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Original article

Synthesis and structure–activity relationships of *N*-6 substituted analogues of 9-hydroxy-4-phenylpyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-diones as inhibitors of Wee1 and Chk1 checkpoint kinases

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Abstract

A series of *N*-6 substituted 9-hydroxy-4-phenylpyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-diones were prepared from *N*-substituted (5-methoxyphenyl)ethenylindoles. The target compounds were tested for their ability to inhibit the G2/M cell cycle checkpoint kinases, Wee1 and Chk1. Analogues with neutral or cationic *N*-6 side chains were potent dual inhibitors. Acidic side chains provided potent (average IC_{50} 0.057 μM) and selective (average ratio 223-fold) Wee1 inhibition. Co-crystal structures of inhibitors bound to Wee1 show that the pyrrolo[3,4-*c*]carbazole scaffold binds in the ATP-binding site, with *N*-6 substituents involved in H-bonding to conserved water molecules. HT-29 cells treated with doxorubicin and then target compounds demonstrate an active Cdc2/cyclin B complex, inhibition of the doxorubicin-induced phosphorylation of tyrosine 15 of Cdc2 and abrogation of the G2 checkpoint.

Keywords: Phenylpyrrolocarbazole; Wee1; Chk1; G2/M checkpoint abrogation

1. Introduction

There is currently wide interest in the development of drugs that can modulate the cell cycle checkpoints in eukaryotic cells. A key control mechanism at the G2/M checkpoint is inhibitory phosphorylation of Cdc2 Tyr15, mainly by the regulatory kinase Wee1 [1,2]. Removal of this inhibitory phosphate is mediated by the phosphatase Cdc25C, which itself is inactivated (following DNA damage) by Chk1/Chk2 mediated phosphorylation [3–5]. Thus inhibition of Wee1 and/or Chk1 kinases can result in abrogation of the G2 checkpoint induced by DNA damage, preferentially enhancing the cytotoxic effects of DNA damaging agents within p53-negative cells (which lack the ability to repair DNA damage at a functional G1/S checkpoint).

While several small molecule inhibitors of Chk1 have been reported, including the indolocarbazole staurosporine (**1**) [4], UCN-01 (**2**) [4], Go6976 (**3**) [6] and isogranulatimide (**4**) [7], the only substantive reports of Wee1 inhibitors concern 6-phenylpyrido[2,3-*d*]pyrimidin-7(8*H*)-ones, e.g., PD 166285 (**5**) [2] and phenyl-substituted analogues [8]. These compounds inhibit a significant number of kinases, most notably c-Src.

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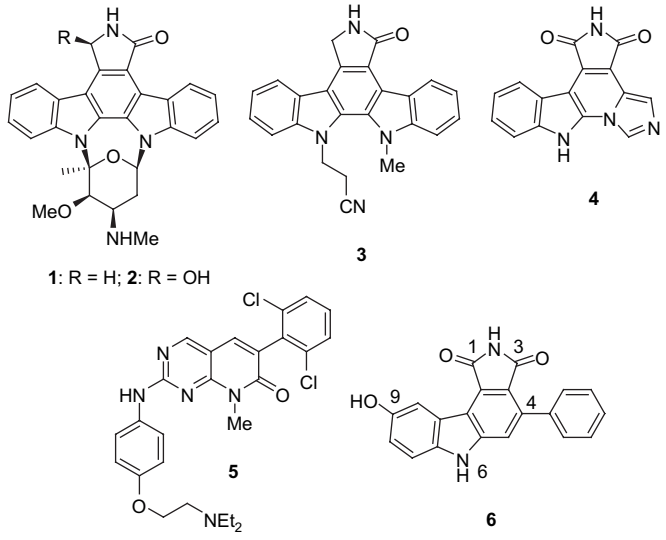
We recently reported that a series of 4-phenylpyrrolocarbazoles (e.g., **6**) were potent inhibitors of Wee1 (IC_{50} 97 nM) and Chk1 (IC_{50} 47 nM), without concomitant c-Src activity [9]. Structure–activity studies around this lead showed that small lipophilic groups (e.g., Cl) at the 2′-position of the pendant phenyl ring enhanced selectivity for Wee1, by increasing potency for Wee1 kinase (compare compounds **6** and **7** in Table 1) and lowering activity against the Chk1 enzyme. Larger lipophilic *N*-6 substituents were also found to improve selectivity for Wee1, again by reducing Chk1 activity (compare compounds **7**, **10** and **11**, Table 1). Since these substitutions did not appreciably alter Wee1 activity, this improved Wee1 selectivity was considered to be due to intolerance of hydrophobic bulk at this site in the Chk1 enzyme rather than favourable lipophilic interactions with Wee1. A general drawback of this series of Wee1 inhibitors was their poor aqueous solubility, and the above result suggested the possibility of utilising the *N*-6 site for the incorporation of hydrophilic, solubilising functionality without loss of potency. In this paper, we explore the influence of a variety of neutral, basic and acidic solubilising groups at the *N*-6 position of the 9-hydroxy-4-phenylpyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-diones on solubility, selectivity of Wee1 inhibition and G2/M checkpoint abrogation in vitro.



2. Chemistry

The 4-phenylpyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-dione ring system was generally constructed as reported previously [9] through Diels–Alder reactions between maleimide and phenyl-substituted dienes, followed by aromatisation. Introduction of side chains at what is eventually the carbazole *N*-6 position was best performed on the phenyl-substituted dienes (**70a–d**) [9,10] usually isolated as an *E*/*Z* mixture of alkene isomers, prior to construction of the 4-phenylpyrrolocarbazole ring system. This prevents an alkylation regioselectivity problem as the imide *N*-2 position is the preferred site of alkylation of the intact chromophore. Thus, **70a–d** [9] were reacted with various electrophiles, typically alkyl bromides, using sodium hydride as the base, to give for example the *N*-substituted indoles (**71a–d** and **75a–c**) (Scheme 1). Deprotection of the *tert*-butyldimethylsilyl-protected alcohols (**71a–d**) or tetrahydropyranyl-protected alcohols (**75a–c**) was carried out at this stage or after the Diels–Alder and aromatisation steps that form the 4-phenylpyrrolocarbazole ring system. To this end acid-promoted deprotection of **71c** and **75b**,**c** gave **72** and **76b**,**c**, respectively. Diels–Alder reaction of the *N*-substituted dienes (**71a**,**b**,**d**, **72**, **75a**, **76b**,**c**) with maleimide was performed in refluxing toluene, with a catalytic amount of tin (II) chloride, to give the respective Diels–Alder adducts as mixtures of diastereomers. These were aromatised directly using either excess activated MnO_2 or 2–5 equivalents of DDQ to give the *N*-6 substituted 4-phenylpyrrolocarbazoles, which were further subjected to acid-promoted deprotection where necessary to give **73a–d** and **77a–c**. Finally, 9-*O*-demethylation to give compounds **12–17** of Table 1 was achieved using either pyridine hydrochloride at 200 °C or 2–10 equivalents of BBr_3 in CH_2Cl_2 at room temperature.

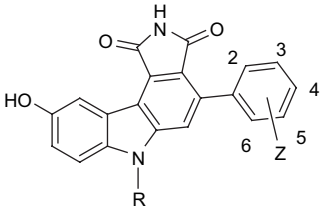
Similarly, alkylation of the diene (**70b**) with acrylonitrile and epibromohydrin gave nitrile **78** and epoxide **79**, respectively (Scheme 2). Treatment of the epoxide **79** with methylamine and dimethylamine gave **80** and **81**, respectively. Diels–Alder reaction of **78**, **80** and **81** followed by aromatisation and 9-*O*-demethylation then gave compounds **22**, **57** and **58** of Table 1.

We have previously reported [9] that late stage alkylation at the *N*-6 position could be achieved selectively by protection of the *N*-2 position with a 2,4-dimethoxybenzyl group, such as in 4-phenylpyrrolocarbazole **85** (Scheme 3). Potassium carbonate mediated alkylation of **85** with allyl bromide gave **86**, which was dihydroxylated with osmium tetroxide in the presence of *N*-methylmorpholine-*N*-oxide to give diol **87**. Imide deprotection was achieved with trifluoroacetic acid and anisole at 90 °C. Subsequent 9-*O*-demethylation with BBr_3 gave compound **26** of Table 1.

Compounds **27–53** of Table 1, bearing basic *N*-6 substituents tethered with an ethyl or propyl side chain, were synthesised by amine displacement of mesylates **88a–c** and bromides **89a–c**, respectively, via both singleton and array synthesis methods (Scheme 4). Mesylates **88a–c** were prepared by reaction of the ethyl alcohols (**77a–c**) with methanesulfonyl chloride, followed by 9-*O*-demethylation with BBr_3. Interestingly, application of the same procedure to the propyl alcohols (**73a–c**) gave predominantly the propyl bromides (**89a–c**), resulting from displacement of mesylate by bromide during the BBr_3 mediated demethylation. In instances where this displacement was incomplete, an additional treatment with lithium bromide in ethyl acetate at 50 °C was performed on the mixture to increase the yield of bromide.

Reaction of **89b** with sodium cyanide in DMSO gave compound **24** of Table 1 (Scheme 5), while reaction with sodium methoxide in refluxing methanol gave the corresponding methyl ether together with concomitant imide ring opening and subsequent anhydride formation, resulting in **90**. Treatment of **90** with ammonium formate at 140 °C gave compound **25** of Table 1.

Table 1
Inhibitory activity of 4,6-disubstituted 9-hydroxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-diones



No	R	Z	IC_{50} (μM)			Sol[b]	Calc. p*K*a[c]	Syn[d]
			Wee1[a]	Chk1[a]	Ratio			
Neutral N-6 side chains								
6[e]	H	H	0.097	0.047	0.48			
7[e]	H	2-Cl	0.011	0.44	40			
8[e]	H	2,6-diCl	0.028	1.2	43			
9[e]	Me	H	0.14	0.056	0.40			
10[e]	Me	2-Cl	0.057	0.22	3.9			
11[e]	*n*-Bu	2-Cl	0.059	35	593			
12	$(CH_2)_2OH$	H	0.025	0.088	3.5			S
13	$(CH_2)_2OH$	2-Cl	0.045	0.42	9.3	<3		S
14	$(CH_2)_2OH$	2,6-diCl	0.008	0.58	73	<3		S
15	$(CH_2)_3OH$	H	0.20	0.059	0.30	<3		S
16	$(CH_2)_3OH$	2-Cl	0.009	0.17	19	<3		S
17	$(CH_2)_3OH$	2,6-diCl	0.007	0.41	59	<3		S
18	$(CH_2)_3OH$	2-OMe	0.030	0.018	0.60	<3		S
19	$(CH_2)_2CONH_2$	H	0.021	0.035	1.7	<3		S
20	$(CH_2)_2CONH_2$	2-Cl	0.006	0.054	9.0			S
21	$(CH_2)_2CONH_2$	2,6-diCl	0.33	0.36	1.1	<3		S
22	$(CH_2)_2CN$	2-Cl	0.015	0.20	13	<3		S
23	$(CH_2)_2COOMe$	2-Cl	0.030	0.20	6.7	<3		S
24	$(CH_2)_3CN$	2-Cl	0.033	0.23	7.0			S
25	$(CH_2)_3OMe$	2-Cl	0.027	0.18	6.7	<3		S
26	$CH_2CH(OH)CH_2OH$	2-Cl	0.023	0.077	3.3	12		S
Basic N-6 side chains								
27	$(CH_2)_2NMe_2$	H	0.20	0.054	0.27	18	9.4	S
28	$(CH_2)_2NMe_2$	2-Cl	0.096	0.11	1.1	8.5	9.4	A
29	$(CH_2)_2NMe_2$	2,6-diCl	0.17	0.85	5.0	7.4	9.4	A
30	$(CH_2)_2$Nmorpholide	H	0.14	0.28	2.0	14	7.4	S
31	$(CH_2)_2$Nmorpholide	2-Cl	0.064	0.77	12	<3	7.4	A
32	$(CH_2)_2$Nmorpholide	2,6-diCl	0.11	4.3	39		7.4	A
33	$(CH_2)_2$Nimidazolide	H	0.23	0.25	1.1		7.1	S
34	$(CH_2)_2$Nimidazolide	2-Cl	0.092	0.27	2.9		7.1	A
35	$(CH_2)_2$Nimidazolide	2,6-diCl	0.12	3.4	28	<3	7.1	A
36	$(CH_2)_3NHMe$	H	0.28	0.002	0.01		10.6	S
37	$(CH_2)_3NHMe$	2-Cl	0.069	0.012	0.17	5.2	10.6	A
38	$(CH_2)_3NHMe$	2,6-diCl	0.11	0.005	0.05	7.3	10.6	A
39	$(CH_2)_3NMe_2$	H	0.36	0.021	0.06		9.6	S
40	$(CH_2)_3NMe_2$	2-Cl	0.10	0.029	0.29		9.6	A
41	$(CH_2)_3NMe_2$	2,6-diCl	0.14	0.096	0.69		9.6	A
42	$(CH_2)_3$Nmorpholide	H	0.29	0.38	1.3	<3	7.7	S
43	$(CH_2)_3$Nmorpholide	2-Cl	0.071	1.1	15		7.7	A
44	$(CH_2)_3$Nmorpholide	2,6-diCl	0.064	5.9	92		7.7	A
45	$(CH_2)_3$Nimidazolide	H	0.11	0.036	0.33	<3	7.2	S
46	$(CH_2)_3$Nimidazolide	2-Cl	0.054	0.055	1.0		7.2	A
47	$(CH_2)_3$Nimidazolide	2,6-diCl	0.059	1.3	22	3.2	7.2	A
48	$(CH_2)_3$N(4-Mepiperazine)	H	0.30	0.20	0.67	28	7.9	A
49	$(CH_2)_3$N(4-Mepiperazine)	2-Cl	0.082	0.83	10		7.9	A
50	$(CH_2)_3$N(4-Mepiperazine)	2,6-diCl	0.062	1.7	27		7.9	A
51	$(CH_2)_3NHPh$	H	0.093	0.21	2.3		4.9	S
52	$(CH_2)_3NHPh$	2-Cl	0.074	4.7	64		4.9	A
53	$(CH_2)_3NHPh$	2,6-diCl	0.067	0.73	11		4.9	A
54	$(CH_2)_2CONH(CH_2)_2NMe_2$	H	0.17	0.027	0.16	>60	8.9	S

Table 1 (*continued*)

No	R	Z	IC_{50} (μM)			Sol[b]	Calc. p*K*a[c]	Syn[d]
			Wee1[a]	Chk1[a]	Ratio			
55	$(CH_2)_2CONH(CH_2)_2NMe_2$	2-Cl	0.035	0.053	1.5	>60	8.9	S
56	$(CH_2)_2CONH(CH_2)_2NMe_2$	2,6-diCl	0.014	0.75	54	>60	8.9	S
57	$CH_2CH(OH)CH_2NHMe$	2-Cl	0.057	0.003	0.05		9.5	S
58	$CH_2CH(OH)CH_2NMe_2$	2-Cl	0.058	0.030	0.52	>60	8.9	S
Acids/acid isosteres/misc.								
59	$(CH_2)_2CO_2H$	H	0.023	0.41	18	>60	4.6	S
60	$(CH_2)_2CO_2H$	2-Cl	0.009	4.4	489	>60	4.6	S
61	$(CH_2)_2CO_2H$	2,6-diCl	0.39	>10	>26	33	4.6	S
62	$(CH_2)_2CONHSO_2Me$	2-Cl	0.012	2.5	209	>60	4.6	S
63	$(CH_2)_2CONHSO_2Ph$	2-Cl	0.007	8.0	1143	47	5.0	S
64	$(CH_2)_2C$tetrazole	2-Cl	0.021	1.4	67	>60	5.3	S
65	$(CH_2)_2S$triazole	2-Cl	0.024	0.42	18	3		S
66	$(CH_2)_2SO$triazole	2-Cl	0.009	0.12	13	18		S
67	$(CH_2)_2SO_2$triazole	2-Cl	0.019	0.077	4.1	16	6.6	S
68	$(CH_2)_3CO_2H$	2-Cl	0.013	0.12	9.2		4.8	S
69	$(CH_2)_3C$tetrazole	2-Cl	0.016	0.63	39	>60	5.4	S

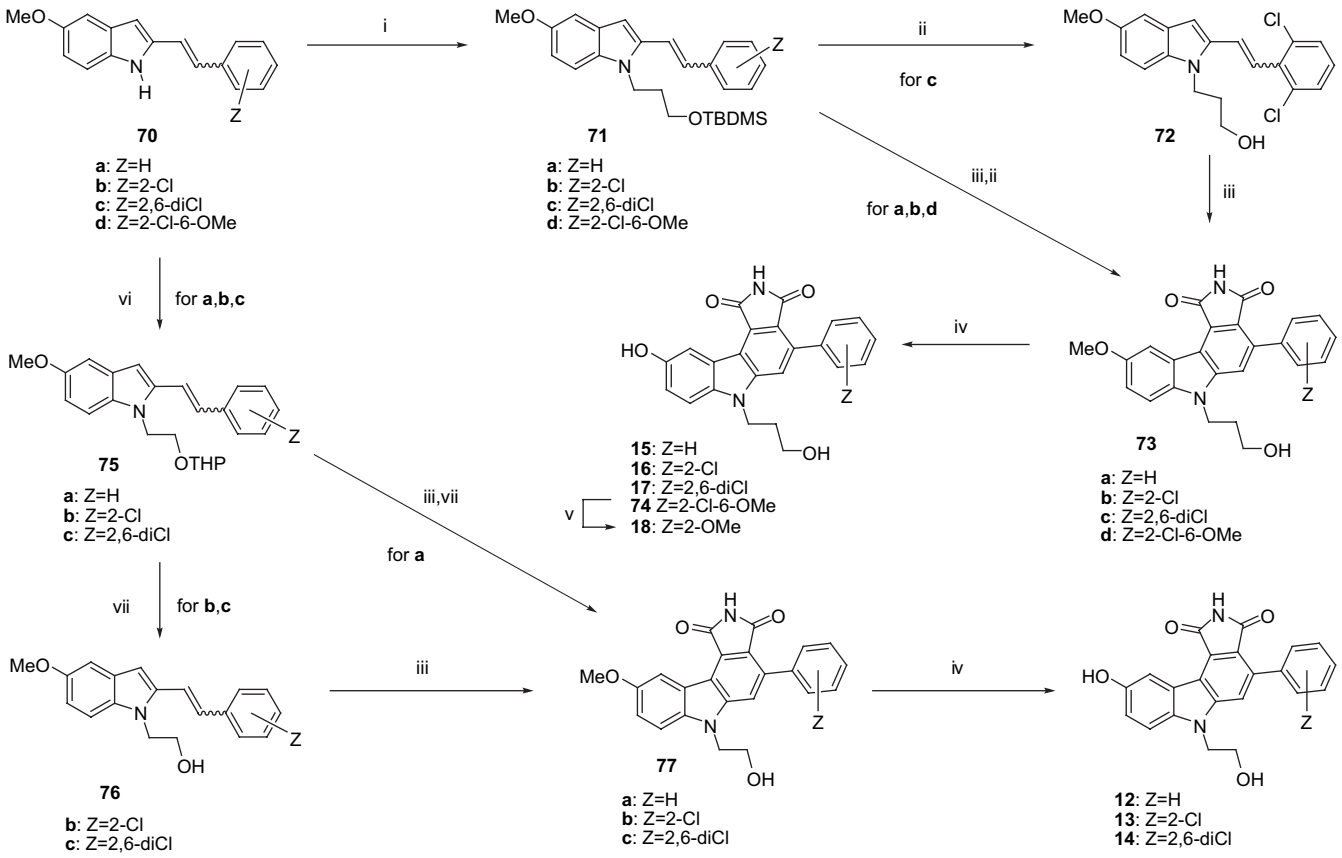
[a] IC_{50}: concentration of drug (μM) to inhibit the phosphorylation of a model polyornithine–tyrosine copolymer by Wee1 kinase and of a GST–Cdc25 substrate by Chk1 kinase. For active compounds, values are an average of two or more separate determinations; variation was generally ±30%.

[b] High throughput aqueous solubility screen (μg/mL) where <3 = poor solubility, 3–60 = moderate solubility, >60 = acceptable solubility.

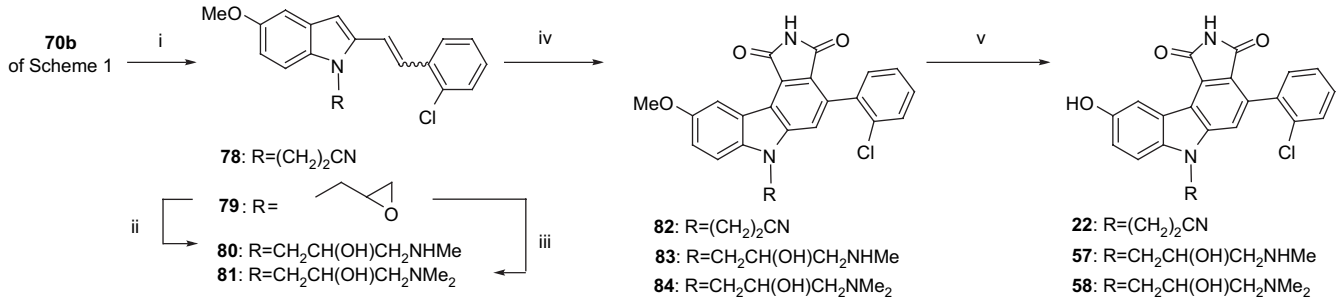
[c] Calculated using ACDLabs log *P* suite version 5.0 with the chromophore set as carbazole.

[d] S, singleton synthesis; A, array synthesis.

[e] Ref. [9].



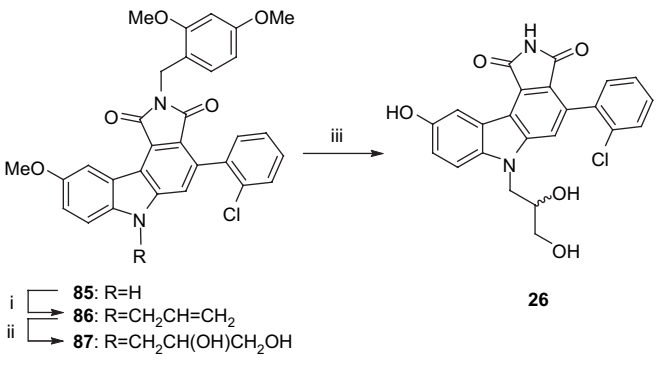
Scheme 1. (i) NaH, DMF, $Br(CH_2)_3OTBDMS$, rt; (ii) 1–3 N HCl, MeOH/co-solvent, rt; (iii) (a) maleimide, cat. $SnCl_2$, toluene, reflux; (b) MnO_2, *p*-dioxane, reflux or DDQ, toluene/*p*-dioxane, reflux; (iv) BBr_3, CH_2Cl_2, 0 °C to rt or pyridine·HCl, 200 °C; (v) H_2, Pd/C, MeOH/EtOAc, rt; (vi) NaH, DMF, $Br(CH_2)_2OTHP$, rt; (vii) *p*-TsOH, MeOH, 50 °C or 2 N HCl, MeOH/THF, rt.



Scheme 2. (i) DBU, CH_3CN, acrylonitrile, rt (for **78**); or NaH, DMF, epibromohydrin, rt (for **79**); (ii) $MeNH_2$, THF, 60 °C; (iii) Me_2NH, THF, 60 °C; (iv) (a) maleimide, cat. $SnCl_2$, toluene, reflux; (b) MnO_2, *p*-dioxane, reflux or DDQ, toluene/*p*-dioxane, reflux; (v) BBr_3, CH_2Cl_2, 0 °C to rt or pyridine·HCl, 200 °C.

Two-step oxidation of alcohols **73a–c** with Dess–Martin periodinane and then sodium chlorite gave the carboxylic acids (**91a–c**) (Scheme 6). Conversion of these to the acid chlorides with oxalyl chloride/DMF, followed by reaction with ammonia or *N*,*N*-dimethylethylenediamine, gave the primary amides (**92a–c**) and solubilised amides (**93a–c**), respectively. 9-*O*-Demethylation of the primary amides (**92a–c**) was best achieved with BBr_3, giving compounds **19–21** of Table 1 (pyridine hydrochloride at 200 °C caused significant amide hydrolysis). Conversely, 9-*O*-demethylation of the solubilised amides (**93a–c**) was best achieved with pyridine hydrochloride at 200 °C, giving compounds **54–56** of Table 1 (amide hydrolysis appeared to be slower, while formation of the HBr salt of starting material and subsequent insolubility was problematic when BBr_3 was used). 9-*O*-Demethylation of **91a–c** with either BBr_3 or pyridine hydrochloride at 200 °C gave compounds **59–61** of Table 1. Standard esterification of **60** in methanolic HCl gave compound **23** of Table 1. Alkylation of the diene **70b** [9] with ethyl bromobutyrate, followed by Diels–Alder reaction with maleimide, aromatisation with DDQ and ester saponification gave the acid **94**. Subsequent 9-*O*-demethylation with BBr_3 gave compound **68** of Table 1.

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI·HCl)-promoted coupling of methanesulfonamide and benzenesulfonamide with **91b** in the presence of 3 equivalents of 4-dimethylaminopyridine, gave the acyl sulfonamides (**95** and **96**, respectively) (Scheme 7). BBr_3 demethylation of these gave compounds **62** and **63** of Table 1.



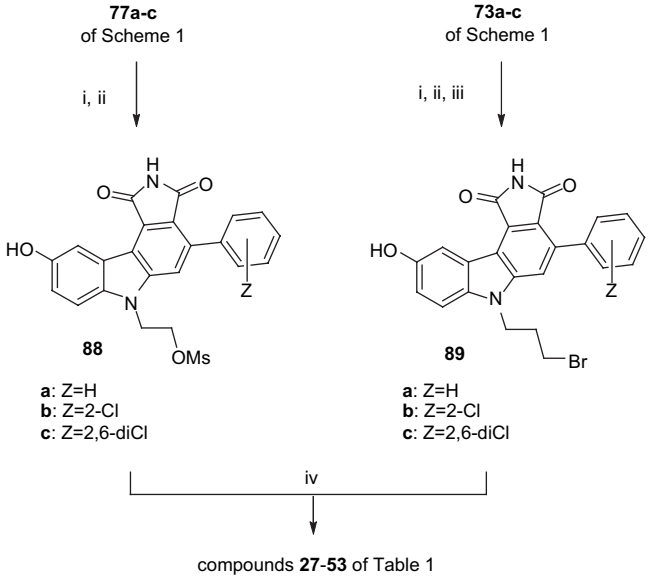
Scheme 3. (i) K_2CO_3, DMF, allyl bromide, 90 °C; (ii) OsO_4, NMMO, acetone/water, rt; (iii) (a) TFA, anisole, 90 °C; (b) BBr_3, CH_2Cl_2, 0 °C to rt.

Reaction of the nitriles (**22** and **24**) with azidotrimethyl tin in DMF/toluene at reflux gave tetrazoles **64** and **69** of Table 1, respectively. Mesylation of the alcohol **77b** to give mesylate **97** and subsequent displacement with 1*H*-1,2,4-triazole-5-thiol in *p*-dioxane at reflux gave the sulfur-linked triazole (**98**). 9-*O*-Demethylation of this with BBr_3 gave compound **65** of Table 1. Oxidation of **65** with 35% hydrogen peroxide in THF containing glacial acetic acid at either room temperature or for a prolonged period at 50 °C gave compounds **66** and **67** of Table 1, respectively.

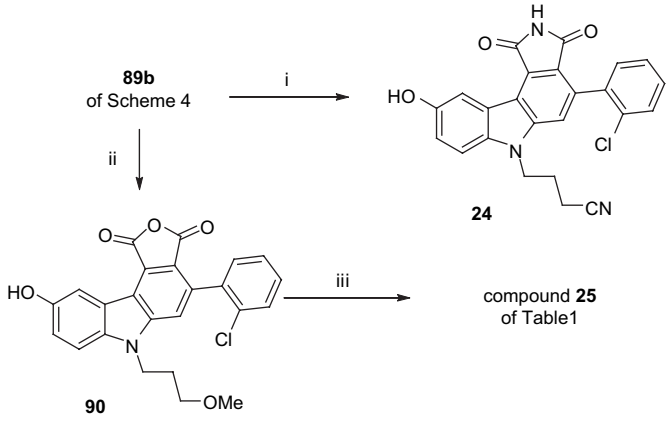
3. Results and discussion

3.1. Isolated enzyme activity

Table 1 gives the structures and enzyme inhibitory activities of 9-hydroxy-4-phenylpyrrolocarbazole analogues bearing a wide range of *N*-6 substituents. The goal of this work was to understand the influence of *N*-6 substitution on the potency and selectivity of Wee1/Chk1 inhibition, while providing compounds with sufficient solubility to be studied for their ability

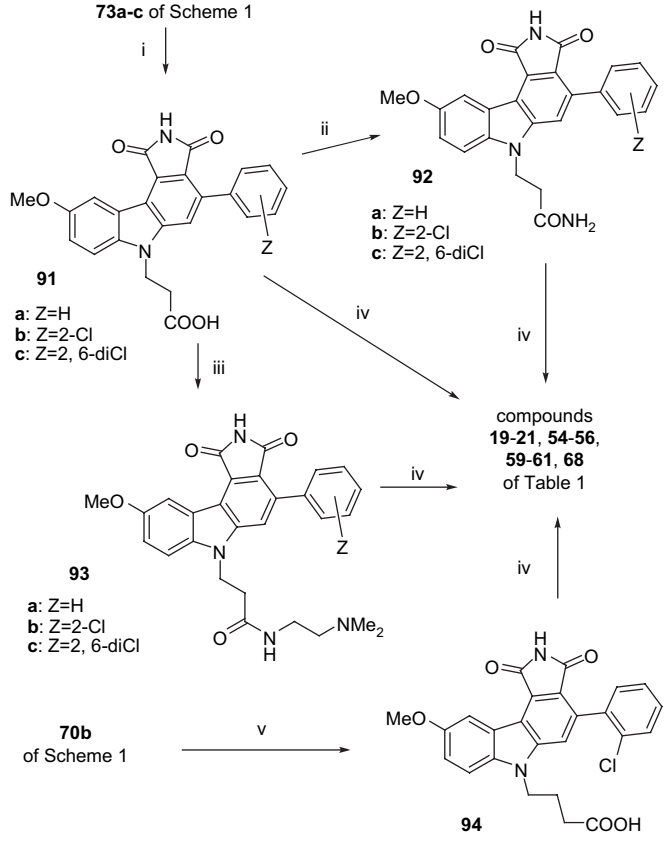


Scheme 4. (i) MsCl, Et_3N, THF, 0 °C; (ii) BBr_3, CH_2Cl_2, 0 °C to rt; (iii) LiBr, EtOAc, 50 °C; (iv) R_1R_2NH, DMA, rt to 80 °C.

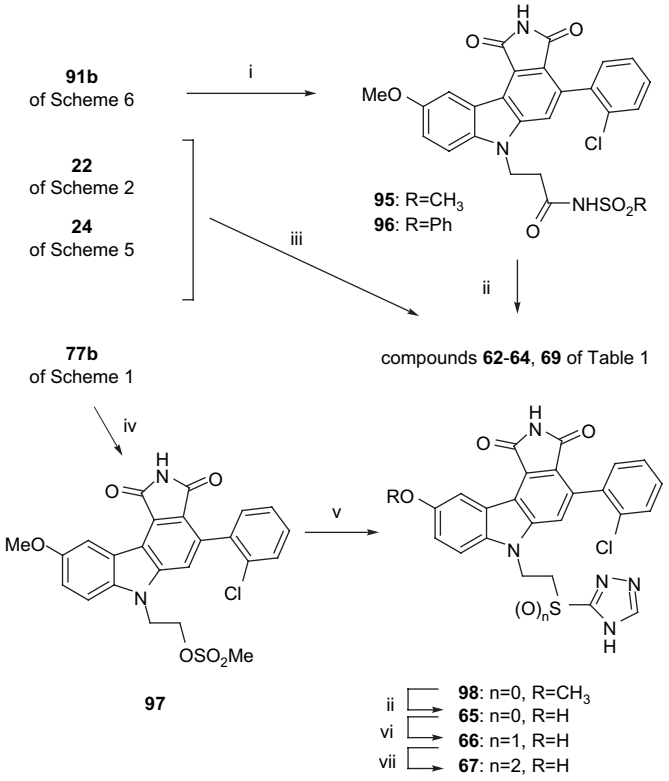


Scheme 5. (i) NaCN, DMSO, rt; (ii) NaOMe, MeOH, reflux; (iii) NH_4OAc, 140 °C.

to abrogate the G2/M checkpoint in vitro. Wee1 selective and dual Wee1/Chk1 analogues were desired for comparative purposes. We have previously reported [9] that substitution of the 4-phenyl ring with 2′-chloro or 2′,6′-dichloro substituents increases potency for Wee1, and selectivity over Chk1. While a broad range of phenyl substituents was explored in the



Scheme 6. (i) (a) Dess–Martin periodinane, THF, rt; (b) $NaClO_2$, 2-methyl-2-butene, NaH_2PO_4, *t*-BuOH/THF/water, rt; (ii) (a) ClCOCOCl, DMF, THF, rt; (b) NH_3, THF, rt; (iii) (a) ClCOCOCl, DMF, THF, rt; (b) $Me_2N(CH_2)_2NH_2$, THF, rt; (iv) pyridine·HCl, 200 °C or BBr_3, CH_2Cl_2, 0 °C to rt; (v) (a) $Br(CH_2)_3COOEt$, NaH, DMF, rt; (b) maleimide, cat. $SnCl_2$, toluene, reflux; (c) DDQ, *p*-dioxane, reflux; (d) 2 M KOH, MeOH, rt.



Scheme 7. (i) $CH_3SO_2NH_2$ ($PhSO_2NH_2$), DMAP, EDCI·HCl, DMF, rt; (ii) BBr_3, CH_2Cl_2, 0 °C to rt; (iii) Me_3SnN_3, DMF/toluene, reflux; (iv) MsCl, Et_3N, THF, 0 °C; (v) 1*H*-1,2,4-triazole-5-thiol, Et_3N, *p*-dioxane, reflux; (vi) 35% H_2O_2, HOAc, THF, rt; (vii) 35% H_2O_2, HOAc, THF, rt to 50 °C.

present study our findings were consistent with the previously reported SAR for the 4-phenyl ring. Therefore only phenyl, 2′-chloro and 2′,6′-dichloro are reported herein, with the notable exception of a single 2′-methoxy analogue (**18**), which was successfully co-crystallised with a construct of Wee1 providing valuable information about interactions between the kinase ATP-binding site and the 2′-methoxy and *N*-6-substituents (see later).

As noted above, previous studies had suggested that larger lipophilic *N*-6 substituents allowed retention of nanomolar Wee1 activity, while also improving selectivity with respect to Chk1 activity (compare compounds **7**, **10** and **11**). Compounds **12–26** bearing neutral substituents of increased polarity also showed retention of nanomolar Wee1 activity, but these substituents were less effective at retaining high selectivity over Chk1 (compare **11** with **16**, **24** and **25**). A number of analogues bearing alcohol (**14**, **16** and **17**) or primary amide (**20**) functionalities possessed very potent activity for inhibition of Wee1 (IC_{50} values of 6–9 nM), but these neutral polar groups did not confer significant increases in aqueous solubility.

Compounds **27–58** explore a more extensive range of cationic *N*-6 side chains, again in combination with 2′-chloro and 2′,6′-dichloro substitution of the 4-phenyl ring. Introduction of a cationic *N*-6 side chain resulted in similar or modestly reduced (<2-fold) Wee1 potency, when compared to the corresponding *N*-6 methyl substituted compound. The nature of

the pendant amine, in terms of steric bulk or pKa, had little influence. Conversely, increased inhibition of Chk1 was noted with small, strongly basic amine substituents. Methylamine pendant on a three carbon chain provides a particular potency advantage (see compounds **36**–**38**). It is interesting to note that the known Chk1 inhibitory natural product staurosporine (**1**) and its derivative UCN-01 (**2**) also possess a *C*-3 methylamine motif (as part of a glycosyl moiety) in this relative position. The reported crystal structure of UCN-01 bound in the ATP-binding site of Chk1 [11] indicates that the glycosyl moiety is positioned in the ribose-binding pocket, with the protonated methylamine residue forming hydrogen bonds to Glu91 and Glu134. It seems likely that a similar interaction between the methylamine moieties of compounds **36**–**38** and the Chk1 protein are providing the potency advantage observed for these compounds. Compounds **54**–**58** of Table 1 represent an attempt to combine the preferred alcohol and amide functionalities discussed above with cationic side chains capable of providing improved solubility. These compounds were potent dual inhibitors of Wee1 and Chk1, with the exception of **56**, which was a selective Wee1 inhibitor (54-fold). The aqueous solubility of amines **27**–**53** at pH 6.9 was generally poor, while the combination side chains **54**–**58** gave improved solubility. Most of the amines did show improved solubility when measured at lower pH (data not shown), with compound **55**, for example, having a solubility of 1.2 mg/mL at pH 5.4 (acetate buffer).

Compounds **59**–**64** and **67**–**69** of Table 1 contain acid and acid isostere bearing side chains, with calculated pKa values ranging from 4.6 to 6.6. Compounds **65** and **66** are intermediates in the synthesis of compound **67** and were also included for kinase screening. The *C*-2 carboxylic acids (**59**–**61**) explored the effect of phenyl ring substitution in this series, while compounds **62**–**69** contained the 2′-chlorophenyl substitution pattern that provides the highest Wee1 potency. As a sub-class, the compounds bearing an acidic side chain (**59**–**64**, **67**–**69**) had the highest potency against Wee1 (average IC_{50} 0.057 μM) and the highest Chk1/Wee1 ratios (average ratio 223-fold). This finding is consistent with unfavourable electrostatic interactions between the acidic side chains of the inhibitors and the carboxylate side chains of Glu91 and Glu134 present in this region of the Chk1 protein [11]. Several of these compounds also had good solubilities.

3.2. Crystal structures of N-6 substituted inhibitors bound to Wee1 kinase

We have previously reported the structure, at 1.8 Å resolution, of an inhibitor bound co-crystal of **6** and the $Wee1^{291-575}$ construct containing residues 291–575 of the human Wee1 enzyme [12]. The pyrrolocarbazole ring system binds in the ATP-binding site of the kinase with the pendant 4-phenyl ring twisted out of the plane of the chromophore (Fig. 1a). Key hydrogen bond contacts are made between the 9-hydroxyl group (donor) and Cys379(CO), the 1-carbonyl group (acceptor) and Cys379(NH), the 2-NH imide group (donor) and Glu377(CO) and the 3-carbonyl group (acceptor) and

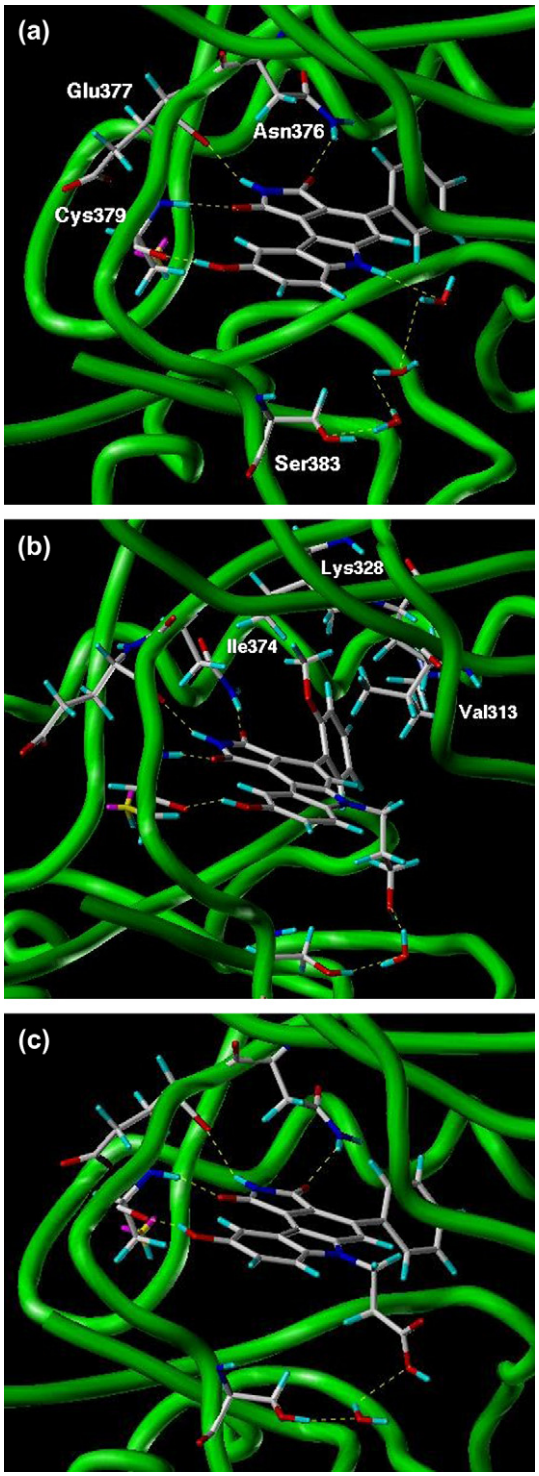


Fig. 1. Crystal structures of compound **6** [12] (a), **18** (b) and **59** (c) bound in the ATP-binding site of a construct of Wee1 kinase. Important hydrogen bonds between the ligand, protein and ordered water molecules in the active site are indicated with dotted orange lines (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Asn376 side chain. Of particular relevance to this discussion is that the carbazole 6-NH group donates an H-bond to the first of an ordered lattice of three water molecules that link through to Ser383. The 5-fold loss of potency observed following

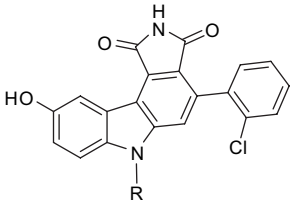
simple alkyl substitution at this *N*-6 position (compare **7** with **10** and **11**) is consistent with unfavourable disruption of this lattice. We determined the co-crystal structures of both the *N*-6 propyl alcohol **18** (Fig. 1b) and the *N*-6 propanoic acid **59** (Fig. 1c) with the Wee1$^{291-575}$ construct, to 2.2 Å and 1.9 Å resolution, respectively. Both inhibitors form identical H-bond contacts from the pyrrolocarbazole chromophore to the protein as does **6**. As predicted by modelling studies [9] the 2′-methoxy group on the 4-phenyl ring of **18** is directed towards a lipophilic pocket bounded by Ile374, Lys328 and Val313. In both structures, the presence of an *N*-6 substituent has resulted in displacement of the first two ordered water molecules seen in the structure of **6**. Displacement of the second ordered water by the terminal oxygen functionality in the *N*-6 side chain places this oxygen atom (in both inhibitors) in a position to form an H-bond contact with the remaining third ordered water, which bridges to Ser383. This finding is consistent with the most potent *N*-6 substituted Wee1 inhibitors of Table 1 generally possessing an H-bond acceptor as the fourth atom of the substituent chain (compare **10** with **16**, **20**, **22**, **60**, **63**, **66**).

3.3. Cell-based assays for target modulation

To study the influence of the *N*-6 substituents on cellular activity, a subset of compounds from Table 1 were selected. The 4-phenyl substituent was kept constant as 2′-chloro (on average the most potent for Wee1 inhibition). The *N*-6 substituents included neutral hydrophilic (compounds **16**, **20**, **22**), basic (compounds **31**, **37**, **55**, **58**) and acidic moieties (compounds **62**, **64**, **68**, **69**). The compounds selected ranged from 6-fold Chk1 selective to 209-fold Wee1 selective.

The compounds were evaluated in four in vitro assays measuring target modulation and G2 checkpoint abrogation in HT-29 cells (p53-negative) following sub-lethal DNA damage by doxorubicin. The histone H1 kinase assay (HKA) has been employed by Suganuma et al. to characterise the G2/M checkpoint abrogation of peptide inhibitors of human Chk1 [3]. This assay indirectly measures the effect of test compounds on the kinase activity of the Cdc2/cyclin B complex for phosphorylation of histone H1, one of its physiological substrates. Thus strong phosphorylation of histone H1 represents an active Cdc2/cyclin B complex, where Tyr15 on Cdc2 is non-phosphorylated. Wee1 inhibition blocks the phosphorylation of Tyr15 on Cdc2, directly resulting in an active Cdc2/cyclin B complex, while Chk1 inhibition blocks phosphorylation of the phosphatase Cdc25C, ensuring it remains active to cleave the phosphate from Tyr15 of Cdc2, also resulting in an active Cdc2/cyclin B complex. The IC_{50} gives the concentration of drug required to permit a 50% activation of the Cdc2/cyclin B complex in cells, compared to controls (Table 2). This is reported as 50% inhibition of the regulatory kinases of the Cdc2/cyclin B complex. The mitotic index assay used a polyclonal antibody to quantify the M-phase specific histological markers, which were used to determine the fraction of cells (% of total) found in mitosis (Table 2). Thirdly, Western blots were used to directly assess the Tyr15 phosphorylation (PY15) on Cdc2 kinase (the natural

Table 2
In vitro data for selected *N*-6-substituted 4-(2-chlorophenyl)-9-hydroxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-diones



No	R	IC_{50} (μM)				Mitotic index[d] (%)
		Wee1[a]	Chk1[a]	HKA[b]	PY15[c]	
16	$(CH_2)_3OH$	0.009	0.17	0.65	0.25–2.5	62 at 1.25 μM
20	$(CH_2)_2CONH_2$	0.006	0.054	>3.4	>10	47 at 2.50 μM
22	$(CH_2)_2CN$	0.015	0.20	>3.4	<0.25	53 at 2.50 μM
31	$(CH_2)_2$Nmorpholide	0.064	0.77	1.7	0.25	65 at 2.50 μM
37	$(CH_2)_3NHMe$	0.069	0.012	0.71	<0.25	59 at 1.25 μM
55	$(CH_2)_2CONH(CH_2)_2NMe_2$	0.035	0.053	1.3	<0.25	65 at 2.50 μM
58	$CH_2CH(OH)CH_2NMe_2$	0.058	0.030	>3.4	0.25	81 at 2.50 μM
62	$(CH_2)_2CONHSO_2Me$	0.012	2.5	>3.4	0.25	Not active
64	$(CH_2)_2$Ctetrazole	0.021	1.4	>3.4	2.5	Not active
68	$(CH_2)_3COOH$	0.013	0.12	>3.4	>10	30 at 2.50 μM
69	$(CH_2)_3$Ctetrazole	0.016	0.63	>3.4	>10	Not active

[a] As for Table 1.
[b] Histone H1 kinase assay; IC_{50} is the concentration of drug (μM) to inhibit the phosphorylation of histone H1 by Cdc2/cyclin B in HT-29 cells.
[c] IC_{50} is the approximate concentration of drug required to produce a 50% reduction in phosphorylation of tyrosine 15 on Cdc2 kinase in HT-29 cells (from western blots).
[d] Fraction of doxorubicin pre-treated HT-29 cells (% of total) found in mitosis after treatment with an abrogator at the stated concentration.

Wee1 substrate) using a phosphospecific antibody (the signal was normalised to the total amount of Cdc2 detected). Here the IC_{50} value gives the approximate concentration of drug required to produce a 50% reduction of this phosphorylation in HT-29 cells, compared to controls (Table 2). Finally, the clonogenic survival of HT-29 cells that were treated with selected compounds of Table 1 (at a range of doses), with and without doxorubicin, was also measured (Fig. 2).

Compounds with neutral *N*-6 side chains (**16**, **20**, **22**) had poor HKA activity, with the exception of **16**, which had an IC_{50} of 0.65 μM. Compound **16** also gave a robust abrogation of the G2 checkpoint at a concentration of 1.25 μM, as measured in the mitotic index assay, with 62% of doxorubicin-damaged cells found in mitosis. This compound inhibited Cdc2 Tyr15 phosphorylation with an IC_{50} between 0.25 and 2.5 μM and gave a 45% reduction in clonogenic survival selectively in doxorubicin treated HT-29 cells at a non-toxic dose of 1 μM (Fig. 2).

Compounds with basic *N*-6 side chains (**31**, **37**, **55**, **58**) showed improved HKA activity. This is likely to be due to improved solubility and cellular uptake. All of these compounds showed potent inhibition of Cdc2 Tyr15 phosphorylation with approximate IC_{50}s of 0.25 μM or less, and abrogation of the G2 checkpoint in combination with doxorubicin. In the latter assay, the dual Wee1/Chk1 inhibitor **37** was the most dose-potent compound, giving 59% of cells in mitosis at 1.25 μM, while **31**, **55** and **58** gave 65, 65 and 81% of cells in mitosis, respectively, at 2.5 μM (Table 2). Compounds **31** (12-fold Wee1 selective) and **55** (dual Wee1/Chk1 inhibitor) were studied in the clonogenic survival assay. Both showed significant cytotoxicity as single agents at the highest dose tested (10 μM), while compound **31** appeared to have the better therapeutic index, giving a 77% reduction in clonogenic survival selectively in doxorubicin treated HT-29 cells at a non-toxic dose of 2.5 μM (Fig. 2).

Compounds with acid and acid isostere *N*-6 side chains (**62**, **64**, **68**, **69**) were uniformly inactive in the cell-based HKA assay. While **62** and **64** showed inhibition of phosphorylation of Cdc2 Tyr15 with approximate IC_{50}s of 0.25 μM and 2.5 μM, respectively, compounds **68** and **69** were inactive in this assay. All the compounds were essentially inactive in the mitotic index assay. Compounds **62**, **63** and **69** were also evaluated in the clonogenic survival assay; both **62** and **63** were inactive over all concentrations tested, while **69** was too toxic as a single agent to draw any conclusions on G2 checkpoint abrogation (Fig. 2). The general lack of activity for this anionic sub-class of compounds across a range of cellular assays may be due to poor cellular permeability or protein binding, typical of acid moieties.

3.4. Kinase selectivity

Selected compounds were screened against a panel of 28 kinases at a concentration of 10 μM [13], as shown in Table 3. Compounds **62** and **69** bearing acidic *N*-6 side chains were the most selective for Wee1. Compounds bearing alcohol and amine *N*-6 side chains (**7**, **16**, **17**, **36**, **37**, **55**) were less selective, showing inhibition of a number of other kinases. As a class, the *N*-6 substituted 9-hydroxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-diones appear also to be inhibitors of MAPK-activated protein kinase 1 (MAPKAP-K1a), AMP-activated protein kinase (AMPK) and checkpoint kinase 1 (Chk1) (as described above). A number of compounds tested also showed activity against 3-phosphoinositide-dependent protein kinase 1 (PDK1), phosphorylase kinase and lymphocyte kinase (Lck). Representative compounds were also screened against c-Src, and as reported previously [9] for compound **6**, nearly all were inactive (IC_{50}s: **7**, >50; **26**, 2.5; **27**, 31; **33**, 27; **39**, 5.6; **42**, 46; **48**, 17; **59**, 13 μM). Introduction of side chains at the *N*-6 position of the 4-phenylpyrrolocarbazole chromophore allows access to the ribose-binding pocket within the ATP-binding domain. It would appear that appending polar functionality in this region from a relatively flexible chain might be counter-productive in terms of kinase selectivity. This lack of selectivity may in part be responsible for the single agent

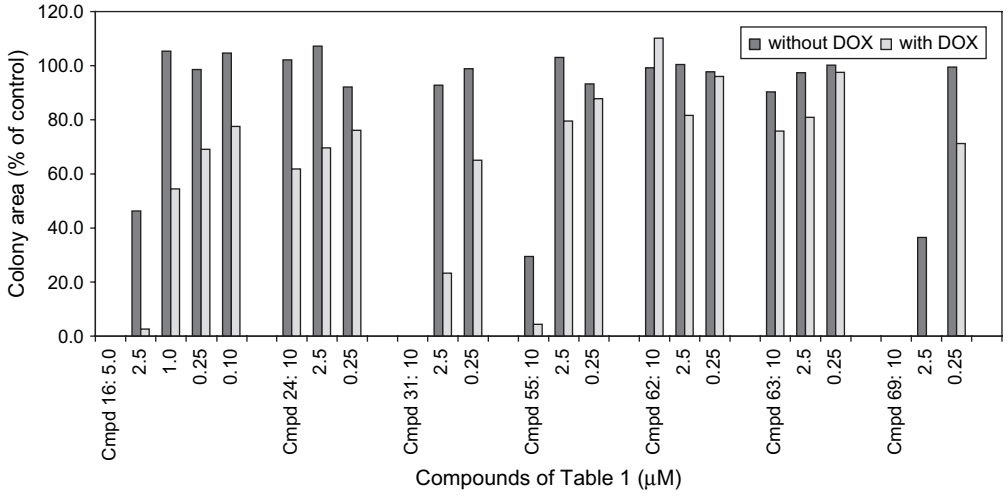


Fig. 2. Soft agar clonogenic assay of HT-29 cells treated with compounds of Table 1, with and without doxorubicin.

Table 3
Kinase counter-screening of representative compounds

Compounds of Table 1	Kinase[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
7	49	54	84	52	69	53	95	13	93	58	6	72	58	0	26	19	44	35	29	8	2	101	8	11	33	38	17	2
16	13	36	83	50	49	18	72	NT	93	25	58	33	20	0	12	37	36	4	38	4	11	104	18	5	28	38	79	1
17	72	14	43	25	13	2	14	6	90	33	21	65	12	11	55	11	26	37	13	4	6	95	8	22	21	4	14	4
36	9	11	50	81	83	77	95	5	100	3	76	49	5	0	12	5	7	61	20	0	1	62	1	13	82	14	29	45
37	20	42	51	73	61	51	62	0	71	3	102	5	0	0	2	20	6	72	22	0	5	88	1	27	52	10	28	30
55	60	114	99	111	108	125	106	4	90	27	98	8	39	24	15	41	51	93	100	0	4	111	32	8	92	48	78	46
62	21	54	79	83	89	62	96	46	102	81	55	77	32	60	63	67	52	17	58	18	7	100	72	16	60	54	22	27
69	12	101	88	87	99	96	110	11	100	59	66	48	63	13	31	3	66	1	112	14	1	103	61	17	45	27	94	29

Kinase activity at 10 μM as a % of non-treated control, where red = 20% or less; yellow = 21–50%; and green = 51% or greater (actual values in cell) (for interpretation of the references to colour in this legend, the reader is referred to the web version of this article). Kinases tested [13]: (1) MKK1; (2) MAPK2/ERK2; (3) JNK/SAPK1c; (4) SAPK2a/p38; (5) SAPK2b/p38β2; (6) SAPK3/p38γ; (7) SAPK4/p38δ; (8) MAPKAP-K1a; (9) MAPKAP-K2; (10) MSK1; (11) PRAK; (12) PKA; (13) PKCα; (14) PDK1; (15) PKBα; (16) SGK; (17) S6K1; (18) GSK3β; (19) ROCK-II; (20) AMPK; (21) Chk1; (22) CK2; (23) PHOS. kinase; (24) Lck; (25) CSK; (26) CDK2/cyclinA; (27) CK1; (28) DYRK1A.

cytotoxicity observed for some of these compounds in the clonogenic survival assay described above.

4. Conclusions

The influence of a range of neutral, basic and acidic solubilising groups at the *N*-6 position of the 9-hydroxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-dione class of Wee1/Chk1 kinase inhibitors has been investigated in combination with previously described optimal substitutions in the 4-phenyl ring [9]. As would be expected, the most basic or acidic side chains provided the biggest improvements in aqueous solubility. Compounds bearing neutral (hydrophilic) or amine side chains were dual inhibitors of Wee1 and Chk1, although this could be influenced by appropriate substitution in the 4-phenyl ring, with 2′-chloro and 2′,6′-dichloro analogues, respectively, being successively more Wee1 selective. Compounds with acidic side chains were the most selective for Wee1 over Chk1. Crystal structures of compounds **6**, **18** and **59** bound in the ATP-binding domain of Wee1 kinase have highlighted an organized lattice of water molecules bridging from the carbazole 6-NH group of the unsubstituted inhibitor **6** through to Ser383. Favourable displacement of (or H-bonding interaction to) water molecules in this lattice by an *N*-6 side chain provides improved potency for inhibition of Wee1 kinase. Examples of both *N*-6 alcohol and *N*-6 amine bearing compounds (but not the acidic compounds), at concentrations ranging from 0.25 to 2.5 μM, resulted in an active Cdc2/cyclin B complex in doxorubicin treated HT-29 cells, capable of phosphorylating histone H1, abrogated the G2 checkpoint and inhibited Cdc2 Tyr15 phosphorylation. They also produced a reduction in clonogenic survival selectively in doxorubicin treated HT-29 cells. Counter-screening in a panel of 28 kinases indicated significant off-target potency. Further studies to improve the aqueous solubility, cellular activity and kinase counter-screening profile of this class of Wee1/Chk1 kinase inhibitors will be reported in due course.

5. Experimental protocols

Combustion analyses were performed by the Microchemical Laboratory, University of Otago, Dunedin, NZ. Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values. Melting points were determined using an Electrothermal Model 9200 digital melting point apparatus, and are as read. NMR spectra were measured on a Bruker DRX-400 spectrometer or a Bruker Avance-400 spectrometer, and are referenced to Me_4Si. High resolution mass spectra were recorded on a Varian VG-70SE spectrometer at nominal 5000 resolution. Liquid chromatography-mass spectrometry (LCMS) was performed on an Agilent 1100 LC system interfaced with an Agilent MSD mass detector. Mass detection was performed with an APCI source, using simultaneous positive and negative ion acquisition. Compound purities were determined by HPLC using simultaneous diode array UV detection. Unless otherwise indicated, compounds were purified by flash column chromatography on Silica gel 60 support (Scharlau, 230–400 mesh ASTM), using the indicated eluents.

5.1. Procedures of Scheme 1

5.1.1. Procedure 1: sodium hydride mediated indole alkylation

Sodium hydride (1.05 g of a 50% dispersion in mineral oil, 0.022 mol) was added to a solution of 2-[(*E*,*Z*)-2-(2-chlorophenyl)ethenyl]-5-methoxy-1*H*-indole (**70b**) [9] (4.13 g, 0.014 mol) in DMF (30 mL) and the solution was stirred at room temperature for 5 min. (3-Bromopropoxy)-*tert*-butyldimethylsilane (4.04 g, 0.016 mol) was added and stirring was continued for 2 h. The solution was diluted with water and extracted with EtOAc. The combined organic layers were washed well with brine and the organic phase was dried over anhydrous Na_2SO_4. The drying agent was removed by filtration and the solution was concentrated to dryness, to

give 1-(3-{[*tert*-butyl(dimethyl)silyl]oxy}propyl)-2-[(*E*,*Z*)-2-(2-chlorophenyl)ethenyl]-5-methoxy-1*H*-indole (**71b**) as an oily solid (4.98 g), which was used without further purification.

5.1.2. Procedure 2: Diels–Alder reaction then aromatisation with MnO_2

A mixture of **71b** (4.98 g, 0.011 mol), maleimide (1.83 g, 0.019 mol) and powdered $SnCl_2$ (40 mg, 0.21 mmol) in toluene (80 mL) was heated at reflux for 16 h. The cooled solution was added to aqueous $NaHCO_3$ (100 mL) and extracted with EtOAc. The crude Diels–Alder adduct was then concentrated to dryness and re-dissolved in *p*-dioxane (100 mL), before activated MnO_2 (20 g, 0.23 mol) was added and the resulting suspension was heated at reflux for 8 h. Filtration through Celite, concentration under reduced pressure and chromatography on silica, eluting with MeOH/EtOAc (1:9), gave 6-(3-{[*tert*-butyl(dimethyl)-silyl]oxy}propyl)-4-(2-chlorophenyl)-9-methoxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-dione (4.87 g, 67% from **70b**) as a yellow solid; mp (Et_2O) 199–201 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.12 (br, 1H), 8.52 (d, $J = 2.6$ Hz, 1H), 7.75 (s, 1H), 7.66 (d, $J = 8.9$ Hz, 1H), 7.57 (dd, $J = 8.3$, 2.2 Hz, 1H), 7.5–7.4 (m, 3H), 7.30 (dd, $J = 8.9$, 2.6 Hz, 1H), 4.53 (t, $J = 6.4$ Hz, 2H), 3.90 (s, 3H), 3.55 (t, $J = 5.8$ Hz, 2H), 1.94 (m, 2H), 0.77 (s, 9H), −0.06 (s, 3H); which was used directly.

5.1.3. Procedure 3: TBDMS deprotection

*5.1.3.1. 4-(2-Chlorophenyl)-6-(3-hydroxypropyl)-9-methoxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**73b**).* Treatment of the above TBDMS-protected alcohol (4.87 g, 8.85 mmol) with 3 N HCl (50 mL) in 1:1 THF/MeOH (200 mL) at room temperature for 2 h was followed by removal of most of the solvents under reduced pressure. The residue was extracted with EtOAc, washed well with water, and concentrated to a volume of 60 mL. Petroleum ether was added to precipitate the product, which was filtered off and triturated several times with Et_2O. The solid was crystallised from THF/petroleum ether to give **73b** (3.77 g, 88%) as a yellow powder; mp 228–230 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.12 (br s, 1H), 8.52 (d, $J = 2.5$ Hz, 1H), 7.80 (s, 1H), 7.70 (d, $J = 8.9$ Hz, 1H), 7.58 (dd, $J = 8.1$, 2.2 Hz, 1H), 7.53–7.42 (m, 3H), 7.31 (dd, $J = 8.9$, 2.5 Hz, 1H), 4.63 (br s, 1H), 4.53 (t, $J = 6.9$ Hz, 2H), 3.91 (s, 3H), 3.40 (m, 2H), 1.91 (m, 2H). Anal. $C_{24}H_{19}ClN_2O_4$ (C, H, N).

5.1.4. Procedure 4: boron tribromide demethylation

*5.1.4.1. 4-(2-Chlorophenyl)-9-hydroxy-6-(3-hydroxypropyl)-pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**16**).* Boron tribromide (11.5 mL of a 1.0 M solution in CH_2Cl_2, 0.011 mol) was added to a stirred solution of **73b** (1.00 g, 2.30 mmol) in dry CH_2Cl_2 (100 mL) under N_2 at 0 °C, and then the mixture was stirred at room temperature for 3 h. Saturated aqueous $NaHCO_3$ solution was added and the solution was diluted with water and extracted with EtOAc. The organic phase was dried (Na_2SO_4), the drying agent was removed and the solution was concentrated to dryness, adsorbed onto silica and chromatographed. Elution with EtOAc/petroleum ether (1:1), followed by EtOAc, gave **16** (0.90 g, 93%), which crystallised from THF/petroleum ether as a yellow/orange powder; mp 291–294 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.05 (br s, 1H), 9.36 (s, 1H), 8.38 (d, $J = 2.5$ Hz, 1H), 7.74 (s, 1H), 7.59–7.54 (m, 2H), 7.52–7.42 (m, 3H), 7.14 (dd, $J = 8.7$, 2.5 Hz, 1H), 4.62 (m, 1H), 4.49 (t, $J = 6.8$ Hz, 2H), 3.41 (m, 2H), 1.90 (m, 2H). Anal. $C_{23}H_{17}ClN_2O_4 \cdot 1/4H_2O$ (C, H, N).

5.1.5. Procedure 5: Diels–Alder reaction then aromatisation with DDQ

Alkylation of 5-methoxy-2-[(*E*,*Z*)-2-phenylethenyl]-1*H*-indole (**70a**) [10] (1.93 g, 7.74 mmol) with 2-(2-bromoethoxy)-tetrahydro-2*H*-pyran, according to Procedure 1, gave crude 5-methoxy-2-[(*E*,*Z*)-2-phenylethenyl]-1-[2-(tetrahydro-2*H*-pyran-2-yloxy)ethyl]-1*H*-indole (**75a**) which was reacted with maleimide (0.79 g) following Procedure 2 to give a crude Diels–Alder adduct, that was used directly. To the crude Diels–Alder adduct, in toluene/*p*-dioxane (4:1, 250 mL) was added DDQ (2.83 g, 12.5 mmol). The resulting solution was heated at reflux for 3 h. The cooled solution was diluted with aqueous $NaHCO_3$ (500 mL) and extracted with EtOAc. The combined organics were washed with aqueous $NaHCO_3$ (several times) and then brine, before being dried over anhydrous Na_2SO_4 and evaporated to dryness to give a crude mixture of 6-(2-hydroxyethyl)-9-methoxy-4-phenylpyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-dione (**77a**) (and partially THP protected material) that was used directly.

5.1.6. Procedure 6: THP deprotection

*5.1.6.1. 6-(2-Hydroxyethyl)-9-methoxy-4-phenylpyrrolo[3,4-c]-carbazole-1,3(2H,6H)-dione (**77a**).* The crude mixture of **77a** obtained above was then dissolved in MeOH (100 mL) and treated with *p*-toluenesulfonic acid monohydrate (30 mg, 0.16 mmol) warming at 50 °C for 3 h. The resulting solution was diluted with water and extracted with EtOAc, then the product was adsorbed onto silica and chromatographed, eluting with EtOAc/hexane (1:2 to 1:1), to give **77a** as a yellow powder (0.51 g, 17%); mp 262–264 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.09 (br s, 1H), 8.56 (d, $J = 2.6$ Hz, 1H), 7.83 (s, 1H), 7.66 (m, 3H), 7.47 (m, 3H), 7.28 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.86 (t, $J = 5.5$ Hz, 1H), 4.55 (t, $J = 5.3$ Hz, 2H), 3.90 (s, 3H), 3.78 (m, 2H). Anal. $C_{23}H_{18}N_2O_4$ (C, H, N).

5.1.7. Procedure 7: pyridine hydrochloride demethylation

*5.1.7.1. 9-Hydroxy-6-(2-hydroxyethyl)-4-phenylpyrrolo[3,4-c]-carbazole-1,3(2H,6H)-dione (**12**).* Compound **77a** (135 mg, 0.35 mmol) was heated in molten pyridine hydrochloride (15 g) at 200 °C under N_2. After 15 min, the reaction mixture was cooled and diluted with water to precipitate the product, which was collected by filtration, washed with water and dried, then chromatographed on silica gel, eluting with EtOAc/hexane, to give **12** (90 mg, 69%) as an orange powder; mp 298–301 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.04 (br s, 1H), 9.31

(br s, 1H), 8.40 (d, $J = 2.5$ Hz, 1H), 7.79 (s, 1H), 7.64 (m, 2H), 7.55 (d, $J = 8.8$ Hz, 1H), 7.47 (m, 3H), 7.10 (dd, $J = 8.8$, 2.5 Hz, 1H), 4.85 (t, $J = 5.3$ Hz, 1H), 4.51 (t, $J = 5.4$ Hz, 2H), 3.77 (m, 2H). Anal. $C_{22}H_{16}N_2O_4 \cdot 3/4H_2O$ (C, H), N: calcd, 7.26; found, 6.76.

*5.1.8. 4-(2-Chlorophenyl)-6-(2-hydroxyethyl)-9-methoxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**77b**)*

Alkylation of 2-[(*E*,*Z*)-2-(2-chlorophenyl)ethenyl]-5-methoxy-1*H*-indole (**70b**) [9] (1.92 g, 6.77 mmol) with sodium hydride (0.39 g of 60% dispersion in mineral oil, 8.12 mmol) and 2-(2-bromoethoxy)tetrahydro-2*H*-pyran (1.56 g, 7.44 mmol) following Procedure 1, gave crude 2-[(*E*,*Z*)-2-(2-chlorophenyl)ethenyl]-5-methoxy-1-[2-(tetrahydro-2*H*-pyran-2-yloxy)ethyl]-1*H*-indole (**75b**) which was dissolved in MeOH/THF (1:1, 120 mL) and treated with 2 N HCl (15 mL), stirring at room temperature for 3 h. The solution was then diluted with saturated aqueous $NaHCO_3$ and concentrated under reduced pressure to precipitate crude 2-{2-[(*E*,*Z*)-2-(2-chlorophenyl) ethenyl]-5-methoxy-1*H*-indol-1-yl}ethanol (**76b**) (1.90 g, 86%), which was collected by filtration and dried in vacuo. This solid was reacted directly with maleimide and aromatised with MnO_2 for 18 h, following Procedure 2 described above, to give **77b** (72%) as a yellow powder; mp 255–257 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.10 (br s, 1H), 8.52 (d, $J = 2.6$ Hz, 1H), 7.80 (s, 1H), 7.71 (d, $J = 9.0$ Hz, 1H), 7.59–7.56 (m, 1H), 7.52–7.43 (m, 4H), 7.29 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.84 (t, $J = 5.0$ Hz, 1H), 4.53 (t, $J = 5.2$ Hz, 2H), 3.90 (s, 3H), 3.77 (m, 2H). Anal. $C_{23}H_{17}ClN_2O_4$ (C, H, N).

*5.1.9. 4-(2-Chlorophenyl)-6-(2-hydroxyethyl)-9-hydroxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**13**)*

Deprotection of **77b** with BBr_3, following Procedure 4, gave **13** (87%) as a yellow/orange powder; mp 265 °C (dec). ^{1}H NMR [$(CD_3)_2SO$] δ 11.04 (br s, 1H), 9.33 (br s, 1H), 8.38 (d, $J = 2.4$ Hz, 1H), 7.75 (s, 1H), 7.60–7.56 (m, 2H), 7.52–7.43 (m, 4H), 7.12 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.83 (t, $J = 5.5$ Hz, 1H), 4.49 (t, $J = 5.2$ Hz, 2H), 3.77 (dt, $J = 5.5$, 5.2 Hz, 2H). Anal. $C_{22}H_{15}ClN_2O_4 \cdot 1/2H_2O$ (C, H, N).

*5.1.10. 4-(2,6-Dichlorophenyl)-6-(2-hydroxyethyl)-9-methoxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**77c**)*

Alkylation of 2-[(*E*)-2-(2,6-dichlorophenyl)ethenyl]-5-methoxy-1*H*-indole (**70c**) [9] (3.0 g, 10.6 mmol) with 2-(2-bromoethoxy)tetrahydro-2*H*-pyran, according to Procedure 1, gave crude 2-[(*E*)-2-(2,6-dichlorophenyl)ethenyl]-5-methoxy-1-[2-(tetrahydro-2*H*-pyran-2-yloxy)ethyl]-1*H*-indole (**75c**). Treatment of this with 2 N HCl in MeOH/THF, following Procedure 3, gave crude 2-{2-[(*E*)-2-(2,6-dichlorophenyl) ethenyl]-5-methoxy-1*H*-indol-1-yl}ethanol (**76c**) which was reacted directly with maleimide (1.26 g) according to Procedure 2 and aromatised with DDQ following Procedure 5. Trituration in MeOH then gave **77c** as a yellow solid (2.14 g, 44%); mp 229–231 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.17 (br s, 1H), 8.50 (d, $J = 2.6$ Hz, 1H), 7.86 (s, 1H), 7.72 (d, $J = 9.0$ Hz, 1H), 7.62 (m, 2H), 7.51 (dd, $J = 8.9$, 7.4 Hz, 1H), 7.31 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.83 (t, $J = 5.3$ Hz, 1H), 4.54 (t, $J = 5.2$ Hz, 2H), 3.91 (s, 3H), 3.77 (m, 2H). Anal. $C_{23}H_{16}Cl_2N_2O_4$ (C, H, N).

*5.1.11. 4-(2,6-Dichlorophenyl)-9-hydroxy-6-(2-hydroxyethyl)pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**14**)*

Demethylation of **77c** (0.10 g, 0.22 mmol) with BBr_3, following Procedure 4, gave **14** (49 mg, 50%) as an orange/yellow powder; mp 254–257 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.10 (br s, 1H), 9.35 (s, 1H), 8.36 (d, $J = 2.4$ Hz, 1H), 7.80 (s, 1H), 7.61 (m, 3H), 7.50 (dd, $J = 8.7$, 7.3 Hz, 1H), 7.13 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.82 (t, $J = 5.5$ Hz, 1H), 4.49 (t, $J = 5.2$ Hz, 2H), 3.75 (m, 2H). Anal. $C_{22}H_{14}Cl_2N_2O_4 \cdot 1/2H_2O$ (C, H, N).

*5.1.12. 6-(3-Hydroxypropyl)-9-methoxy-4-phenylpyrrolo-[3,4-c]carbazole-1,3(2H,6H)-dione (**73a**)*

Alkylation of 5-methoxy-2-[(*E*,*Z*)-2-phenylethenyl]-1*H*-indole (**70a**) [10] (6.85 g, 27.5 mmol) with 3-bromopropoxy-*tert*-butyldimethylsilane, following Procedure 1, gave crude 1-(3-{[*tert*-butyl(dimethyl)silyl]oxy}propyl)-5-methoxy-2-[(*E*,*Z*)-2-phenylethenyl]-1*H*-indole (**71a**) which was reacted successively with maleimide and DDQ following Procedures 2 and 5, respectively, to give crude 6-(3-{[*tert*-butyl(dimethyl)-silyl]oxy}propyl)-9-methoxy-4-phenylpyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-dione. This product was dissolved in MeOH (300 mL) containing 1 N HCl (50 mL), and the solution was kept at room temperature for 3 h, then partitioned between water and EtOAc. The organic layer was dried and evaporated, and the residue was chromatographed on silica gel, eluting with CH_2Cl_2 to CH_2Cl_2/EtOAc (7:3), to give **73a** (2.55 g, 23%); mp (Et_2O) 241–243 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.10 (br s, 1H), 8.56 (d, $J = 2.6$ Hz, 1H), 7.82 (s, 1H), 7.67 (m, 3H), 7.47 (m, 3H), 7.30 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.66 (t, $J = 4.9$ Hz, 1H), 4.55 (t, $J = 6.9$ Hz, 2H), 3.90 (s, 3H), 3.39 (m, 2H), 1.93 (m, 2H). Anal. $C_{24}H_{20}N_2O_4$ (C, H, N).

*5.1.13. 9-Hydroxy-6-(3-hydroxypropyl)-4-phenylpyrrolo-[3,4-c]carbazole-1,3(2H,6H)-dione (**15**)*

Demethylation of **73a** (60 mg, 0.15 mmol) with BBr_3 at room temperature for 3 h, following Procedure 4, gave **15** (45 mg, 78%); mp 274–276 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.05 (br s, 1H), 9.33 (s, 1H), 8.41 (d, $J = 2.4$ Hz, 1H), 7.77 (s, 1H), 7.64 (m, 2H), 7.56 (d, $J = 8.8$ Hz, 1H), 7.46 (m, 3H), 7.12 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.64 (t, $J = 4.9$ Hz, 1H), 4.51 (t, $J = 6.9$ Hz, 2H), 3.39 (m, 2H), 1.92 (m, 2H). Anal. $C_{23}H_{18}N_2O_4$ (C, H, N).

*5.1.14. 4-(2,6-Dichlorophenyl)-6-(3-hydroxypropyl)-9-methoxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**73c**)*

Alkylation of 2-[(*E*)-2-(2,6-dichlorophenyl)ethenyl]-5-methoxy-1*H*-indole (**70c**) [9] (3.0 g, 9.43 mmol) with 3-bromopropoxy-*tert*-butyldimethylsilane, according to Procedure 1, gave crude 1-(3-{[*tert*-butyl(dimethyl)silyl]oxy}propyl)-2-[(*E*)-2-(2,6-dichlorophenyl)ethenyl]-5-methoxy-1*H*-indole (**71c**) which was dissolved in MeOH/CH_2Cl_2 (3:1, 300 mL) and treated with 1 N HCl (60 mL). This solution was stirred at room temperature for 2 h, then diluted with saturated aqueous $NaHCO_3$ and concentrated under reduced pressure

to precipitate crude 3-{2-[(*E*)-2-(2,6-dichlorophenyl)ethenyl]-5-methoxy-1*H*-indol-1-yl}-1-propanol (**72**), which was collected by filtration and dried in vacuo. Crude **72** thus obtained was reacted directly with maleimide (1.20 g) and then aromatised with DDQ, following Procedures 2 and 5, respectively. Chromatography on silica, eluting with MeOH/CH_2Cl_2 (2:98–5:95), gave **73c** (2.4 g, 54%) as a yellow powder; mp 254–256 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.18 (br s, 1H), 8.51 (d, $J = 2.6$ Hz, 1H), 7.86 (s, 1H), 7.72 (d, $J = 9.0$ Hz, 1H), 7.63 (m, 2H), 7.51 (dd, $J = 8.8$, 7.4 Hz, 1H), 7.34 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.62 (t, $J = 5.0$ Hz, 1H), 4.54 (t, $J = 6.9$ Hz, 2H), 3.91 (s, 3H), 3.42 (m, 2H), 1.90 (m, 2H). FABMS found M^+: 468.0630, 470.0628, 472.0626. $C_{24}H_{18}Cl_2N_2O_4$ requires 468.0644, 470.0614, 472.0585.

5.1.15. 4-(2,6-Dichlorophenyl)-9-hydroxy-6-(3-hydroxypropyl)pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (*17*)

Demethylation of **73c** (0.36 g, 0.77 mmol) with BBr_3, according to Procedure 4, gave **17** (0.32 g, 92%) as an orange/yellow powder; mp 215–218 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.12 (br s, 1H), 9.39 (s, 1H), 8.37 (d, $J = 2.5$ Hz, 1H), 7.80 (s, 1H), 7.61 (m, 3H), 7.51 (dd, $J = 8.7$, 7.2 Hz, 1H), 7.16 (dd, $J = 8.8$, 2.5 Hz, 1H), 4.61 (t, $J = 5.0$ Hz, 1H), 4.49 (t, $J = 6.9$ Hz, 2H), 3.42 (m, 2H), 1.88 (m, 2H). Anal. $C_{23}H_{16}Cl_2N_2O_4$ (C, H, N).

5.1.16. 4-(2-Chloro-6-methoxyphenyl)-6-(3-hydroxypropyl)-9-methoxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (*73d*)

Reaction of 2-[(*E*,*Z*)-2-(2-chloro-6-methoxyphenyl)ethenyl]-5-methoxy-1*H*-indole (**70d**) [9] with 3-bromopropoxy-*tert*-butyldimethylsilane following Procedure 1, gave 1-(3-{[*tert*-butyl(dimethyl)silyl]oxy}propyl)-2-[(*E*,*Z*)-2-(2-chloro-6-methoxyphenyl)ethenyl]-5-methoxy-1*H*-indole (**71d**), which was used without further purification. Reaction of **71d** with maleimide and then with DDQ, following Procedures 2 and 5, respectively, gave 6-(3-{[*tert*-butyl(dimethyl)silyl]oxy}propyl)-4-(2-chloro-6-methoxyphenyl)-9-methoxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-dione, which was reacted directly with 2 N HCl in THF/MeOH (4:1) according to Procedure 3, to give **73d** (76%) as an orange powder; mp 224–227 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.05 (br s, 1H), 8.51 (d, $J = 2.6$ Hz, 1H), 7.75 (s, 1H), 7.69 (d, $J = 8.9$ Hz, 1H), 7.44 (dd, $J = 8.2$, 8.2 Hz, 1H), 7.31 (dd, $J = 8.9$, 2.6 Hz, 1H), 7.18 (d, $J = 8.2$ Hz, 1H), 7.13 (d, $J = 8.2$ Hz, 1H), 4.63 (t, $J = 4.9$ Hz, 1H), 4.51 (t, $J = 6.8$ Hz, 2H), 3.91 (s, 3H), 3.68 (s, 3H), 3.41 (m, 2H), 1.89 (m, 2H). Anal. $C_{25}H_{22}N_2O_5$ (C, N), H: calcd, 5.15; found, 4.53.

5.1.17. 4-(2-Chloro-6-methoxyphenyl)-9-hydroxy-6-(3-hydroxypropyl)pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (*74*)

Deprotection of **73d** with BBr_3 in CH_2Cl_2 for 90 min, following Procedure 4, gave **74** (64%) as an orange powder; mp 270–273 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.01 (br s, 1H), 9.35 (br s, 1H), 8.37 (d, $J = 2.4$ Hz, 1H), 7.70 (s, 1H), 7.58 (d, $J = 8.7$ Hz, 1H), 7.44 (dd, $J = 8.3$, 8.3 Hz, 1H), 7.20–7.11 (m, 3H), 4.61 (m, 1H), 4.47 (t, $J = 6.9$ Hz, 2H), 3.68 (s, 3H), 3.42 (m, 2H), 1.88 (m, 2H). Anal. $C_{24}H_{19}ClN_2O_5$ (C, H, N).

5.1.18. 9-Hydroxy-6-(3-hydroxypropyl)-4-(2-methoxyphenyl)-pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (*18*)

A solution of **74** (0.20 g, 0.44 mmol), potassium acetate (0.20 g) and 5% Pd/C in EtOAc/MeOH (50 mL, 1:1) was hydrogenated at 60 psi for 7 h. The catalyst was filtered off and the filtrate concentrated to dryness, partitioned between EtOAc and water, and evaporated. The residue was chromatographed on silica, eluting with EtOAc/petroleum ether (2:1), to give **18** (51%) as an orange powder (from THF/petroleum ether); mp 296–300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 10.93 (br s, 1H), 9.31 (br s, 1H), 8.38 (d, $J = 2.4$ Hz, 1H), 7.69 (s, 1H), 7.55 (d, $J = 8.8$ Hz, 1H), 7.42 (m, 1H), 7.35 (dd, $J = 7.5$, 1.7 Hz, 1H), 7.13–7.08 (m, 2H), 7.05 (dd, $J = 7.2$, 7.2 Hz, 1H), 4.62 (t, $J = 4.8$ Hz, 1H), 4.48 (t, $J = 6.8$ Hz, 2H), 3.68 (s, 3H), 3.40 (m, 2H), 1.90 (m, 2H). Anal. $C_{24}H_{20}N_2O_5$ (C, H, N).

5.2. Procedures of Scheme 2

5.2.1. 3-{2-[(*E*)-2-(2-Chlorophenyl)ethenyl]-5-methoxy-1*H*-indol-1-yl}propanenitrile (*78*)

A solution of 2-[(*E*)-2-(2-chlorophenyl)ethenyl]-5-methoxy-1*H*-indole (**70b**) [9] (1.5 g, 5.25 mmol) in CH_3CN (30 mL) was treated with acrylonitrile (2.42 mL, 36.8 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (20 drops). The resulting solution was stirred at room temperature under nitrogen for 18 h, then diluted with water and extracted with EtOAc. The organic layer was dried (Na_2SO_4), the drying agent was removed and the solution was concentrated to dryness. Chromatography on silica, eluting with EtOAc/hexane (1:1), followed by trituration in MeOH gave **78** (1.36 g, 77%) as an off-white solid; mp 136–138 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.05 (dd, $J = 7.9$, 1.5 Hz, 1H), 7.50 (m, 4H), 7.41 (m, 1H), 7.33 (m, 1H), 7.04 (d, $J = 2.4$ Hz, 1H), 6.90 (s, 1H), 6.81 (dd, $J = 9.0$, 2.4 Hz, 1H), 4.68 (t, $J = 6.6$ Hz, 2H), 3.77 (s, 3H), 2.93 (t, $J = 6.6$ Hz, 2H). Anal. $C_{20}H_{17}ClN_2O$ (C, H, N).

5.2.2. 3-(4-(2-Chlorophenyl)-9-methoxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)propanenitrile (*82*)

Reaction of **78** (1.30 g, 3.86 mmol) with maleimide (0.49 g), and aromatisation of the product with MnO_2 following Procedure 2, gave crude material that was chromatographed on silica gel, eluting with EtOAc/hexane (1:1). Trituration from MeOH gave **82** (1.33 g, 80%) as a yellow powder; mp 287–288 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.16 (br s, 1H), 8.53 (d, $J = 2.6$ Hz, 1H), 8.02 (s, 1H), 7.83 (d, $J = 9.0$ Hz, 1H), 7.59 (m, 1H), 7.49 (m, 3H), 7.33 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.86 (t, $J = 6.7$ Hz, 2H), 3.91 (s, 3H), 3.04 (t, $J = 6.7$ Hz, 2H). Anal. $C_{24}H_{16}ClN_3O_3 \cdot 3/4H_2O$ (C, H, N).

*5.2.3. 3-(4-(2-Chlorophenyl)-9-hydroxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)propanenitrile (**22**)*

Demethylation of **82** (0.15 g, 0.35 mmol) with 1 N BBr_3 in CH_2Cl_2, according to Procedure 4, followed by trituration in MeOH, gave **22** (0.13 g, 89%) as an orange/yellow powder; mp 332–336 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.10 (br s, 1H), 9.42 (br s, 1H), 8.39 (d, $J = 2.4$ Hz, 1H), 7.96 (s, 1H), 7.69 (d, $J = 8.8$ Hz, 1H), 7.58 (m, 1H), 7.48 (m, 3H), 7.15 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.82 (t, $J = 6.7$ Hz, 2H), 3.02 (t, $J = 6.7$ Hz, 2H). Anal. $C_{23}H_{14}ClN_3O_3$ (C, H, N).

*5.2.4. 4-(2-Chlorophenyl)-9-hydroxy-6-[2-hydroxy-3-(methylamino)propyl]pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**57**)*

Alkylation of 2-[(*E*)-2-(2-chlorophenyl)ethenyl]-5-methoxy-1*H*-indole (**70b**) [9] (0.25 g, 0.88 mmol) with epibromohydrin, following Procedure 1, gave crude epoxide **79**, which was used directly. The epoxide **79** was dissolved in THF (2 mL) and aqueous methylamine (0.5 mL, 40% solution) was added. The resulting solution was heated at 60 °C for 6 h, then diluted with water and extracted with EtOAc to give crude 1-{2-[(*E*)-2-(2-chlorophenyl)ethenyl]-5-methoxy-1*H*-indol-1-yl}-3-(methylamino)-2-propanol (**80**). This material was triturated in Et_2O/hexane and reacted directly with maleimide (0.13 g, 1.5 mmol), following Procedure 2 (with the addition of *p*-dioxane as a co-solvent). DDQ aromatisation of the crude Diels–Alder adduct, according to Procedure 5, gave 4-(2-chlorophenyl)-6-[2-hydroxy-3-(methylamino)-propyl]-9-methoxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-dione (**83**), which was demethylated with pyridine hydrochloride, according to Procedure 7. Chromatography on silica, eluting with EtOAc/MeOH/Et_3N (4:1:trace), and then crystallisation from EtOAc/hexane gave **57** (61 mg, 15%) as a yellow powder; mp 188–191 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 10.9 (br s, 1H), 9.36 (br s, 1H), 8.37 (d, $J = 2.4$ Hz, 1H), 7.77 (s, 1H), 7.58 (m, 2H), 7.47 (m, 3H), 7.13 (dd, $J = 8.8$, 2.4 Hz, 1H), 5.09 (br s, 1H), 4.50 (m, 1H), 4.34 (m, 1H), 3.97 (m, 1H), 2.77–2.63 (m, 2H), 2.28 (d, $J = 2.1$ Hz, 3H). Anal. $C_{24}H_{20}ClN_3O_4 \cdot 2/3H_2O$ (C, H, N).

*5.2.5. 4-(2-Chlorophenyl)-6-[3-(dimethylamino)-2-hydro-xypropyl]-9-hydroxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**58**)*

Epoxide **79** (0.88 mmol) was dissolved in THF (2 mL) and aqueous dimethylamine (0.5 mL, 40% solution) was added. The resulting solution was heated at 60 °C for 6 h, then diluted with water and extracted with EtOAc to give 1-{2-[(*E*)-2-(2-chlorophenyl)ethenyl]-5-methoxy-1*H*-indol-1-yl}-3-(dimethylamino)-2-propanol (**81**). This crude material was triturated in Et_2O/hexane and then reacted directly with maleimide (0.13 g, 1.5 mmol), following Procedure 2. DDQ aromatisation of the crude Diels–Alder adduct, according to Procedure 5, gave 4-(2-chlorophenyl)-6-[3-(dimethylamino)-2-hydroxypropyl]-9-methoxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-dione (**84**), which was demethylated with pyridine hydrochloride following Procedure 7. Chromatography on silica, eluting with EtOAc/MeOH/Et_3N (4:1:trace), followed by crystallisation from EtOAc/hexane, gave **58** (165 mg, 40%) as a yellow powder; mp 225–228 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.04 (br s, 1H), 9.34 (br s, 1H), 8.38 (d, $J = 2.4$ Hz, 1H), 7.75 (s, 1H), 7.57 (m, 2H), 7.47 (m, 3H), 7.13 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.91 (br s, 1H), 4.50 (m, 1H), 4.34 (m, 1H), 4.00 (m, 1H), 2.35 (m, 1H), 2.24 (m, 1H), 2.17 (s, 6H). Anal. $C_{25}H_{22}ClN_3O_4 \cdot 1/2H_2O$ (C, H, N).

5.3. Procedures of Scheme 3

*5.3.1. 6-Allyl-4-(2-chlorophenyl)-2-(2,4-dimethoxybenzyl)-9-methoxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**86**)*

To a solution of 4-(2-chlorophenyl)-2-(2,4-dimethoxybenzyl)-9-methoxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-dione (**85**) [9] (150 mg, 0.28 mmol) in DMF (10 mL) under nitrogen was added K_2CO_3 (0.39 g, 2.80 mmol) and allyl bromide (72 μL, 0.84 mmol). The resulting suspension was warmed to 90 °C with stirring for 3 h, then diluted with water and extracted with EtOAc. The organic phase was dried (Na_2SO_4), the drying agent was removed, and the solution was concentrated to dryness. Chromatography on silica, eluting with EtOAc/hexane (1:2), followed by crystallisation from Et_2O/hexane, gave **86** (90 mg, 57%) as a yellow powder; mp 171–173 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.51 (d, $J = 2.6$ Hz, 1H), 7.84 (s, 1H), 7.67 (d, $J = 8.9$ Hz, 1H), 7.58 (m, 1H), 7.53–7.46 (m, 3H), 7.32 (dd, $J = 8.9$, 2.6 Hz, 1H), 6.97 (d, $J = 8.4$ Hz, 1H), 6.57 (d, $J = 2.4$ Hz, 1H), 6.45 (dd, $J = 8.4$, 2.4 Hz, 1H), 5.99 (m, 1H), 5.18 (d, $J = 4.9$ Hz, 2H), 5.14 (dd, $J = 10.3$, 1.3 Hz, 1H), 4.99 (dd, $J = 17.2$, 1.3 Hz, 1H), 4.69 (s, 2H), 3.90 (s, 3H), 3.80 (s, 3H), 3.72 (s, 3H). Anal. $C_{33}H_{27}ClN_2O_5$ (C, H, N).

*5.3.2. 4-(2-Chlorophenyl)-6-(2,3-dihydroxypropyl)-2-(2,4-dimethoxybenzyl)-9-methoxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**87**)*

To a solution of **86** (80 mg, 0.14 mmol) in acetone/water (4:1, 20 mL) was added *N*-methylmorpholine *N*-oxide (33 mg, 0.28 mmol) and osmium tetroxide (176 μL, 4% solution in water, ~0.028 mmol). The reaction mixture was stirred at room temperature for 18 h, then diluted with 1 N HCl and extracted with EtOAc. The organic phase was dried, the drying agent was removed, and the solution was concentrated to dryness. Chromatography on silica, eluting with EtOAc/hexane (2:1), followed by crystallisation from Et_2O/hexane, gave **87** (80 mg, 94%) as a yellow powder; mp 151–156 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.50 (d, $J = 2.6$ Hz, 1H), 7.82 (s, 1H), 7.69 (br d, $J = 9.0$ Hz, 1H), 7.58 (m, 1H), 7.52–7.46 (m, 3H), 7.30 (dd, $J = 9.0$, 2.6 Hz, 1H), 6.95 (d, $J = 8.4$ Hz, 1H), 6.57 (d, $J = 2.4$ Hz, 1H), 6.44 (dd, $J = 8.4$, 2.4 Hz, 1H), 5.02 (d, $J = 5.0$ Hz, 1H), 4.87 (br s, 1H), 4.69 (s, 2H), 4.55 (m, 1H), 4.38 (m, 1H), 3.89 (m, 4H), 3.80 (s, 3H), 3.72 (s, 3H), 3.41 (m, 2H). Anal. $C_{33}H_{29}ClN_2O_7 \cdot 1/4H_2O$ (C, H, N).

*5.3.3. 4-(2-Chlorophenyl)-6-(2,3-dihydroxypropyl)-9-hydroxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**26**)*

To a solution of **87** (75 mg, 0.13 mmol) in anisole (0.5 mL) was added TFA (2.0 mL). The reaction vessel was sealed and

heated to 90 °C in an oil bath for 18 h before the TFA was removed under reduced pressure. The residue was diluted with water and extracted with Et_2O (three times). The combined organic extracts were washed thoroughly with 1 N KOH and brine, then dried over anhydrous Na_2SO_4 and concentrated in vacuo. The aqueous layer was acidified by the addition of concentrated HCl, extracted with Et_2O and worked up as above. The combined crude material was triturated in Et_2O/hexane and then dissolved in CH_2Cl_2 (20 mL) and demethylated with BBr_3, according to Procedure 4. Chromatography of the product on silica, eluting with EtOAc/hexane (1:1 to 1:0), gave **26** (31 mg, 57%) as an orange powder; mp 305–309 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.04 (br s, 1H), 9.33 (s, 1H), 8.38 (d, $J = 2.4$ Hz, 1H), 7.73 (s, 1H), 7.57 (m, 2H), 7.47 (m, 3H), 7.13 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.99 (d, $J = 5.5$ Hz, 1H), 4.83 (t, $J = 5.4$ Hz, 1H), 4.50 (m, 1H), 4.32 (m, 1H), 3.87 (br s, 1H), 3.45–3.36 (m, 2H). Anal. $C_{23}H_{17}ClN_2O_5$ (C, H, N).

5.4. Procedures of Scheme 4

5.4.1. Procedure 8: preparation of C-2 mesylates/C-3 bromides for amine displacement

*5.4.1.1. 2-(9-Hydroxy-1,3-dioxo-4-phenyl-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)ethyl methanesulfonate (**88a**).* Alcohol **77a** (0.21 g, 0.53 mmol) in dry THF (30 mL) and Et_3N (0.37 mL) under nitrogen was cooled to 0 °C and treated dropwise with MsCl (45 μL, 0.58 mmol). After 1 h, the reaction was partitioned between saturated aqueous $NaHCO_3$ and EtOAc. The organic layer was evaporated and the crude product was demethylated with BBr_3, following Procedure 4. Chromatography of the product on silica gel, eluting with EtOAc/hexane (1:1 to 4:1), gave **88a** (0.19 g, 80%); mp (EtOAc/hexane) 271–276 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.08 (s, 1H), 9.37 (br s, 1H), 8.40 (d, $J = 2.4$ Hz, 1H), 7.86 (s, 1H), 7.67 (m, 2H), 7.60 (d, $J = 8.8$ Hz, 1H), 7.46 (m, 3H), 7.13 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.86 (t, $J = 4.8$ Hz, 2H), 4.57 (t, $J = 4.8$ Hz, 2H), 2.97 (s, 3H). FABMS found $[M + H]^+$: 451.0958. $C_{23}H_{18}N_2O_6S$ requires 451.0964.

*5.4.1.2. 2-(4-(2-Chlorophenyl)-9-hydroxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)ethyl methanesulfonate (**88b**).* A solution of alcohol **77b** (1.10 g, 2.61 mmol) was mesylated and demethylated with BBr_3 (7 h) according to Procedure 8, then chromatography of the product on silica gel, eluting with EtOAc/hexane (1:1 to 3:1), gave **88b** (1.05 g, 65%) as a yellow solid; mp (EtOAc/hexane) 266 °C (dec). ^{1}H NMR [$(CD_3)_2SO$] δ 11.09 (br s, 1H), 9.40 (s, 1H), 8.39 (d, $J = 2.5$ Hz, 1H), 7.84 (s, 1H), 7.62 (d, $J = 8.9$ Hz, 1H), 7.58 (m, 1H), 7.48 (m, 3H), 7.14 (dd, $J = 8.9$, 2.5 Hz, 1H), 4.85 (t, $J = 5.0$ Hz, 2H), 4.54 (m, 2H), 2.93 (s, 3H).

*5.4.1.3. 2-(4-(2,6-Dichlorophenyl)-9-hydroxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)ethyl methanesulfonate (**88c**).* Alcohol **77c** (1.0 g, 2.2 mmol) was mesylated and demethylated with BBr_3 (30 h), according to Procedure 8. Chromatography of the product on silica gel, eluting with EtOAc/hexane (1:1 to 4:1), gave **88c** (0.95 g, 83%); mp (EtOAc/hexane) 255–260 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.16 (br s, 1H), 9.43 (br s, 1H), 8.38 (d, $J = 2.5$ Hz, 1H), 7.91 (s, 1H), 7.63 (m, 3H), 7.51 (dd, $J = 8.7$, 7.3 Hz, 1H), 7.16 (dd, $J = 8.9$, 2.5 Hz, 1H), 4.85 (t, $J = 4.9$ Hz, 2H), 4.53 (t, $J = 4.9$ Hz, 2H), 2.88 (s, 3H). Anal. $C_{23}H_{16}Cl_2N_2O_6S$ (C, H, N).

*5.4.1.4. 6-(3-Bromopropyl)-9-hydroxy-4-phenylpyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**89a**).* Alcohol **72a** (0.50 g, 1.25 mmol) was treated with MsCl in THF/Et_3N and the crude product was demethylated with BBr_3 (0 °C for 18 h), following Procedure 8. This product was dissolved in EtOAc (100 mL) containing LiBr (1.0 g), and the solution was kept at 50 °C for 2 h, then worked up and chromatographed on silica gel, eluting with EtOAc/hexane (1:1 to 4:1), to give **89a** (0.54 g, 97%); mp (EtOAc/hexane) 280–282 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.07 (s, 1H), 9.36 (s, 1H), 8.41 (d, $J = 2.2$ Hz, 1H), 7.83 (s, 1H), 7.66 (m, 2H), 7.58 (d, $J = 8.8$ Hz, 1H), 7.47 (m, 3H), 7.14 (dd, $J = 8.8$, 2.2 Hz, 1H), 4.57 (t, $J = 6.9$ Hz, 2H), 3.53 (t, $J = 6.5$ Hz, 2H), 2.32 (m, 2H). Anal. $C_{23}H_{17}BrN_2O_3$ (C, H, N).

*5.4.1.5. 6-(3-Bromopropyl)-4-(2-chlorophenyl)-9-hydroxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**89b**).* Reaction of alcohol **73b** with MsCl, followed by demethylation with BBr_3, following Procedure 8, gave **89b** (81%) as an orange powder; mp 278 °C (dec). ^{1}H NMR [$(CD_3)_2SO$] δ 11.08 (s, 1H), 9.39 (s, 1H), 8.39 (d, $J = 2.4$ Hz, 1H), 7.81 (s, 1H), 7.60 (d, $J = 8.7$ Hz, 1H), 7.60–7.58 (m, 1H), 7.53–7.43 (m, 3H), 7.16 (dd, $J = 8.7$, 2.4 Hz, 1H), 4.55 (t, $J = 6.9$ Hz, 2H), 3.56–3.49 (m, 2H), 2.34–2.24 (m, 2H). Anal. $C_{23}H_{16}BrClN_2O_3$ (C, H, N).

*5.4.1.6. 6-(3-Bromopropyl)-4-(2,6-dichlorophenyl)-9-hydroxypyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**89c**).* Alcohol **73c** (0.77 g, 1.68 mmol) was mesylated and demethylated with BBr_3, according to Procedure 8. This product was dissolved in EtOAc (100 mL) containing LiBr (1.0 g), and the solution was kept at 50 °C for 2 h, then worked up. Chromatography on silica gel, eluting with EtOAc/hexane (1:1 to 4:1), gave **89c** (0.70 g, 80%); mp (EtOAc/hexane) 273–276 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.15 (br s, 1H), 9.42 (s, 1H), 8.38 (d, $J = 2.4$ Hz, 1H), 7.87 (s, 1H), 7.62 (m, 3H), 7.51 (dd, $J = 8.9$, 7.6 Hz, 1H), 7.18 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.56 (t, $J = 6.9$ Hz, 2H), 3.51 (t, $J = 6.7$ Hz, 2H), 2.27 (m, 2H). Anal. $C_{23}H_{15}BrCl_2N_2O_3$ (C, H, N).

5.4.2. Procedure 9: amine displacement of C-2 mesylates/C-3 bromides

*5.4.2.1. 6-[3-(Dimethylamino)propyl]-9-hydroxy-4-phenylpyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**39**).* A solution of **89a** (0.12 g, 0.27 mmol) in dimethylacetamide (4 mL) was treated with $NHMe_2$ (25 mol equivalents, 0.85 mL of a 40% aqueous solution) and the reaction was stirred in a sealed vessel at 80 °C for 18 h, then diluted with water. The resulting

solution was acidified with conc. HCl and the pH was then adjusted to ca. 9 with solid K_2CO_3. The resulting precipitate was either collected by filtration and washed with water, or extracted with EtOAc, dried, and the organic layer evaporated. Chromatography on silica gel, eluting with MeOH/CH_2Cl_2 (1:9 to 1:4), gave **39** (52 mg, 47%) as a yellow powder; mp (EtOAc/hexane) 185–189 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.05 (s, 1H), 9.34 (s, 1H), 8.41 (d, $J = 2.4$ Hz, 1H), 7.78 (s, 1H), 7.64 (m, 2H), 7.56 (d, $J = 8.9$ Hz, 1H), 7.47 (m, 3H), 7.14 (dd, $J = 8.9$, 2.4 Hz, 1H), 4.48 (t, $J = 6.6$ Hz, 2H), 2.16 (t, $J = 6.5$ Hz, 2H), 2.08 (s, 6H), 1.90 (m, 2H). Anal. $C_{25}H_{23}N_3O_3 \cdot 1/4H_2O$ (C, H, N).

Compounds **27–38**, **40–53** were similarly prepared according to Procedure 9 (Supplementary material).

5.5. Procedures of Scheme 5

5.5.1. 4-(4-(2-Chlorophenyl)-9-hydroxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)butanenitrile (*24*)

To a solution of **89b** (0.13 g, 0.27 mmol) in DMSO (2 mL) was added a solution of NaCN (15 mg, 0.30 mmol) in DMSO (2 mL) dropwise over 15 min. After 30 min the reaction was diluted with water and given a standard EtOAc work-up. Chromatography on silica, eluting with EtOAc/hexane (1:4 to 1:1), followed by crystallisation from EtOAc/hexane, gave **24** (68 mg, 59%) as an orange powder; mp 262–266 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.08 (br s, 1H), 9.39 (s, 1H), 8.39 (d, $J = 2.4$ Hz, 1H), 7.83 (s, 1H), 7.59 (m, 2H), 7.49 (m, 3H), 7.15 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.50 (t, $J = 7.3$ Hz, 2H), 2.57 (m, 2H), 2.07 (m, 2H). FABMS found $[M + H]^+$: 430.0927, 432.0916. $C_{24}H_{16}ClN_3O_3$ requires 430.0958, 432.0929.

5.5.2. 4-(2-Chlorophenyl)-9-hydroxy-6-(3-methoxypropyl)-pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (*25*)

A solution of **89b** (50 mg, 0.103 mmol) and sodium methoxide (35.2 mg, 0.65 mmol) in MeOH (0.5 mL) and *p*-dioxane (8 mL) was refluxed for 3 h. The solution was acidified with 2 N HCl, then extracted with EtOAc. The organic layer was dried (Na_2SO_4), the drying agent was removed and the solution was concentrated to dryness and then chromatographed on silica. Elution with EtOAc gave 4-(2-chlorophenyl)-9-hydroxy-6-(3-methoxypropyl)-1*H*-furo[3,4-*c*]carbazole-1,3(6*H*)-dione (**90**) as an orange powder which was used directly. ^{1}H NMR [$(CD_3)_2SO$] δ 9.57 (br s, 1H), 8.24 (d, $J = 2.3$ Hz, 1H), 8.00 (s, 1H), 7.66–7.61 (m, 2H), 7.58–7.48 (m, 3H), 7.22 (dd, $J = 9.0$, 2.3 Hz, 1H), 4.56 (t, $J = 6.7$ Hz, 2H), 3.22 (t, $J = 6.0$ Hz, 2H), 3.13 (s, 3H), 2.02 (m, 2H).

Compound **90** was added to molten ammonium acetate (10 g) at 140 °C and the mixture was warmed at this temperature for 3 h. Water was added and the resulting precipitate was filtered off, adsorbed onto silica from a THF solution, and chromatographed. Elution with EtOAc/petroleum ether (1:1) gave **25** (32 mg, 71%) an orange powder; mp 260–262 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.07 (br s, 1H), 9.42 (br s, 1H), 8.39 (d, $J = 2.4$ Hz, 1H), 7.69 (s, 1H), 7.60–7.44 (m, 5H), 7.15 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.49 (t, $J = 6.6$ Hz, 2H), 3.22 (t, $J = 6.0$ Hz, 2H), 3.13 (s, 3H), 1.99 (m, 2H). FABMS found $[M + H]^+$: 437.1088, 435.1090. $C_{24}H_{20}ClN_2O_4$ requires 437.1082, 435.1112.

5.6. Procedures of Scheme 6

5.6.1. Procedure 10: Dess–Martin/sodium chlorite oxidation

Dess–Martin periodinane (1.91 g, 4.5 mmol) was added to a stirred solution of **73a** (1.20 g, 3.0 mmol) in THF (100 mL) under nitrogen. After 1 h at room temperature, a solution of saturated aqueous $Na_2S_2O_8$ and saturated aqueous $NaHCO_3$ (1:1, 100 mL) was added, and the mixture was stirred vigorously for 15 min, then extracted with EtOAc. The organic layer was dried (Na_2SO_4) and evaporated to dryness. The crude aldehyde was dissolved in *t*-BuOH/THF (9:1, 230 mL) and treated with 2-methyl-2-butene (12.0 mmol, 6.0 mL of a 2 M solution in THF), followed by sodium chlorite (1.09 g, 12.0 mmol) and NaH_2PO_4 (2.5 g, 18.0 mmol) in water (100 mL) and *t*-BuOH (4 mL). The resulting solution was stirred at room temperature for 18 h, then diluted with brine and extracted with EtOAc. The organic layer was dried and evaporated, and the residue was chromatographed on silica gel, eluting with EtOAc/MeOH (1:0 to 9:1), to give 3-(9-methoxy-1,3-dioxo-4-phenyl-2,3-dihydropyrrolo[3,4-*c*]carbazol-6(1*H*)-yl)propanoic acid (**91a**) (0.86 g, 69%), which was used directly; mp (EtOAc/hexane) 252–255 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.2 (br s, 1H), 11.13 (br s, 1H), 8.55 (d, $J = 2.6$ Hz, 1H), 7.89 (s, 1H), 7.68 (m, 3H), 7.47 (m, 3H), 7.28 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.73 (t, $J = 6.6$ Hz, 2H), 3.90 (s, 3H), 2.76 (t, $J = 6.6$ Hz, 2H).

5.6.2. Procedure 11: primary carboxamide formation

*5.6.2.1. 3-(9-Methoxy-1,3-dioxo-4-phenyl-2,3-dihydropyrrolo-[3,4-c]carbazol-6(1H)-yl)propanamide (**92a**).* To a solution of **91a** (100 mg, 0.24 mmol) in dry THF (20 mL) under nitrogen was added one drop of DMF, followed by oxalyl chloride (84 μL, 0.96 mmol) dropwise. The resulting solution was stirred at room temperature for 2 h, then evaporated to dryness in vacuo. Dry benzene (20 mL) was added to the residue and the suspension was again evaporated to dryness in vacuo, then dissolved in dry THF (20 mL) and flushed with nitrogen. To this solution was added a saturated solution of ammonia gas in THF (20 mL). The resulting solution was stirred at room temperature for 1 h, then diluted with brine and extracted with EtOAc. The organic layer was dried and evaporated, and the residue was chromatographed on silica gel, eluting with CH_2Cl_2/MeOH (95:5), to give **92a** (61 mg, 62%) as a yellow powder; mp 266–270 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.11 (br s, 1H), 8.55 (d, $J = 2.6$ Hz, 1H), 7.87 (s, 1H), 7.68 (m, 3H), 7.48 (m, 3H), 7.33 (br s, 1H), 7.29 (dd, $J = 9.0$, 2.6 Hz, 1H), 6.85 (br s, 1H), 4.71 (t, $J = 6.5$ Hz, 2H), 3.90 (s, 3H), 2.60 (t, $J = 6.5$ Hz, 2H). Anal. $C_{24}H_{19}N_3O_4 \cdot 1/2H_2O$ (C, H, N).

5.6.3. 3-(9-Hydroxy-1,3-dioxo-4-phenyl-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)propanamide (*19*)

Reaction of **92a** (56 mg, 0.14 mmol) with molten pyridine hydrochloride, according to Procedure 7, followed by

chromatography on silica, eluting with EtOAc/hexane (4:1) to EtOAc/MeOH (9:1), gave **19** (11 mg, 20%) as an orange powder; mp 284–288 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.05 (br s, 1H), 9.33 (br s, 1H), 8.40 (d, $J = 2.5$ Hz, 1H), 7.82 (s, 1H), 7.67 (m, 2H), 7.56 (d, $J = 8.8$ Hz, 1H), 7.47 (m, 3H), 7.34 (br s, 1H), 7.12 (dd, $J = 8.8$, 2.5 Hz, 1H), 6.86 (br s, 1H), 4.67 (t, $J = 6.5$ Hz, 2H), 2.58 (t, $J = 6.5$ Hz, 2H). FABMS found $[M + H]^+$: 400.1307. $C_{23}H_{17}N_3O_4$ requires 400.1297.

Compounds **20** and **21** were similarly prepared according to Procedures 10, 11, and 4 (Supplementary material).

5.6.4. Procedure 12: solubilised carboxamide formation

*5.6.4.1. N-[2-(Dimethylamino)ethyl]-3-(9-methoxy-1,3-dioxo-4-phenyl-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)propanamide (**93a**).* Reaction of **91a** (100 mg, 0.24 mmol) with oxalyl chloride, according to Procedure 11, gave the crude acid chloride which was dissolved in dry THF (20 mL), flushed with nitrogen, and treated with *N*,*N*-dimethylethylenediamine (0.105 mL, 0.96 mmol) at room temperature for 2 h. The reaction was diluted with water, basified with solid K_2CO_3, and extracted with EtOAc. The organic layer was dried and evaporated, and the residue was chromatographed on silica gel, eluting with EtOAc/MeOH/Et_3N (1:0:0 to 3:1:trace), to give **93a** (85 mg, 73%) as a yellow powder; mp (EtOAc/hexane) 206–210 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.11 (br s, 1H), 8.54 (d, $J = 2.6$ Hz, 1H), 7.81 (s, 1H), 7.73 (t, $J = 5.6$ Hz, 1H), 7.67 (m, 3H), 7.48 (m, 3H), 7.29 (dd, $J = 8.9$, 2.6 Hz, 1H), 4.73 (t, $J = 6.3$ Hz, 2H), 3.89 (s, 3H), 2.93 (dd, $J = 6.8$, 5.6 Hz, 2H), 2.59 (t, $J = 6.3$ Hz, 2H), 1.90 (s, 6H), 1.84 (t, $J = 6.8$ Hz, 2H). Anal. $C_{28}H_{28}N_4O_4 \cdot H_2O$ (C, H, N).

*5.6.5. N-[2-(Dimethylamino)ethyl]-3-(9-hydroxy-1,3-dioxo-4-phenyl-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)-propanamide (**54**)*

Compound **93a** (70 mg, 0.14 mmol) was demethylated with pyridine hydrochloride at 200 °C for 10 min, following Procedure 7. The reaction mixture was diluted with water, basified with conc. ammonia and extracted with EtOAc. The organic layer was dried and evaporated, and the residue was chromatographed on silica gel, eluting with EtOAc/MeOH/Et_3N (1:0:0 to 3:1:trace), to give **54** (35 mg, 53%); mp (EtOAc/hexane) 176–180 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.06 (br s, 1H), 9.35 (br s, 1H), 8.39 (d, $J = 2.5$ Hz, 1H), 7.77 (s, 1H), 7.74 (t, $J = 5.6$ Hz, 1H), 7.66 (m, 2H), 7.53 (d, $J = 8.8$ Hz, 1H), 7.48 (m, 3H), 7.12 (dd, $J = 8.8$, 2.5 Hz, 1H), 4.69 (t, $J = 6.4$ Hz, 2H), 2.94 (m, 2H), 2.58 (t, $J = 6.4$ Hz, 2H), 1.91 (s, 6H), 1.87 (m, 2H). Anal. $C_{27}H_{26}N_4O_4 \cdot 3/4H_2O$ (C, H, N).

Compounds **55**, **56**, and **59–61** were similarly prepared according to Procedures 11, 12 and 7 (or 4) (Supplementary material).

*5.6.6. Methyl 3-(4-(2-chlorophenyl)-9-hydroxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)propanoate (**23**)*

Gaseous HCl was bubbled through a stirred solution of **60** (35 mg, 0.08 mmol) in MeOH (10 mL) for 30 s. The resulting solution was stirred for 30 min at room temperature, then diluted with water and extracted with EtOAc. Chromatography of the product on silica, eluting with EtOAc/hexane (1:1), followed by crystallisation from EtOAc/hexane, gave **23** (25 mg, 70%) as an orange powder; mp 218–220 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.07 (br s, 1H), 9.39 (br s, 1H), 8.38 (d, $J = 2.5$ Hz, 1H), 7.79 (s, 1H), 7.57 (m, 2H), 7.51–7.44 (m, 3H), 7.14 (dd, $J = 8.8$, 2.5 Hz, 1H), 4.71 (t, $J = 6.8$ Hz, 2H), 3.48 (s, 3H), 2.83 (t, $J = 6.8$ Hz, 2H). Anal. $C_{24}H_{17}ClN_2O_5 \cdot 3/4H_2O$ (C, H, N).

*5.6.7. 4-(4-(2-Chlorophenyl)-9-methoxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)butanoic acid (**94**)*

Alkylation of 2-[(*E*,*Z*)-2-(2-chlorophenyl)ethenyl]-5-methoxy-1*H*-indole (**70b**) [9] (0.40 g, 1.41 mmol) with NaH (0.14 g of a 50% dispersion in mineral oil, 2.82 mmol) and ethyl bromobutyrate in DMF (20 mL), according to Procedure 1, followed by reaction with maleimide and aromatisation with DDQ, following Procedures 2 and 5, respectively, gave the crude ester product. This crude product was dissolved in MeOH (100 mL) and treated with 2 M KOH (2 mL) at room temperature for 2 h. Dilution with water and acidification with 1 N HCl gave a precipitate that was collected by filtration, dried and chromatographed on silica gel, eluting with EtOAc. Trituration of the purified fractions with Et_2O/hexane, gave **94** as a yellow powder (0.10 g, 16%); mp 251–254 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.12 (br s, 1H), 11.12 (br s, 1H), 8.53 (d, $J = 2.6$ Hz, 1H), 7.85 (s, 1H), 7.72 (d, $J = 9.0$ Hz, 1H), 7.58 (m, 1H), 7.53–7.45 (m, 3H), 7.32 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.50 (t, $J = 7.3$ Hz, 2H), 3.91 (s, 3H), 2.30 (m, 2H), 1.97 (m, 2H). FABMS found $[M + H]^+$: 463.1025, 465.1034. $C_{25}H_{19}ClN_2O_5$ requires 463.1061, 465.1031.

*5.6.8. 4-(4-(2-Chlorophenyl)-9-hydroxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)butanoic acid (**68**)*

Demethylation of **94** (32 mg, 0.07 mmol) with 1 N BBr_3 in CH_2Cl_2, following Procedure 4, gave **68** (6 mg, 19%) as an orange powder; mp 274–277 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.0 (v br s, 1H), 11.06 (br s, 1H), 9.38 (br s, 1H), 8.38 (d, $J = 2.4$ Hz, 1H), 7.80 (s, 1H), 7.60 (d, $J = 8.9$ Hz, 1H), 7.58 (m, 1H), 7.52–7.43 (m, 3H), 7.14 (dd, $J = 8.9$, 2.4 Hz, 1H), 4.45 (t, $J = 7.5$ Hz, 2H), 2.27 (m, 2H), 1.95 (m, 2H). FABMS found $[M + H]^+$: 449.0874, 451.0879. $C_{24}H_{17}ClN_2O_5$ requires 449.0904, 451.0875.

5.7. Procedures of Scheme 7

5.7.1. Procedure 13: acylsulfonamide formation

*5.7.1.1. N-[3-(4-(2-Chlorophenyl)-9-methoxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)propanoyl]methanesulfonamide (**95**).* To a stirred solution of **91b** (0.20 g, 0.45 mmol), 4-dimethylaminopyridine (DMAP) (165 mg, 1.35 mmol) and methanesulfonamide (86 mg, 0.90 mmol) in DMF (10 mL) under nitrogen was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI·HCl) (259 mg, 1.35 mmol). The resulting mixture was stirred at room temperature for 18 h and then diluted with water,

acidified by the addition of 1 N HCl, and extracted with EtOAc. The organic phase was dried (Na_2SO_4), the drying agent was removed, and the solution was concentrated to dryness. Chromatography on silica, eluting with EtOAc/hexane (4:1) to EtOAc/MeOH (1:0 to 9:1), gave **95** (151 mg, 64%) as a yellow powder; mp 293–295 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.76 (br s, 1H), 11.14 (br s, 1H), 8.52 (d, $J = 2.6$ Hz, 1H), 7.87 (s, 1H), 7.73 (d, $J = 9.0$ Hz, 1H), 7.58 (m, 1H), 7.53–7.46 (m, 3H), 7.32 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.74 (t, $J = 6.9$ Hz, 2H), 3.91 (s, 3H), 3.08 (s, 3H), 2.81 (t, $J = 6.9$ Hz, 2H). Anal. $C_{25}H_{20}ClN_3O_6S$ (C, H, N).

5.7.2. *N-[3-(4-(2-Chlorophenyl)-9-hydroxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)-propanoyl]methanesulfonamide (**62**)*

Reaction of **95** (130 mg, 0.25 mmol) with BBr_3, according to Procedure 4, followed by chromatography on silica, eluting with MeOH/CH_2Cl_2 (5:95–1:9), gave **62** (83 mg, 66%) as an orange powder; mp 290–296 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.75 (br s, 1H), 11.08 (br s, 1H), 9.38 (s, 1H), 8.38 (d, $J = 2.4$ Hz, 1H), 7.80 (s, 1H), 7.59 (m, 2H), 7.53–7.46 (m, 3H), 7.14 (dd, $J = 8.9$, 2.4 Hz, 1H), 4.69 (t, $J = 6.9$ Hz, 2H), 3.04 (s, 3H), 2.76 (t, $J = 6.9$ Hz, 2H). Anal. $C_{24}H_{18}ClN_3O_6S \cdot 3/4H_2O$ (C, H, N).

Compound **63** was similarly prepared according to Procedures 13 and 4 (Supplementary material).

5.7.3. *Procedure 14: tetrazole formation*

*5.7.3.1. 4-(2-Chlorophenyl)-9-hydroxy-6-[2-(1H-tetrazol-5-yl)-ethyl]pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**64**).* To a solution of **22** (0.68 g, 1.63 mmol) in toluene/DMF (5:1, 120 mL) was added azidotrimethyl tin (0.67 g, 3.26 mmol). The resulting solution was heated at reflux for 24 h, then a further portion of azidotrimethyl tin (0.34 g, 1.63 mmol) was added. After a further 24 h at reflux, the reaction was diluted with water and extracted with EtOAc. Chromatography on silica, eluting with EtOAc/hexane (3:1) to EtOAc/MeOH (9:1), followed by crystallisation from EtOAc/hexane, gave **64** (0.54 g, 72%) as an orange powder; mp 230–234 °C (dec). ^{1}H NMR [$(CD_3)_2SO$] δ 11.06 (br s, 1H), 9.37 (br s, 1H), 8.36 (d, $J = 2.4$ Hz, 1H), 7.61 (s, 1H), 7.56 (m, 1H), 7.48 (m, 4H), 7.10 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.82 (t, $J = 6.9$ Hz, 2H), 3.32 (obscured t, $J = 6.9$ Hz, 2H). Anal. $C_{23}H_{15}ClN_6O_3 \cdot 3½H_2O$ (C, H, N).

5.7.4. *2-(4-(2-Chlorophenyl)-9-methoxy-1,3-dioxo-2,3-dihydropyrrolo[3,4-c]carbazol-6(1H)-yl)ethyl-methanesulfonate (**97**)*

Alcohol **77b** (1.10 g, 2.61 mmol) was dissolved in dry THF (80 mL) under nitrogen. The resulting solution was cooled to 0 °C and Et_3N (2.0 mL) was added, followed by MsCl (263 μL, 3.40 mmol) dropwise. After 30 min a further portion of MsCl (50 μL) was added and then, after another 30 min, the reaction was diluted with saturated aqueous $NaHCO_3$ and extracted with EtOAc. The organic layer was dried (Na_2SO_4), the drying agent was removed and the solution was concentrated to dryness. Crystallisation from EtOAc/hexane gave **97** (0.96 g, 74%) as a yellow solid; mp 254 °C (dec). ^{1}H NMR [$(CD_3)_2SO$] δ 11.15 (s, 1H), 8.53 (d, $J = 2.6$ Hz, 1H), 7.90 (s, 1H), 7.74 (d, $J = 9.0$ Hz, 1H), 7.58 (m, 1H), 7.48 (m, 3H), 7.33 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.89 (t, $J = 5.0$ Hz, 2H), 4.56 (m, 2H), 3.91 (s, 3H), 2.94 (s, 3H). FABMS found $[M + H]^+$: 499.0694, 501.0696. $C_{24}H_{19}ClN_2O_6S$ requires 499.0731, 501.0701.

5.7.5. *4-(2-Chlorophenyl)-9-methoxy-6-[2-(4H-1,2,4-triazol-3-ylsulfanyl)ethyl]pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**98**)*

A solution of **97** (0.25 g, 0.50 mmol), 1*H*-1,2,4-triazole-5-thiol (76 mg, 0.75 mmol) and Et_3N (2.0 mL) in *p*-dioxane (50 mL) under nitrogen was heated at reflux for 48 h, then diluted with water and extracted with EtOAc. The organic phase was dried, the drying agent was removed and the solution was concentrated to dryness. Chromatography on silica, eluting with EtOAc/hexane (1:1 to 1:0), followed by crystallisation from EtOAc/hexane, gave **98** (0.24 g, 95%) as a yellow powder; mp 211–213 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 14.09 (br s, 1H), 11.13 (br s, 1H), 8.52 (d, $J = 2.6$ Hz, 1H), 8.43 (br s, 1H), 7.88 (s, 1H), 7.80 (d, $J = 9.0$ Hz, 1H), 7.57 (m, 1H), 7.48 (m, 3H), 7.31 (dd, $J = 9.0$, 2.6 Hz, 1H), 4.83 (t, $J = 7.4$ Hz, 2H), 3.91 (s, 3H), 3.52 (t, $J = 7.4$ Hz, 2H). Anal. $C_{25}H_{18}ClN_5O_3S \cdot 1/4H_2O$ (C, H, N).

5.7.6. *4-(2-Chlorophenyl)-9-hydroxy-6-[2-(4H-1,2,4-triazol-3-ylsulfanyl)ethyl]pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (**65**)*

Reaction of **98** (0.25 g, 0.50 mmol) with BBr_3 in CH_2Cl_2, following Procedure 4, and chromatography of the product on silica gel, eluting with EtOAc/THF (1:0 to 9:1 to 1:1), followed by trituration in MeOH, gave **65** (0.22 g, 90%) as an orange powder; mp 314–319 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 14.10 (br s, 1H), 11.07 (br s, 1H), 9.39 (s, 1H), 8.42 (br s, 1H), 8.38 (d, $J = 2.3$ Hz, 1H), 7.83 (s, 1H), 7.68 (d, $J = 8.8$ Hz, 1H), 7.57 (m, 1H), 7.48 (m, 3H), 7.13 (dd, $J = 8.8$, 2.3 Hz, 1H), 4.79 (t, $J = 7.4$ Hz, 2H), 3.50 (t, $J = 7.4$ Hz, 2H). Anal. $C_{24}H_{16}ClN_5O_3S \cdot 2/5MeOH$ (C, H, N).

5.7.7. *4-(2-Chlorophenyl)-9-hydroxy-6-[2-(1H-1,2,4-triazol-5-ylsulfinyl)ethyl]pyrrolo[3,4-c]-carbazole-1,3(2H,6H)-dione (**66**)*

To a solution of **65** (70 mg, 0.14 mmol) in THF (30 mL) and glacial HOAc (8 mL) was added 35% H_2O_2 (2 mL). The resulting solution was stirred at room temperature for 10 h, then poured onto solid $NaHCO_3$, diluted with water, and extracted with EtOAc. The combined organic layers were dried (Na_2SO_4), partially concentrated under reduced pressure, then adsorbed directly onto silica and chromatographed, eluting with MeOH/CH_2Cl_2 (5:95–1:9). Trituration in Et_2O gave **66** (29 mg, 41%) as an orange powder; mp 237–242 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 14.7 (br s, 1H), 11.09 (br s, 1H), 9.41 (br s, 1H), 8.69 (br s, 1H), 8.38 (d, $J = 2.3$ Hz, 1H), 7.79 (d, $J = 9.6$ Hz, 1H), 7.60–7.46 (m, 5H), 7.15 (dd, $J = 8.8$,

2.3 Hz, 1H), 4.89 (m, 2H), 3.71 (m, 2H). Anal. $C_{24}H_{16}ClN_5O_4S \cdot 1/5Et_2O$ (C, H, N).

5.7.8. 4-(2-Chlorophenyl)-9-hydroxy-6-[2-(1H-1,2,4-triazol-5-ylsulfonyl)ethyl]pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (67)

To a solution of **65** (60 mg, 0.12 mmol) in THF (30 mL) and glacial HOAc (10 mL) was added 35% H_2O_2 (2 mL). The resulting solution was stirred at room temperature for 30 h, then additional 35% H_2O_2 (2 mL) was added and the temperature was increased to 50 °C for 18 h. The reaction was then poured onto solid $NaHCO_3$, diluted with water, and extracted with EtOAc. The combined organic layers were dried, partially concentrated under reduced pressure, then adsorbed directly onto silica and chromatographed, eluting with EtOAc/hexane (1:1 to 1:0). Crystallisation from EtOAc/hexane gave **67** (45 mg, 72%) as an orange powder; mp 301–304 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 14.9 (br s, 1H), 11.07 (br s, 1H), 9.38 (br s, 1H), 8.60 (s, 1H), 8.34 (d, $J = 2.4$ Hz, 1H), 7.71 (s, 1H), 7.56 (m, 2H), 7.48 (m, 3H), 7.12 (dd, $J = 8.8$, 2.4 Hz, 1H), 4.89 (m, 2H), 4.03 (m, 2H). Anal. $C_{24}H_{16}ClN_5O_5S \cdot 1/4EtOAc$ (C, H, N).

5.7.9. 4-(2-Chlorophenyl)-9-hydroxy-6-[3-(1H-tetrazol-5-yl)-propyl]pyrrolo[3,4-c]carbazole-1,3(2H,6H)-dione (69)

A solution of **24** (60 mg, 0.14 mmol) in toluene/DMF (5:1, 35 mL) was treated with azidotrimethyl tin (57 mg, 0.28 mmol) for 72 h, according to Procedure 14. Chromatography on silica gel, eluting with EtOAc/hexane (3:1) to EtOAc/MeOH (9:1), gave **69** (29 mg, 44%); mp (THF/hexane) 269–272 °C. ^{1}H NMR [$(CD_3)_2SO$] δ ~16 (v br s, 1H), 11.07 (s, 1H), 9.38 (br s, 1H), 8.39 (d, $J = 2.5$ Hz, 1H), 7.84 (s, 1H), 7.61 (d, $J = 8.9$ Hz, 1H), 7.57 (m, 1H), 7.53–7.43 (m, 3H), 7.16 (dd, $J = 8.9$, 2.5 Hz, 1H), 4.59 (t, $J = 7.1$ Hz, 2H), 2.93 (m, 2H), 2.20 (m, 2H). Anal. $C_{24}H_{17}ClN_6O_3 \cdot 3/4H_2O \cdot 1/4THF$ (C, H, N).

5.8. Determinations of enzyme inhibition

Wee1 and Chk1 isolated enzyme inhibition assays were performed as reported previously [9].

5.9. Cdc2 histone H1 kinase assay for G2 checkpoint abrogation

This cellular assay is a measure of the effect of the test compounds on the activity of the Cdc2/cyclin B complex on one of its physiological substrates, histone H1. HT-29 cells, 2×10^4 per well (NUNCLON™ cat no. 163320, 96-well plate), were plated in 171 μL media [Dulbecco's Modified Eagle's Medium 4500 mg/L Glucose (DME High Glucose), 1% penicillin and streptomycin, 2% L-glutamate, 10% FBS]. The plate was incubated at 37 °C for 24 h. To each well 9 μL of a 5 μM doxorubicin (DOX) solution was added (250 nM final concentration) and incubated at 37 °C for an additional 16 h. Next, 20 μL of 500 ng/mL nocodazole was added per well, immediately followed by addition of 5 μL test compound. The plate was incubated at 37 °C for 4 h. The plate was removed from the incubator and spun in a Beckman GS-6R Centrifuge for 10 min at 800 rpm and 4 °C. The media was removed and the plate surface dried by blotting. To each well 100 μL of PBS was added. The plate was spun as above. The PBS was removed from the plate and the plate surface was dried. To each well 20 μL of lysis buffer (50 mM Hepes pH 7.5, 250 mM NaCl, 0.1% NP 40, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM EDTA, 1 mM pefabloc, 1 mM DTT, 0.1 mM sodium orthovanadate, 10 μg/mL aprotinin, 20 μM leupeptin) was then added, followed by medium-speed rocking at 4 °C for 45 min. After lysis, 30 μL of kinase assay buffer (50 mM Hepes, 22 mM $MgCl_2$, 1 mM DTT, 166.7 ng/μL histone H1, 83 μM ATP, 0.033 μCi/μL [γ-^{33}P] ATP) was added. The plate was incubated on a 32 °C plate warmer for 25 min. The kinase reaction was stopped by adding 80 μL of 100 mM EDTA, pH 7.8, to each well. The lysate was harvested onto a pre-wetted Wallac P30 filtermat (Wallac 1450–523, glass fiber filter with negatively charged P30 active groups, size 90 × 120 mm) using 75 mM H_3PO_4 for 10 s, followed by a 10 s aspiration step. The filtermat was placed in a 75 mM H_3PO_4 bath and shaken gently for 10 min at room temperature, then placed within the fold of a single sheet of paper towel, and subjected to microwaves on high power for 2–3 min, or until the filtermat was dry. The filtermat was placed in a sample bag (Wallac 1450-432), 5 mL nonaqueous scintillation fluid was added to the sample bag and the bag was sealed. The samples were read in a Wallac 1450 MicroBeta Liquid Scintillation Counter. The data shown in Table 1 demonstrate the cellular effect of a Wee1 and/or Chk1 inhibitor on a physiological substrate of Cdc2/cyclinB, a complex which itself is a substrate of Wee1 kinase.

5.10. Detection of MPM-2 in cultured HT-29 cells ± potential checkpoint abrogators, doxorubicin, and nocodazole

This assay uses polyclonal antibody to quantitate the M-phase specific histological markers in the determination of a mitotic index (fraction of cells found in mitosis). HT-29 cells were grown in Dulbecco's Modified Eagle Medium with high glucose, supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 16 mM HEPES, 8 mM MOPS, and 10% fetal bovine serum. They were incubated at 37 °C in an atmosphere of 5% CO_2 and 100% relative humidity. Cells were seeded in 96-well tissue culture plates at 100 μL per well at a concentration of 4×10^4 per mL. The cells were allowed to attach and begin growing for 24 h. To the test cells 100 μL of doxorubicin (DOX) at 2 μM (final = 1 μM) was added and the cells were incubated for 1 h as above. Following the incubation, the plates were washed twice with growth media. The media was replaced with 100 μL fresh media and incubated for a further 16 h. Serial 2-fold dilutions of potential abrogators were added to the test wells. The rows that received nocodazole (NOC) have 2 μL NOC added at 2.5 μg/mL. The plates were then incubated for an additional 6 h. Following the incubation, the plates were centrifuged for 5 min at 4 °C and 200G.

To each well 100 μL of methanol/acetic acid in a ratio 3:1, was added directly and left at room temperature for 15 min. The media/fixative mixture was removed by suction and replaced with ice cold ethanol/acetic acid in a 20:1 ratio. The plates were stored at 4 °C until stained. The fixed cells were stained for MPM-2 using the Upstate Biotechnology MPM-2 rhodamine detection kit as per the manufacturer's instructions. Briefly, the cells were washed with Dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium, followed by incubation with blocking buffer, consisting of 8% bovine serum albumen (BSA) for a minimum of 1 h. The cells were then washed with PBS one time and treated overnight at 4 °C with primary antibody at 5 μg/mL (100 μL per well), diluted using DPBS with 1% BSA. The cells were then washed twice for 15 min each with DPBS and then incubated with a 1:1000 dilution of rhodamine conjugated goat antimouse IgG in DPBS with 1% BSA for 1 h. The secondary antibody was washed off in three 30 min washes with DPBS. The cells were then counterstained using the Molecular Probes Mycoflour kit to stain nuclei following the instructions for the kit. The stained cells were viewed under fluorescence microscopy using fluorescence filters suitable for detecting rhodamine and DAPI stains. Images were captured using a Spot digital camera and analyzed using ImageQuant to quantitate total nuclei and to count MPM-2 positive cells.

5.11. Western blot determination of phosphotyrosine 15 on Cdc2

These western blot assays measure the phosphorylation state of the physiological substrate of Wee1: tyrosine 15 on Cdc2 kinase. This is accomplished by means of a phosphospecific antibody whose signal is normalised by comparison with the total amount of Cdc2 detected in the samples. HT-29 cells were grown in Dulbecco's Modified Eagle Medium with high glucose, supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 16 mM HEPES, 8 mM MOPS, and 10% fetal bovine serum. The cells were incubated at 37 °C, in 5% CO_2, and 100% relative humidity. Cells were grown and treated in 6-well tissue culture plates. Cells were seeded in 3 mL media at a concentration of 2×10^5 per mL. Once seeded, the cells were allowed to attach 24 h. All treatments were done in duplicate wells. The wells that were treated with doxorubicin (DOX) were exposed to 1 μM DOX for 1 h. DOX was dissolved in sterile distilled water. After the 1 h incubation, the cells were washed twice with 2 mL media and then incubated in 3 mL media for 16 h. After the 16 h incubation, the cells were treated with various concentrations of abrogator ± nocodazole (NOC) at 50 ng/mL. Abrogators were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM and diluted with growth medium before being added to the cells and NOC was dissolved at 1 mg/mL in DMSO and diluted with growth medium before administration to the cells. The cells were incubated 6 h with the abrogator and NOC. The duplicate wells were scraped, on ice, and combined in a 15 mL centrifuge tube. The wells were rinsed with Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium and the rinse combined with the scraped cells. The cells were centrifuged at 200G at 4 °C for 5 min. The supernatant was discarded and the pellets resuspended in 100 μL DPBS. The cell suspension was then transferred to 1.5 mL eppendorf centrifuge tubes and centrifuged at 4 °C for 4 min at 4000 rpm. After the supernatant was removed, the pellet was frozen on dry ice and stored at −80 °C. The pellets were thawed on ice prior to lysis with the lysis buffer, ELB (2.5 mM HEPES (7.5), 150 mM NaCl, 25 μM NaF and 0.5% NP40 supplemented with 1 mM AEBSF, 1 mM sodium orthovanadate, and 1 mM dithiothretol) and complete protease inhibitor cocktail tablets (Roche Biochemicals). The tablets were dissolved in 2 mL distilled water and diluted 1:25 in the lysis buffer. The pellets were suspended in 100 μL complete lysis buffer and incubated on ice for 30 min. Following lysis, the suspension was centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant liquid was collected and the protein concentration determined using the Pierce BCA protein assay kit as per the manufacturer's instructions. The protein concentration was adjusted to 3 mg/mL with DPBS. The samples were then diluted 1:1 with Invitrogen 2 × Tris–glycine sample buffer supplemented with 50 μL/mL 2-mercaptoethanol, boiled for 3 min, and stored frozen at −20 °C. Thirty micrograms of protein per lane were run on Novex pre-cast 12%, 1.5 mm, 10-well, Tris–glycine polyacrylimide gels using Novex running buffer and Invitrogen (see Blue Plus 2) molecular weight standards. The gels were run at 100 V for 30 min, then 125 V for 1.5 h. The proteins were transferred to 0.45 μm pore nitrocellulose membranes using Novex transfer buffer and the Novex X-Cell II blot module. The nitrocellulose membranes were blocked overnight at room temperature. The blocking buffer was 5 mM Tris (8.0), 150 mM NaCl, 0.1% Tween 20,1 mM NaF, 10 mM glycerolphosphate, 100 μM sodium orthovanadate, and 3% bovine serum albumen. After blocking, the gels were cut with a razor blade and treated with biotinylated antiphosphotyrosine 15, diluted 1:5000 in blocking buffer. The membranes were incubated for 2 h at room temperature with constant rocking. The antibody solution was removed and the membranes were washed three times for 20 min each with TNT buffer. TNT buffer consisted of 50 mM Tris (8.0), 150 mM NaCl, and 0.1% Tween 20. Secondary antibody was then added in blocking buffer (Neutravidin HRP at 1:40,000). The blots remained in secondary antibody for 1 h at room temperature, followed by three 20 min washes with TNT buffer. Protein bands were detected using the Amersham Pharmacia ECL detection kit and Kodak Bio Max film as per the manufacturer's instructions. The phosphotyrosine 15 membranes were stripped using the Chemicon International Re-Blot kit as per the manufacturer's instructions. The blots were then washed twice with TNT buffer and once with blocking buffer for 20 min each. Anti Cdc2 (cdk1; Labvision Corporation) were diluted 150 μL per 50 mL blocking buffer and incubated with the blots for 2 h at room temperature, followed by three 20 min washes in TNT buffer. The secondary antibody was Bio Rad goat antimouse HRP and was diluted 1:10,000 in blocking buffer before a 1 h incubation with the

blots at room temperature. Three 20 min washes preceded ECL detection.

5.12. Clonogenic survival assay in HT-29 cells ± doxorubicin

This cellular assay is a measure of the toxicity of the test compounds in the absence and presence of DNA damage induced by a conventional chemotherapeutic agent. HT-29 cells were grown in Dulbecco's Modified Eagle Medium with high glucose, supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 16 mM HEPES, 8 mM MOPS, and 10% fetal bovine serum. The cells were incubated at 37 °C in an atmosphere of 5% CO_2 and 100% relative humidity. Two or three T-75 tissue culture flasks were seeded at about 50% confluency in 30 mL media and incubated for approximately 24 h. Doxorubicin (DOX) (dissolved at 5 mM in distilled water (dH_2O)) was added to the flasks to a final concentration of 1 μM or 500 nM. One flask received no DOX. The cells were allowed to incubate with the DOX for 1 h. All flasks were then washed twice with 20 mL media and allowed to incubate for a further 16 h in 30 mL media. The stock agar (3.2% Seaplaque GTG Agarose (BioWhittaker Molecular Applications)) was suspended in dH_2O and autoclaved for 20 min. The agar was melted prior to use in a microwave oven. The bottom agar was a 1:4 dilution of the stock agar in media, with enough fetal bovine serum added to bring the solution to 10%. One milliliter was plated in each well of a 6-well tissue culture plate and the plates were allowed to harden. The cloning agar (a 1:8 dilution of stock agar in media with fetal bovine serum added to 10%) was prepared and held at 40 °C until used. The cells were trypsinized using Trypsin/EDTA and their concentration adjusted to 7.5×10^4 cells/μL with media. Into sterile 15 mL plastic centrifuge tubes 100 μL of each cell suspension was placed. Twenty-five microliters of each test compound was added to appropriate tubes, followed by the addition of 5 mL of warm cloning agar. The tubes were mixed well and 2 mL of the agar/cell suspension was added to duplicate wells of the 6-well plates that were coated with agar earlier. The plates were swirled and placed in the refrigerator for 5 min. After the plates returned to room temperature, they were incubated for 10 to 14 days, until colonies were visible. The colonies were stained with INT (*p*-iodonitrotetrazolium violet) (dissolved in dH_2O at 1 mg/mL and filter sterilized). To each well 1 mL INT was added and the plates were incubated overnight at 37 °C, 5% CO_2, and 100% relative humidity. The colonies were counted using a Hamamatsu video imaging system and ImageQuant software.

5.13. Crystallography

Wee1 protein was expressed, purified, complexed, crystallised, and X-ray data collected as described previously [12]. The Wee1 complex structures were determined by molecular replacement using the previously published structure as initial model and refinement with Refmac [14]. Refinement proceeded with the replacement of molecule **6** by molecule **18** or **59** as appropriate. Tabulated details of the data collection, structure solution and refinement can be found in Supplementary material. Compound **18** X-ray coordinates; PDB ID 2IO6. Compound **59** X-ray coordinates; PDB ID 2IN6.

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Appendix. Supplementary material

Additional experimental procedures, characterisations, and combustion analytical data. Tabulated details of the data collection, structure solution and refinement for compounds **18** and **59** bound to Wee1 kinase. This material is available online at www.sciencedirect.com. Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2007.07.016.

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