors from the carboxylate side chain of Asp 79, the main-chain amide group of Gly 83 and the hydroxyl side chain of Thr 171, all via the same tightly bound water molecule, are conserved in both structures. Another conserved interaction occurs between the main-chain carbonyl of Leu 34 via another tightly bound water molecule to the inhibitors. These tightly bound water molecules are also involved in the binding of ATP and **1a**.[7] The only direct hydrogen bond interaction between Hsp90 and the inhibitors is between the δ-amino side chain of Lys 44 and the epoxide oxygen of **2**. The rest of the interactions between Hsp90 and the inhibitors are hydrophobic in nature and a common set of residues (Asn 37, Ile 82, Met 84, Leu 173, and Phe 124) are found to be in van der Waals contact with **2** and **3a**. van der Waals contacts for **3a** are somewhat more extensive than those seen for **2** and involve additional hydrophobic residues (Ala 41 and Leu 93).

As the ethyl group in **3a** and chlorine in the more potent **2** occupy the same region of the ATP site, a chlorine containing analogue of **3a** was synthesised. Owing to improved enzyme potency of this compound **3b**, the chlorine substituent was retained subsequently in the majority of analogues.

Following our disclosure of pyrazole inhibitors of Hsp90 ATPase and the X-ray crystal structure of an enzyme–inhibitor complex,[39,40] Kreusch et al.[41] have recently published their independent discovery of related resorcylic pyrazoles that bind to Hsp90 in the same fashion.

From the biochemical data of the small set of analogues, some additional initial SAR could be derived. The necessity of inhibitors to possess both resorcinol hydroxyls was demonstrated by the loss in activity of **23a** and **23b.** The crystal structure of CCT018159 **3a** (Fig. 4) shows extensive H-bonding involving both resorcinol hydroxyls, thus explaining the weaker activity of monophenolic analogues **23a** and **23b**.

Compounds **3d** and **16c**–**16e** indicate that there is extensive room for large flexible groups in place of the dioxan ring of **3a**, especially in the *meta*- and *para*-positions. This has been demonstrated in the crystal structure (2.0 Å resolution) of **16c** (Fig. 5) where the butyronitrile chain can clearly be seen pointing out of the ATP pocket towards solvent. Apart from familiar hydrogen-bonding interactions via two tightly bound water molecules close to the resorcinol ring and the van der Waals contacts seen in **3a**, two further interactions are noted: (1) a van der Waals contact between Gly 121 and the butyronitrile chain in **16c**; and (2) a hydrogen bond interaction via a water molecule between Lys44 and the butyronitrile oxygen atom. It is noted above that Lys 44 also forms a hydrogen bond with the epoxide oxygen of Radicicol **2**.

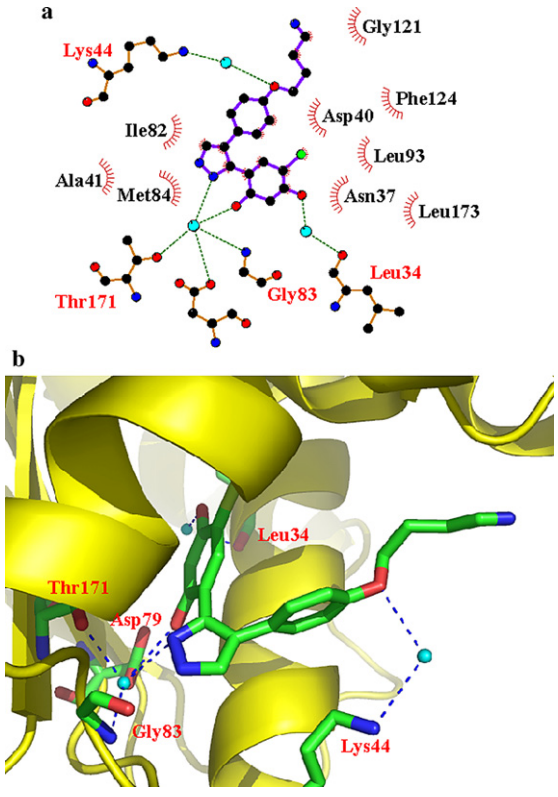



**Figure 5.** Binding interactions of **16c** with yeast Hsp90. (a) Ligplot diagram showing hydrophobic and hydrogen-bonded interactions. Hydrogen-bonding interactions are shown as broken lines. Atoms in van der Waals contact have 'spokes' directed at each other; (b) cartoon showing the hydrogen-bonded interactions represented as broken lines. In both structures (a) and (b), amino acids shown in red are involved in hydrogen bonding.

The $GI_{50}$ results show that the analogues displaying enzyme potency also possess activity against HCT116 cells; however, a relationship between the two is not immediately apparent for these early compounds. By comparing **3b** and **3d** with **16a** and **16c**, we conclude that the presence of 5-methyl on pyrazole appears to have a negligible effect on cellular activity.

In conclusion, we have found a new class of small molecule Hsp90 inhibitors, which have been characterised both by biochemical evaluation and crystallography. Three synthetic routes, of which one is novel, have been employed, which allow access to both the initial hit compound from the high-throughput screen and to a number of structurally diverse derivatives. Initial SAR has revealed the key interactions of the inhibitor and areas where extensive derivatisation is possible. Overall, the results indicate that this novel Hsp90 inhibitor series provides an attractive starting point for hit to lead exploration.

## Supplementary data

Crystallographic coordinates have been deposited with the Protein Data Bank: 2BRC (CCT018159, **3a**) and 2BRE (CCT072453, **16c**).

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