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Synthesis and structure–activity relationships of soluble 8-substituted 4-(2-chlorophenyl)-9-hydroxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-diones as inhibitors of the Wee1 and Chk1 checkpoint kinases

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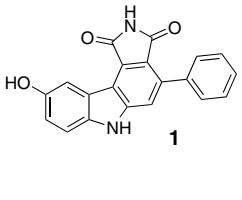
Abstract—Pyrrolo[3,4-*c*]carbazoles bearing solubilising basic side chains at the 8-position retain potent Wee1 and Chk1 inhibitory properties in isolated enzyme assays, and evidence of G2/M checkpoint abrogation in several cellular assays. Co-crystal structure studies confirm that the primary binding to the Wee1 enzyme is as described previously, with the *C*-8 side chains residing in an area of bulk tolerance.



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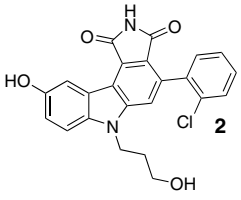

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Drugs that abrogate the G2/M checkpoint are of interest as potential adjunct therapy for cancer, since many cancer cells rely more heavily than do normal cells on the G2/M checkpoint for the repair of drug-induced DNA damage.[1] This checkpoint is mainly controlled by the kinases Wee1 and Chk1, so that inhibition of these kinases can preferentially enhance the cytotoxic effects of DNA-damaging agents. The 4-phenylpyrrolocarbazole **1** is a potent inhibitor of both Wee1 and Chk1 (IC_{50}s of 97 and 47 nM, respectively), and previous SAR studies[2,3] show that analogues with a 2′-Cl substituent on the 4-phenyl ring and a variety of neutral polar *N*-6 side chains retain high potency against both enzymes (e.g., **2** has IC_{50}s of 9 and 170 nM, respectively), but lacked sufficient solubility for in vivo development.[3]

In this paper we explore structure–activity relationships for the analogues bearing hydrogen or acceptable (Me or $(CH_2)_2OH$) side chains at *N*-6, and a variety of solubilising side chains off the *C*-8 position in ring A, where co-crystal structure studies of related compounds bound to Wee1 suggest there is significant bulk tolerance.[2–4] These were joined to the chromophore by linker groups of varying electronic and lipophilic properties (O, CH_2, S, SO, SO_2, SO_2NH and CONH) and bear cationic groups of varying pK_a (pyrrolidine, ~10.5; NMe_2, ~9.5; morpholide, ~7.8).

Schemes 1–3 outline the preparation of the compounds.[5] For the $(CH_2)_4$ side-chain analogues (Scheme 1), prepa-

Keywords: Pyrrolocarbazole; G2/M checkpoint abrogation; Wee1 kinase inhibitor; Chk1 kinase inhibitor; Checkpoint inhibitor.
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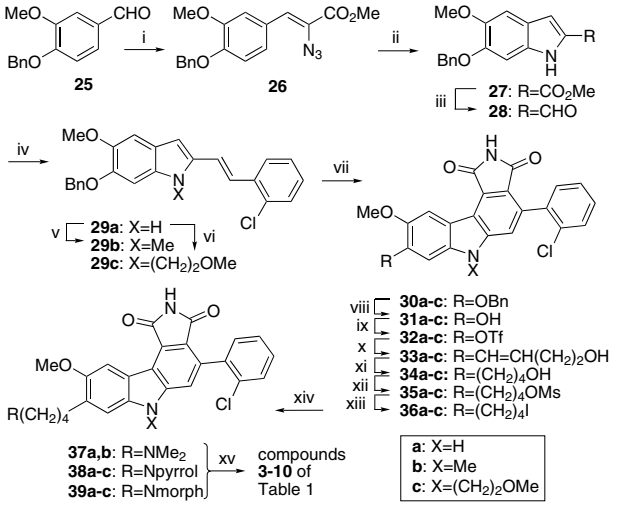
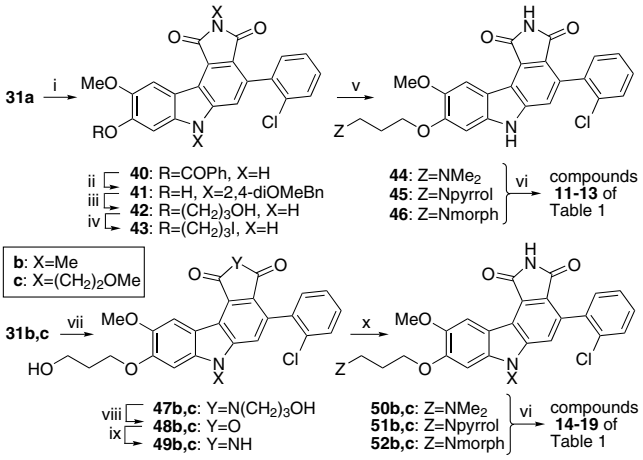
ration of the parent 8-hydroxycarbazoles **31a–c** bearing different *N*-6 groups followed essentially the published route[3] beginning with the 4-benzyloxy benzaldehyde **25**. Elaboration of the subsequent 8-OH group on the carbazole to triflate was followed by Stille reactions to give the butenols **33a–c**. Hydrogenation of these was followed by elaboration of the alcohol groups via their mesylates **35a–c** to the iodides **36a–c**. Reaction of these with the appropriate amines, followed by O-demethylation, gave the desired compounds **3–10** of Table 1.

The $O(CH_2)_3$-linked compounds **11–13** (Scheme 2) were prepared from the phenol **31a**, which was initially protected as the benzoate ester prior to bis *N*-protection with 2,4-dimethoxybenzyl groups and concomitant debenzoylation. The resulting phenol **41** was reacted with 3-bromopropanol and then *N*-deprotected to give alcohol **42**. Conversion of **42** (via the mesylate) to the iodide **43**, followed by reaction of **43** with amines, gave **44–46**. O-Demethylation of these as before then gave the desired compounds **11–13** of Table 1. Compounds **14–19** of Table 1 were prepared by reacting the *N*-6-alkylated phenols **31b**,**c** with 3-bromopropanol to give the diols **47b**,**c**. These were deprotected at the 2-position by sequential base cleavage, followed by acid cyclisation to give the anhydrides **48b**,**c**, from which the imides **49b**,**c** were regenerated by heating with ammonium acetate. Conversion of **49b**,**c** to their respective mesylates, followed by reaction with secondary amines and O-demethylation, then gave compounds **14–19**.

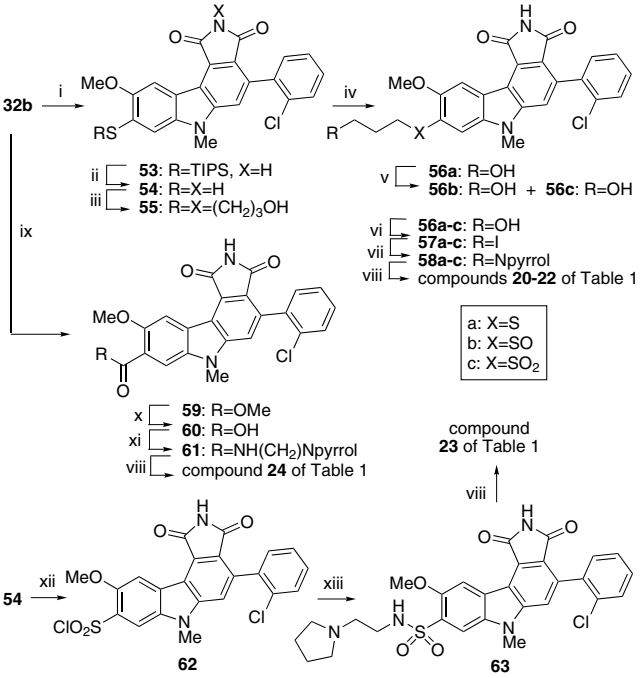
Compounds **20–24** of Table 1 were prepared as outlined in Scheme 3. Reaction of *N*-6-methylated 8-triflate **32b** with NaSTIPS under palladium catalysis followed by TBAF deprotection gave the thiol **54**, which on reaction with 3-bromopropanol gave the di(3-hydroxypropyl)



Scheme 1. Reagents and conditions: (i) $N_3CH_2CO_2Me$, NaOMe, MeOH; (ii) xylene, reflux; (iii) $LiAlH_4$, THF, then MnO_2, $CHCl_3$; (iv) 2-ClBnPPh$_3$Br, 17 N NaOH, CH_2Cl_2; (v) NaH, MeI, DMF; (vi) $MeO(CH_2)_2Br$, NaH, DMF; (vii) maleimide, cat. $SnCl_2$, $PhCH_3$, reflux; (viii) HCl, AcOH; (ix) $(TfO)_2O$, pyridine, THF; (x) (*E*)$Bu_3SnCH{=}CH(CH_2)_2OH$, LiCl, $Pd(PPh_3)_2Cl_2$, DMF; (xi) H_2, PtO_2, THF, MeOH; (xii) MsCl, pyridine, THF; (xiii) NaI, EtOAc; (xiv) R_2NH, DMA; (xv) py·HCl, 200–220 °C.



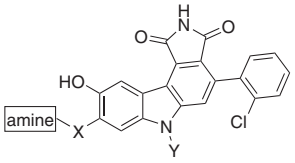
Scheme 2. Reagents and conditions: (i) PhCOCl, Et_3N, THF; (ii) 2,4-diOMeBnOH, Ph_3P, DEAD, THF; (iii) $Br(CH_2)_3OH$, K_2CO_3, DMF, then TFA, $PhOCH_3$, 90 °C; (iv) MsCl, Et_3N, THF, then NaI, EtOAc, 66 °C; (v) ZH, DMA; (vi) BBr_3, DCM, 0 °C; (vii) $Br(CH_2)_3OH$, K_2CO_3, DMF; (viii) 5 N KOH, MeOH, then 2 N HCl, dioxane, 100 °C; (ix) NH_4OAc, 150 °C; (x) MsCl, Et_3N, DCM, then ZH, DMA, 85 °C.



Scheme 3. Reagents and conditions: (i) NaSTIPS, $Pd(PPh_3)_4$, THF, reflux; (ii) TBAF, THF, 0 °C; (iii) $Br(CH_2)_3OH$, K_2CO_3, DMF, 50 °C; (iv) 5 N NaOH, THF, EtOH, then 1 N HCl, 60 °C, then NH_4OAc, 140 °C, then 2 M K_2CO_3, MeOH, CH_2Cl_2; (v) 35% H_2O_2, THF, AcOH, 60 °C; (vi) MsCl, Et_3N, THF, then NaI, EtOAc, reflux; (vii) pyrrolidine, DMA; (viii) BBr_3, CH_2Cl_2; (ix) CO(g), $Pd(OAc)_2$, DPPP, Et_3N, MeOH, DMF; (x) cHCl, dioxane, reflux; (xi) $(COCl)_2$, cat. DMF, THF, then $H_2N(CH_2)_2Npyrrol$, THF; (xii) KNO_3, SO_2Cl_2, MeCN, 0 °C; (xiii) $H_2N(CH_2)_2Npyrrol$, DMA.

intermediate **55**. One-pot deprotection of the 2-position by sequential reaction with NaOH and HCl gave the anhydride, which was converted back to the imide by reaction with molten NH_4OAc as above. This treatment

Table 1. *C*-8 and *N*-6-substituted 4-(2-chlorophenyl)-9-hydroxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-diones



Compound	X	Y	Amine	IC_{50}[a] (nM)	
				Wee1	Chk1
2	—	—	—	9	170
3	$(CH_2)_4$	H	NMe_2	49	55
4	$(CH_2)_4$	H	Npyrrol	50	22
5	$(CH_2)_4$	H	Nmorph	37	71
6	$(CH_2)_4$	Me	NMe_2	34	102
7	$(CH_2)_4$	Me	Npyrrol	36	4
8	$(CH_2)_4$	Me	Nmorph	30	74
9	$(CH_2)_4$	$(CH_2)_2OH$	Npyrrol	24	14
10	$(CH_2)_4$	$(CH_2)_2OH$	Nmorph	19	22
11	$O(CH_2)_3$	H	NMe_2	26	3
12	$O(CH_2)_3$	H	Npyrrol	35	10
13	$O(CH_2)_3$	H	Nmorph	26	41
14	$O(CH_2)_3$	Me	NMe_2	58	24
15	$O(CH_2)_3$	Me	Npyrrol	75	14
16	$O(CH_2)_3$	Me	Nmorph	57	374
17	$O(CH_2)_3$	$(CH_2)_2OH$	NMe_2	18	134
18	$O(CH_2)_3$	$(CH_2)_2OH$	Npyrrol	24	63
19	$O(CH_2)_3$	$(CH_2)_2OH$	Nmorph	15	290
20	$S(CH_2)_3$	Me	Npyrrol	20	44
21	$SO(CH_2)_3$	Me	Npyrrol	33	7
22	$SO_2(CH_2)_3$	Me	Npyrrol	160	240
23	$SO_2NH(CH_2)_2$	Me	Npyrrol	46	57
24	$CONH(CH_2)_2$	Me	Npyrrol	15	242

[a] IC_{50} values were determined for both Wee1 and Chk1 inhibition by the published methods cited. Values are the average of two or more independent determinations, with a variance of ±30%.

gave some acetate of the *C*-8 propanol side chain which was hydrolysed using K_2CO_3 to give **56a**. Treatment of **56a** with H_2O_2 in THF gave a mixture of the sulfoxide **56b** and the sulfone **56c**, which were separated by chromatography. Reaction of **56a–c** with MsCl then NaI, followed by reaction with pyrrolidine, gave compounds **58a–c**, which were O-demethylated to give **20–22**. Conversion of triflate **32b** to the ester **59**, followed by hydrolysis, activation of the acid **60** with $(COCl)_2$ and reaction with *N*-(2-aminoethyl)pyrrolidine gave, **61**, which was O-demethylated to **24**. Finally, thiol **54** was converted to the chlorosulfonyl derivative **62** with KNO_3/SO_2Cl_2, and this was reacted with *N*-(2-aminoethyl)pyrrolidine and O-demethylated to give **23**.

Table 1 gives the structures of the compounds evaluated, and their potencies (IC_{50}s) for preventing phosphorylation by human Wee1 kinase of a model polyornithine–tyrosine copolymer substrate and phosphorylation by Chk1 kinase of a GST-Cdc25 substrate.[2] IC_{50} values are defined as the concentration of inhibitor required to reduce by 50% the level of ^{32}P (from added [^{32}P]-ATP) incorporated into the peptide substrate.

Compounds **3–10**, with a $(CH_2)_4$ linker chain, explored the effects on enzyme inhibition of variations in *N*-6 substituent and the pK_a of the solubilising amine. Wee1 activity varied little, but the more strongly basic pyrrolidine analogues **4**, **7** and **9** were the most potent Chk1 inhibitors. Compounds **11–19**, with an isosteric $O(CH_2)_3$ linker chain, also showed only a small (5-fold) variation in Wee1 activity, but compounds **16**, **17** and **19** displayed significantly lower Chk1 potencies. Compounds **20–24** explored a wider variety of linker groups, particularly more electron-withdrawing ones. In this series, because of the above results, the amine solubilising group was restricted to pyrrolidine and the *N*-6 group to methyl. With the exception of **22**, the Wee1 data again showed little variation, but the Chk1 data were more variable, with the SO_2- and CONH-linked compounds **22** and **24** being much less potent than the others.

We have previously reported the structure, at 1.8 Å resolution, of an inhibitor bound co-crystal of **1** and the Wee1(291–575) construct containing residues 291–575 of the human Wee1 enzyme.[4] We have further reported inhibitor bound co-crystal structures of two *N*-6 substituted pyrrolocarbazole Wee1/Chk1 inhibitors (2.2 and 1.9 Å resolution) against this same Wee1(291–575) construct.[3] In all examples 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. 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 Asn376 side chain. In the present study, Wee1(291–575) inhibitor bound co-crystal structures of compounds **14** (PBD deposition number 3BI6) and **24** (PBD deposition number 3BIZ) were obtained according to methods previously reported.[4] As expected, hydrogen bond contacts between the pyrrolocarbazole scaffold and the hinge region of Wee1 kinase were identical to those previously described,[3,4] and the *C*-8 side chains appeared to reside in an area of bulk tolerance.

Table 2. Cellular data for selected compounds of Table 1

Compound	Amine	IC_{50} (μM)		MI[c] (%/μM)
		HKA[a]	PY15[b]	
2		0.65	0.25–2.5	62%/1.25
3	NMe_2	0.88	0.25	70%/0.31
4	Npyrrol	0.78	0.20	59%/0.31
5	Nmorph	1.23	0.25	47%/0.31
14	NMe_2	0.51	0.01	86%/0.31
15	Npyrrol	0.23	0.03	51%/1.25
17	NMe_2	0.75	0.25	51%/0.31
18	Npyrrol	0.86	0.03–0.25	48%/0.31
19	Nmorph	0.53	0.25	50%/0.63

[a] 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.
[b] PY15: 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).
[c] MI: mitotic index assay; % of doxorubicin pre-treated HT-29 cells in mitosis after treatment with an abrogator at the stated concentration.

Selected compounds were studied in a variety of cellular assays[3] to determine their pharmacodynamic effects (Table 2) in HT-29 (p53-negative) colon cancer cells. The histone H1 kinase assay (HKA) has previously been employed to characterise the G2/M checkpoint abrogation of peptide inhibitors of human Chk1.[6] This assay indirectly measures the effect of test compounds on the kinase activity of the Cdc2/cyclin B complex for phosphorylation of histone H1.[3] The IC_{50} measures the concentration of drug required to permit a 50% activation of the Cdc2/cyclin B complex in cells, compared to controls (reported as 50% inhibition of the regulatory kinases of the Cdc2/cyclin B complex). Tyr15 phosphorylation (PY15) on Cdc2 kinase (the natural Wee1 substrate) was determined directly by Western blotting, using a phosphospecific antibody, with the signal normalised for the total amount of Cdc2 detected. The IC_{50} value measures the approximate concentration of drug required to produce a 50% reduction of this phosphorylation in HT-29 cells, compared to controls. 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 3. Modification of cisPt IC_{50} values in HT-29 cells with added **14** or **15**

Compound **14**			Compound **15**		
[**14**][a] (μM)	IC_{50}[b] (μM)	Fold dose mod.[c]	[**15**][a] (μM)	IC_{50}[b] (μM)	Fold dose mod.[c]
0	4.2		0	11.7	
0.024	1.5	2.8	0.078	6.2	1.9
0.049	0.61	6.9	0.156	3.2	3.6
0.195	0.13	32	0.313	0.34	34
14 alone	0.23		**15** alone	0.31	

[a] Concentration of added checkpoint abrogator.
[b] IC_{50} value for cell growth inhibition by cisplatin (with added abrogator) or by abrogator alone. Cells were treated continuously for 72 h and then fixed and stained with sulforhodamine B to assess growth.
[c] Fold reduction in IC_{50} with added abrogator over cisplatin alone.

Table 4. Kinase counter-screening of representative compounds

		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
Compounds of Table 1	14	14	96	93	87	86	97	93	1	47	13	137	53	36	53	33	26	13	55	63	2	0	106	7	4	43	47	93	39
	15	20	70	72	55	59	73	73	1	10	16	248	40	18	74	20	27	33	70	42	0	11	84	3	2	39	41	71	18
	18	12	79	101	87	83	88	88	4	82	21	104	33	15	31	28	57	23	31	67	0	0	100	2	0	39	31	84	36
	19	16	80	81	75	78	85	93	7	92	31	111	83	11	20	55	53	41	43	70	0	8	88	9	2	33	49	78	44
	20	20	89	90	68	74	90	89	64	76	55	99	72	32	25	8	15	6	16	53	15	0	97	119	13	71	29	58	35
	24	38	62	99	72	68	78	45	22	57	10	116	43	35	62	16	7	56	22	66	19	0	99	7	1	50	67	95	47

[a] 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). Kinases tested[6]: (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.

Table 5. Modulation of Cdc2 Y15 phosphorylation by compound **14** in HT-29 tumours in nude mice

Dose[b] (mg/kg)	Cdc2 PY15[a]			
	Control	CPT-11 only	**14** only	CPT-11 + **14**
40	100	250	57	124
20	100	298	113	258
10	100	266	73	263

[a] Intensity of PY15 signal in Western blots in relation to normalised controls set at 100%.
[b] Drug given in two doses 3 h apart.

There was little differentiation in these assays between the compounds, including the $(CH_2)_4$- and $O(CH_2)_3$-linked ones, and compound **2**. Compound **14** was considered of particular interest, showing the greatest potency in both the PY15 and MI assays (Table 2) and possessing acceptable aqueous solubility as the HCl salt in water (2.2 mg/mL). This compares[3] with a solubility of <3 μg/mL for compound **2**.

Compounds **14** and **15** were also evaluated for their ability to sensitise tumour cells to the cytotoxic effects of cisplatin, which was used as a typical DNA-damaging agent (Table 3). In HT-29 human colon cancer cells, concentrations of those well below their IC_{50}s for growth inhibition resulted in significant (~4- to 7-fold) increases in the toxicity of cisplatin. The table reports a single experiment that was typical of a number using different cell lines and with different DNA-damaging agents.

Selected compounds were screened against a panel of 28 kinases at a concentration of 10 μM,[7] as shown in Table 4. As a class, the *C*-8 solubilised 9-hydroxypyrrolo[3,4-*c*]carbazole-1,3(2*H*,6*H*)-diones appear also to be inhibitors of MKK1, AMPK, Chk1 (as described above), PHOS kinase and Lck. Significant inhibition of MAPKAP-K1a, MSK1, PKCα, PKBα, SGK and S6K1 was also observed for a number of examples. This lack of selectivity may in part be responsible for the relatively potent single agent cytotoxicity described in Table 3 and the low MTDs achieved in vivo (see below).

The pharmacodynamic effect of compound **14** was also studied in vivo in female *nu/nu* mice bearing HT-29 human tumour xenografts. Animals were treated with CPT-11 (75 mg/kg iv; a high dose, but within the accepted range[8,9]), followed at 24 and 27 h with **14** (iv) at a range of doses. Tumours were excised at 30 h, then disaggregated, and the levels of Cdc2 Y15 phosphorylation were measured by Western blotting, using anti-pTyr15 antibody. The results are given in Table 5, and show that the DNA damage caused by the topo I poison CPT-11 results, as expected, in a substantial increase in phosphorylation on Cdc2 Y15. This can be lowered by **14**, only marginally at 20 mg/kg but substantially (50% reduction) at 40 mg/kg, although the latter dose generated significant oedema at the injection site, and is likely too high for multi-dose regimens.

In summary, the data show that a range of solubilising bases can be tolerated at the 8-position of the carbazole chromophore, providing potent Wee1/Chk1 inhibitors of sufficient solubility to evaluate in cellular and in vivo assays. These compounds were able to inhibit phosphorylation of the target Cdc2 kinase on tyrosine-15, and to substantially potentiate the cytotoxicity of cisplatin in p53-negative cells in culture through abrogation of the G2/M checkpoint.

Acknowledgments

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