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Design, Synthesis and Biological Evaluation of Novel Hsp90 Inhibitors with Reduced Toxicity

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Abstract

The molecular chaperone Heat Shock Protein 90 (Hsp90) is an attractive target for the treatment of multiple diseases and conditions including various cancers, Parkinson's disease, HIV/AIDS and malaria. Multiple Hsp90 inhibitors have progressed to clinical trials including derivatives from the geldanamycin and radicicol family of natural products. However, particularly in the former case, progression was halted due to unacceptable toxicity and solubility issues. Efforts to address this for the geldanamycin family through substitution at the quinone 19-position and subsequent analysis and biological evaluation are described herein.

I declare that the work described in this thesis is predominantly my own (synthetic chemistry and analysis), my own in conjunction directly with others (radicicol synthesis and analysis, manuscript preparation, conformational and trace metal analysis, small molecule X-ray studies) or performed by our collaborators (co-crystallised X-ray analysis, ITC, biological evaluation and related analysis).

Acknowledgments

I would like to thank Parkinson's UK for funding for the research described herein. I would also like to extend my gratitude to Prof. Chris Moody, his research group and the technical team at the University of Nottingham and Prof. David Ross, Dr. David Siegel and the rest of the team at the Colorado University for their key roles in the work described. Finally I would like to thank my family for their love and support, without which none of this would have been possible.

Abbreviations

17-AAG	17-Allylamino-17-demethoxygeldanamycin
17-DMAG	17-(*N*,*N*-Dimethylaminoethylamino-17-demethoxygeldanamycin
ARPE	Adult Retinal Pigment Epithelial
Bu	Butyl
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DMF	*N*,*N*-Dimethylformamide
EOM	Ethoxymethyl
Et	Ethyl
Grubbs II	Grubbs 2nd Generation Catalyst
HATU	Hexafluorophosphate azabenzotriazole tetramethyl uronium
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
Hsp	Heat shock protein
HUVEC	Human umbilical vein endothelial
IPI504	17-Allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride salt
iPr	*iso*-Propyl
ITC	Isothermal titration calorimetry
Me	Methyl
NMR	Nuclear magnetic resonance (spectroscopy)
NQO1	nicotinamide adenine dinucleotide phosphate hydrogen quinone dehydrogenase 1
NRF2	Nuclear factor erythroid 2-related factor 2
ROESY	Rotating frame nuclear Overhauser Effect Spectroscopy
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UCL	University College London

Chapter 1: Introduction

Foreword

In lieu of an introduction chapter, a perspective review article, co-authored by the author of this thesis, is included. This summarises the context of the synthetic research activities and biological evaluation overview for targeting Heat Shock protein 90 (Hsp90) with molecules from the geldanamycin and radicicol families.

Chapter 2: Commentary on Submitted Work

Foreword

The results presented in this habilitation thesis stem from my work during my poctdoctoral stint at the University of Nottingham, UK (2010-2014, financially supported by Parkinson's Disease Society UK) and subsequent research collaborations during predominantly teaching-focussed academic positions (2014-2015, University of Nottingham and 2015-2022 University of Warwick, both financially supported by university research allowance funds).

The work herein can be subdivided into two categories:

1) That based on the geldanamycin natural product.
2) That based on the radicicol natural product.

2.1 Geldanamycin

2.1.1 Rationale for substituting the geldanamycin 19-position

As mentioned in Chapter 1, the polyketide natural product geldanamycin **1** was found to be a potent inhibitor of Hsp90, with implications in therapeutic areas including cancer, neurological diseases, HIV/aids and malaria.[1] Subsequently, several amino-substituted derivatives of geldanamycin (tanespimycin (17-AAG) **2**, alvespimycin (17-DMAG) **3** and retaspimycin/IPI504 [17-AAG hydroquinone hydrochloride] **4**, Figure 1) were evaluated in the clinic against various cancers, but trials were ceased due predominantly to off-target toxicity issues, but also poor solubility.[1]

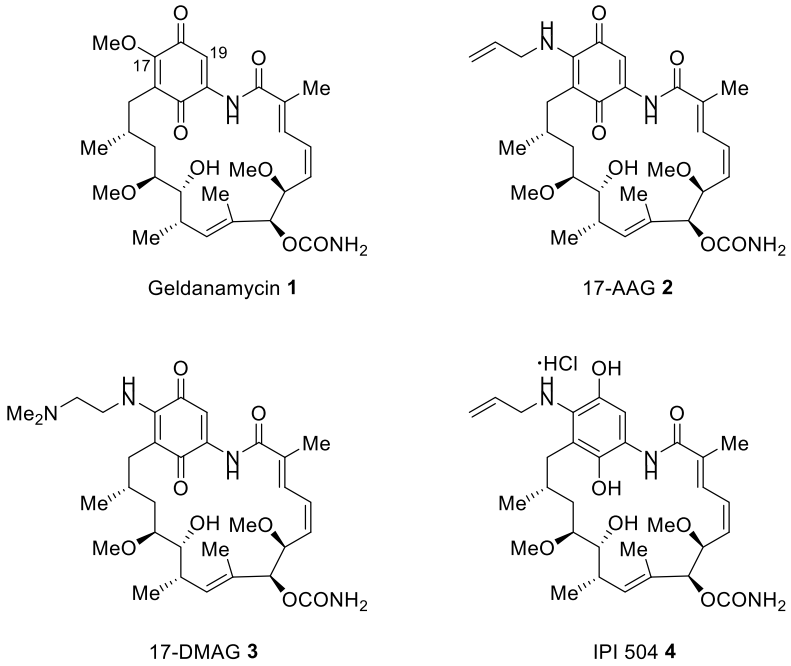
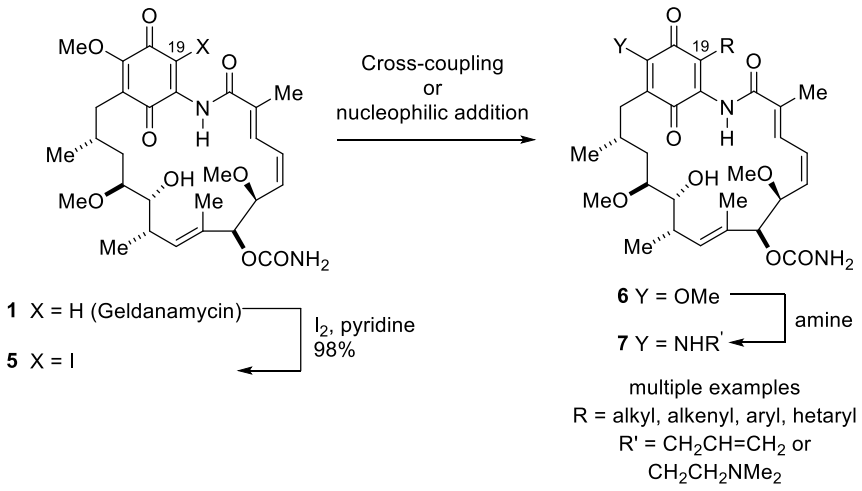


Figure 1: Structure of geldanamycin **1** and its clinically-evaluated derivatives **2**-**4**.

The toxicity issues were thought to be due to one of two factors: 1) quinone redox reactions or 2) conjugation with biological nucleophiles at the quinone 19 position (Figure 1). To test the latter hypothesis, we planned to substitute the 19 position with a C-C bond to an alkyl, aryl or alkenyl group using palladium-catalysed cross-coupling methods. We also wanted to investigate whether the introduction of a 19-substituent would promote the well documented conformational 'switch' of this class of compounds and if this would have any affect on the binding and potency.

2.1.2 Synthetic studies, conformational analysis and binding to Hsp90

From commercially available geldanamycin **1**, we were able to develop several methods for introducing a C-C bonded substituent at position 19, predominantly using Stille or Suzuki cross-coupling methodologies *via* the readily synthesised 19-iodogeldanamycin **5**, but also other metal-promoted and Mannich-type approaches (Scheme 1).[2-4]



Scheme 1: Semisynthetic approaches to the 19-substitution of geldanamycin **1**.

TLC and HPLC analysis indicated that the new compounds **6** were much more polar than geldanamycin **1** itself, despite the introduction of a lipophilic group. The hypothesis for this was a switch in conformation in which the more polar moieties were exposed while the lipophilic portions were contained in the centre (Figure 2).[2]

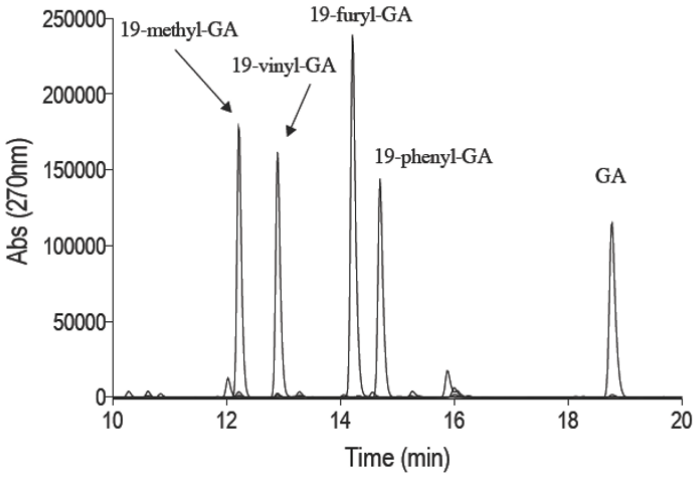
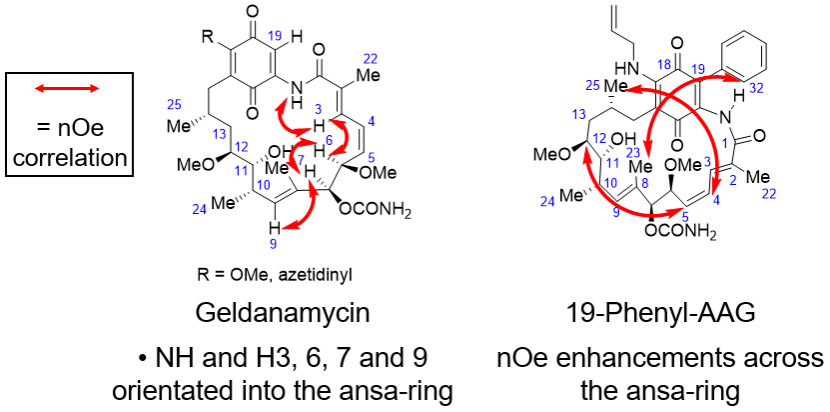


Figure 2: Reverse phase HPLC trace overlay showing the relative polarities of geldanamycin **1** and its more polar 19-substituted derivatives **6**.

This was supported by initial results from ROESY NMR analysis, which showed interactions of proton nuclei across the ansa ring, somewhat different from the parent compounds (Figure 3a). Quantitative analysis of the interatomic distances

and feeding this into computational molecular modelling gave a predicted low-energy conformation with a C-clamp shape and *cis*-amide, as depicted in Figure 3b. This was further supported with an X-ray structure for the 19-furyl derivative (Figure 3c).[2]

a



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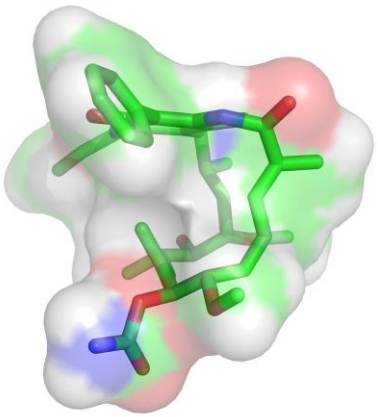

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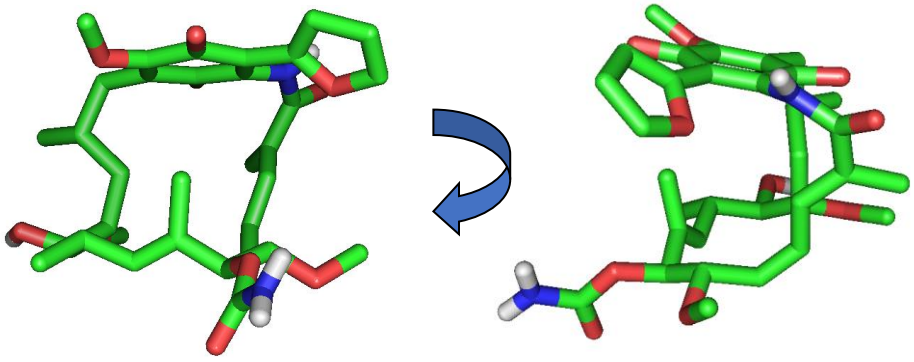


Figure 3: a, nOe correlations observed for parent geldanamycin and 19-substituted derivatives. b, Molecular modelling predicted lowest energy conformation of derivatives **6** (R = Ph, Y = OMe). c, X-ray structure of derivatives **6** (R = 2-furyl, Y = OMe).

On analysing the binding of the 19-substituted derivatives to Hsp90, ITC indicated a slight loss in binding affinity (Table 1) compared to the parent compound, with an entropic penalty observed. Co-crystallisation X-ray analysis showed that although the compounds bound to the Hsp90 active site, a slight steric clash of the position 19-substituent with the protein backbone led to a shift in position of the quinone and the disruption of water bridges, presumably the cause of the variation in the ITC entropic component (Figure 4).[2]

Table 1: ITC analysis on binding to yeast Hsp90 for geldanamycin **1** and its 19-substituted derivatives **6** and **7**.

Compound	K_d / μM
GA	2.9 ± 0.8
19-Me-GA	16.3 ± 4.2
17-AAG	2.5 ± 0.46
19-Me-17-AAG	21.6 ± 4.0
17-DMAG	1.2 ± 0.13
19-Me-17-DMAG	22.4 ± 3.2

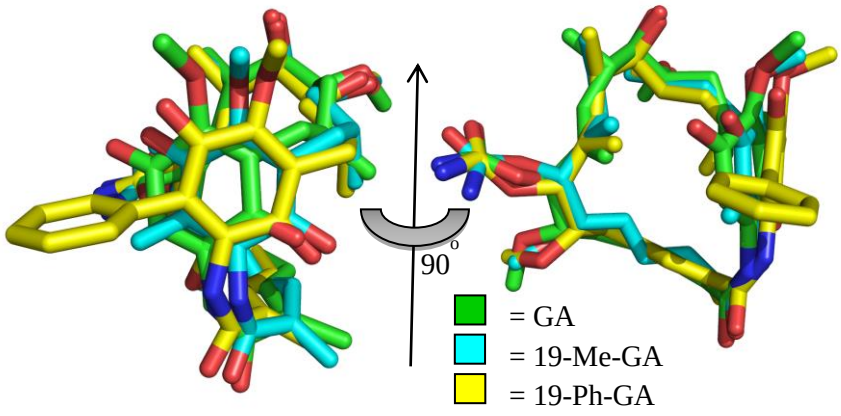


Figure 4: Co-crystallisation X-ray structures of geldanamycin **1** and its more polar 19-substituted derivatives **6** (R = Ph, Me, Y = OMe) with the *N*-terminal domain of yeast Hsp90.

2.1.3 Hsp90 inhibition and toxicity studies

As demonstrated by the calorimetric and co-crystallised X-ray studies, the 19-substituted derivatives bound to the *N*-terminal domain active site of Hsp90, but further proof was obtained from the common biomarkers used to gauge effective inhibition of Hsp90: A decrease in Hsp90 client proteins (including Raf1, Akt and CDK4) and a compensatory increase in other heat shock proteins (e.g. Hsp70 and Hsp27). Initially MDA-468-NQ16 cells that over-express the quinone reductase NQO1 were employed along with the 17-DMAG (alvespimycin) series. This is because previous work by Ross and co-workers demonstrated that NQO1 potentiates Hsp90 inhibition *via* reduction to the more active hydroquinone ansamycin.[5] Figure 5a shows that both 17-DMAG and 19-phenyl 17-DMAG are effective Hsp90 inhibitors as indicated by decreases in Hsp90 client proteins and induction of Hsp70. Interestingly, 19-phenyl 17-DMAG demonstrated somewhat superior activity on Hsp90 client proteins relative to the parent 19-unsubstituted 17-DMAG. The 19-methyl derivative was also found to be an effective Hsp90 inhibitor, with comparable, if not sometimes superior upregulation of heat shock proteins observed (Figure 5b-c).[2]

For proof of principle regarding the toxicity, the compounds were evaluated for their potential to react with nucleophiles at position 19. The parent compounds geldanamycin **1**, 17-AAG **2** and 17-DMAG **3** were reacted with the glutathione model *N*-acetylcysteine methyl ester under basic conditions (DBU, THF) and were found to form the corresponding 19-adducts in modest yield. The 19-substituted derivatives gave consistently no reaction, indicating that they were not prone to the reaction with such nucleophiles.[2] This was subsequently also demonstrated to be true with glutathione itself.[6]

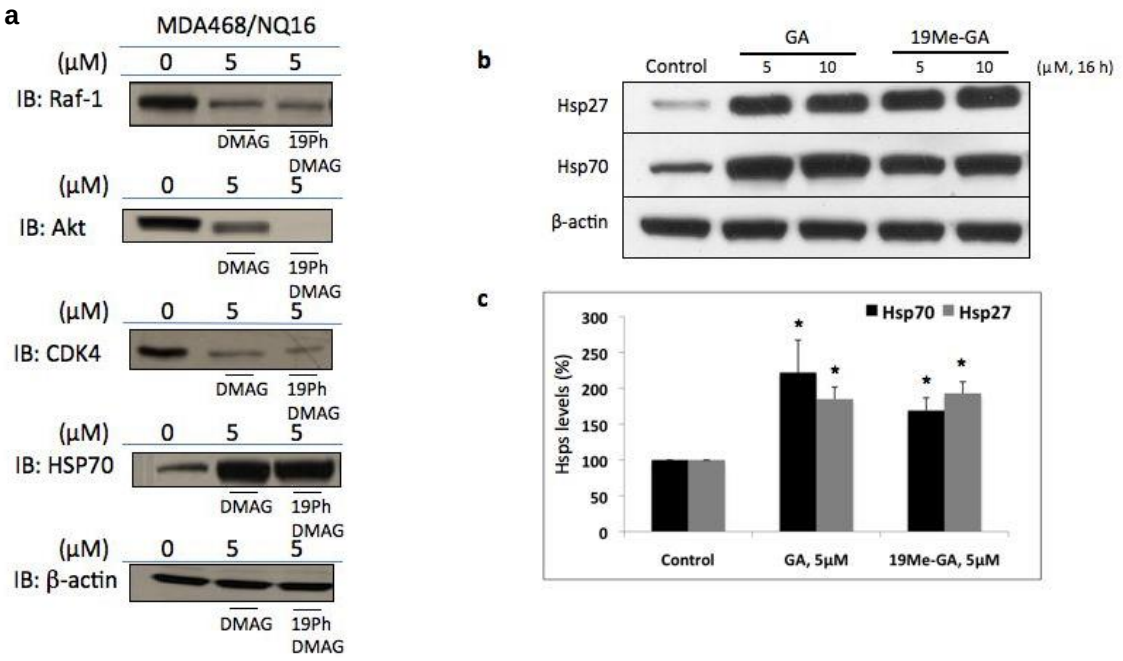


Figure 5. a, Immunoblot analysis of biomarkers of Hsp90 inhibition in MDA468-NQ16 human breast cancer cells treated with either 17-DMAG **3** or 19-phenyl 17-DMAG (**6**, R = Ph, Y = $CH_2CH_2NMe_2$) and immunoblotted for the Hsp90 clients Raf-1, Akt or CDK4 or Hsp70. Analysis of Raf-1 and Hsp70 was performed after 8 h while Akt and CDK-4 were at 48 h. Results are representative of 3 separate determinations. b, Immunoblot analysis of Hsp70 and Hsp27 induction in SH-SY5Y neuroblastoma cells following treatment with either geldanamycin or 19-methyl geldanamycin for 16 h. Results are representative of 3 separate experiments. c, quantitative data from immunoblot images. *, $p<0.05$; compared with control.

The compounds were then subjected to cellular toxicity evaluation compared to the parent compounds in normal endothelial and epithelial cellular systems. Employing human umbilical vein endothelial cells (HUVECs) and retinal pigmented epithelial cells (ARPE-19 cells), the data obtained indicated that for all series tested, 19-substitution

markedly reduces the toxicity. This suggested that the primary toxicity pathway is the conjugation of biological nucleophiles rather than the quinone redox cycling.[2]

2.1.4 Cancer studies

Since the parent compounds were clinically evaluated against various cancer lines, we tested the 19-substituted accordingly. Utilising the Her2-positive BT474 breast cancer cell line, both DMAG and 19-phenyl DMAG resulted in degradation of Hsp90 clients, including Her2, as well as inducing a compensatory increase in Hsp70.[2, 6] The reduced toxicity, coupled with the retained Hsp90 and growth-inhibition activity in human breast cancer cells gave the compounds great potential for further pre-clinical evaluation, albeit with somewhat diminished potency relative to the parent compounds.

2.1.5 Parkinson's studies

As previously mentioned, in addition to their clinical evaluation as anticancer agents, Hsp90 inhibitors have attracted attention as potential therapeutics for neurodegenerative diseases, particularly Parkinson's disease, due to their ability to induce a compensatory increase in other heat shock proteins that has been shown to be beneficial for protection against protein misfolding and aggregation. Using dopaminergic SH-SY5Y neuroblastoma cells (a cellular model for Parkinson's disease), we demonstrated that both geldanamycin and 19-phenyl geldanamycin induce Hsp70 and Hsp27 with high potency.[2, 7] The results obtained showed that the compounds have potential for use as neurodegenerative therapeutics, albeit with significant work still to be done around formulation and delivery of such a drug.

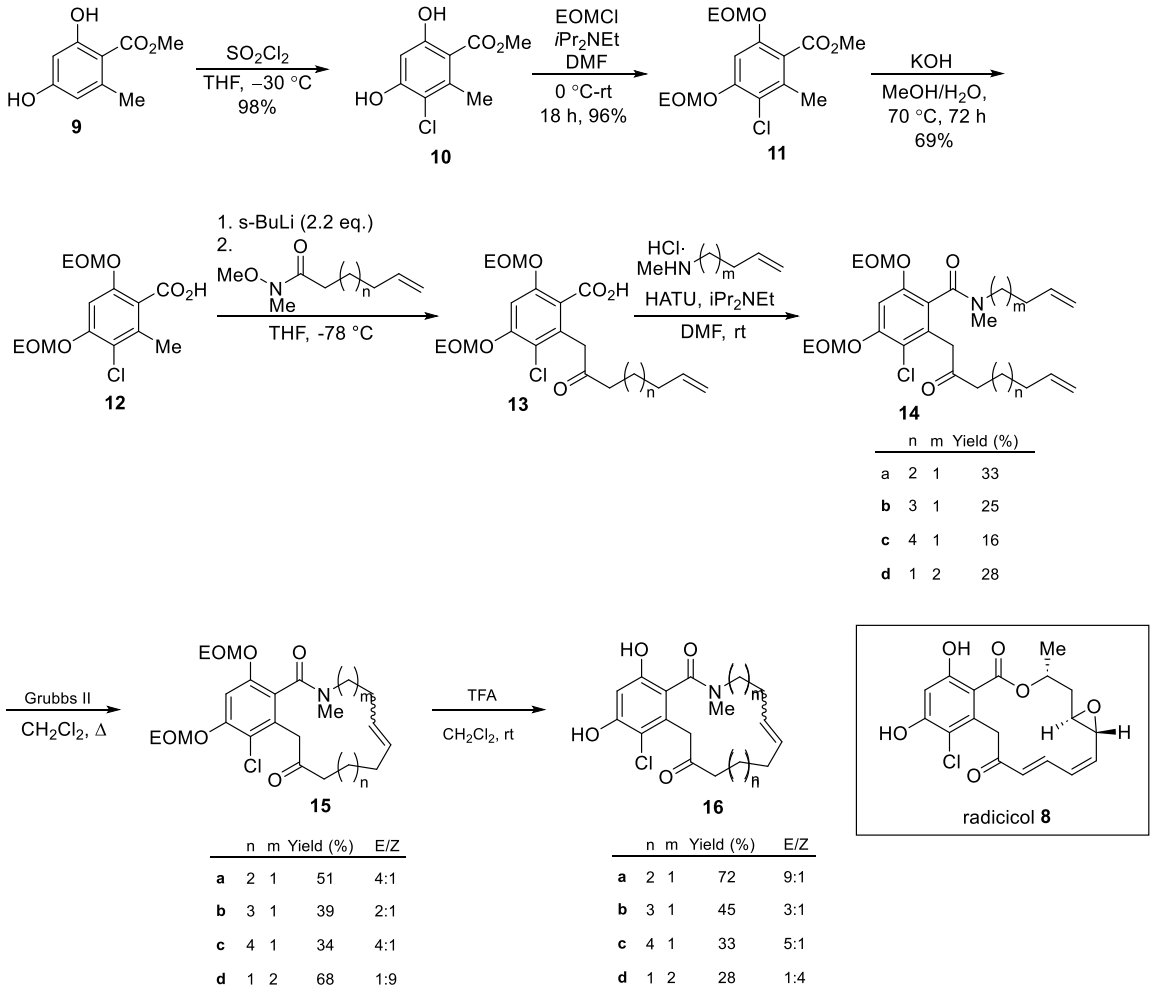
2.1.6 Other therapeutic studies

As mentioned above, Hsp90 inhibition has implications in pathways for HIV and malaria. In collaboration with Ariberto Fassati (UCL), the compounds were incorporated in studies for the link between Hsp90 and HIV reactivation from latency following antiretroviral therapy. Despite the 19-substituted compounds being much less active in provoking the response versus other Hsp90 inhibitors (including the parent compounds), the link was still established and the work published accordingly.[8]

In a similar fashion, the 19-substituted compounds were evaluated against malaria in collaboration with Didier Picard (University of Geneva), although this did not prove to be a fruitful line of investigation.

2.2 Radicicol

As outlined in Chapter 1, as well as geldanamycin, the unrelated natural product radicicol **8** has also been found to be a potent inhibitor of Hsp90, indeed it has inspired much research leading to several drug candidates being evaluated in clinical trials.[1] As part of our interest in Hsp90 inhibitors, we investigated the synthesis and Hsp90 binding of various simplified radicicol analogues, replacing the lactone with a lactam for greater metabolic stability, removing various sensitive functional groups and investigating the effect of varying the size of the macrocycle (Scheme 2).[9]



Scheme 2: Synthesis of macrolactam radicicol analogues **16** of varying ring size.

ITC showed that although the compounds **16** bound to Hsp90, there was a significant enthalpic penalty compared to radicicol itself, albeit the macrolactams were superior relative to previously described macrolactones (Table 2).[9] On co-crystalising the compounds with Hsp90, the resorcinol unit was located with similar interactions as radicicol, but the macrocycle exhibited a significant conformational change (Figure 6).[9] These results emphasise the importance of the radicicol epoxide interaction within the active site, work that further studies could be focussed on within this class of analogues.

Table 1. Isothermal titration calorimetry for macrolactams **16b-d** with yeast Hsp90 *N*-terminal domain.

Ligand	K_d/μM	ΔH/kJmol^{-1}	ΔS/ J mol^{-1}K^{-1}
Radicicol	0.015±0.06	-6726	13.6
16b	144±25	-1012	14.2
16c	41.8±8.7	-834.8	17.3
16d	110±7.9	-1466	13.3

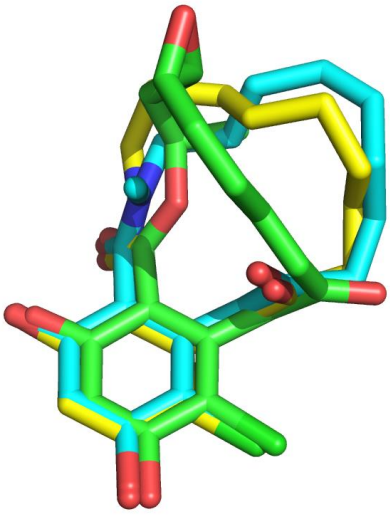


Figure 5. Overlaid crystal structures showing the difference in the conformation and binding of resorcylic acid macrolactams **16** in comparison to radicicol.

2.3 Summary, conclusions and future directions

In conclusion, a series of 19-substituted geldanamycin derivatives has been designed and two, somewhat complementary cross-coupling approaches for their synthesis has been developed. A range of coupling partners can be exploited to tune the 19-substituent as required and the 17-amino derivatives formed that correspond to the parent compounds that have been evaluated in the clinic for cancer therapeutics. Substitution at position 19

has in impact on the conformation of the molecules, forcing a switch from the extended S-shape of the parent molecules to the C-clamp conformation that has been documented upon binding to the Hsp90 *N*-terminal active site. This in turn affects the polarity of the molecules, with the hydrophilic moieties exposed to the solvent.

The 19-substituted geldanamycins were found to suppress the conjugate addition with nucleophiles and as such exhibit significantly reduced toxicity to healthy human cells, that were issues in the previous clinical trials. The compounds are still potent Hsp90 inhibitors, as judged by the heat shock response and depletion of client proteins, and, whilst there is a noticeable drop in binding affinity, the new derivatives can outperform the parent compounds in some aspects of both cancer and Parkinson's studies. This, coupled with the reduced toxicity, gives the substrates considerable potential for use as therapeutics in these areas.

The compounds were also evaluated in HIV and malaria studies but were found to be less effective versus the parent derivative control.

For the radicicol analogues, albeit still able to bind effectively, a pronounced change in the conformation of the macrocycle led to a considerable reduction in the binding affinity to the Hsp90 active site, emphasising the importance of the epoxide interactions for radicicol itself.

Future efforts will be devoted to furthering the potential for the 19-substituted geldanamycin derivatives for both cancer and Parkinson's therapeutics. This will see continued *in vivo* evaluation and optimisation of the 19-substituent for cancer treatment while the Parkinson's efforts will focus on addressing the polar surface area and requirements to cross the blood-brain barrier through structural optimisation.

There are also new directions for the research to take. Geldanamycin and the 17-amino derivatives have been found to be effective against various NRF2-activated cancer lines,

largely in view of the quinone moiety. The 19-substituted derivatives will be investigated in this regard as part of a new collaboration. Furthermore, geldanamycin was also initially found to have notable antimicrobial activity, so this will also be explored for the 19-substituted derivatives as potential new antibiotics.

Overall, with relevance to a wide range of therapeutic applications, as detailed herein, the 19-substituted geldanamycin class of compounds has considerable potential for medicinal use, publications and patents and attracting commercial interest.

2.4 Share of the author of this thesis for individual publications

Introduction section paper	First author (review article).[1]
Paper 1.	First author.[2]
Paper 2.	First author.[3]
Paper 3.	First and corresponding author.[4]
Paper 4.	Collaboration with the corresponding author, providing chemistry direction and research samples. Interpreting the data and contributing to writing the manuscript.[6]
Paper 5.	Collaboration with the corresponding author, providing chemistry direction and research samples. Interpreting the data and contributing to writing the manuscript.[7]
Paper 6.	Collaboration with the corresponding author providing research samples.[8]
Paper 7.	Corresponding author.[9]

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Chapter 3: Published Work