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A Casein kinase 1/Checkpoint kinase 1 pyrazolo-pyridine protein kinase inhibitor as novel activator of the p53 pathway

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ABSTRACT

Reactivation of the wild-type p53 pathway is one key goal aimed at developing targeted therapeutics in the cancer research field. Although most p53 protein kinases form 'p53-activating' signals, there are few kinases whose action can contribute to the inhibition of p53, as Casein kinase 1 (CK1) and Checkpoint kinase 1 (CHK1). Here we report on a pyrazolo-pyridine analogue showing activity against both CK1 and CHK1 kinases that lead to p53 pathway stabilisation, thus having pharmacological similarities to the p53-activator Nutlin-3. These data demonstrate the emerging potential utility of multivalent kinase inhibitors.

Inactivation of p53 tumour suppressor functions is a common feature of human cancer cells, which has led to extensive drug therapy research focused on targeting specific pathways around p53.[1] This inactivation can be achieved genetically by p53 gene mutation resulting in an attenuation of the tumour suppressor functions of p53 or by post-translational attenuation of wild-type p53 function through the over-production of 'p53-inactivating' pathways. There are over four-hundred p53-interacting proteins, many of which can function as p53-inhibitors, though the clinical relevance of the vast majority of these protein–protein interactions is undefined. However, there are a few well-characterised p53 inhibitors that have been the target of drug development programmes whose leads can stimulate p53. For example such inhibitory proteins include the ubiquitin ligase Murine double minute chromosome 2 (MDM2) that normally mediates p53 protein degradation,[2] the histone deacetylase Sirtuin, whose deacetylation transcriptionally attenuates p53,[3] and the protein Casein kinase 1 (CK1) that cooperates with MDM2 to mediate p53 protein degradation.[4]

MDM2 mediated degradation of p53 and its druggability forms a core paradigm for inhibiting protein–protein interactions. Small molecule *cis*-imidazole analogues like Nutlin-3 can bind to MDM2 and disrupt its many regulatory protein–protein interactions. A key pharmacological feature of Nutlin-3 is its ability to both stabilise p53 protein and destabilise the cell-cycle transcription factor E2F-1 through disruption of MDM2:p53 and MDM2:E2F-1 protein-interactions that co-ordinately blocks cell cycle progression.[5] In searching for protein kinases that interact with MDM2 and which are pharmacologically similar to MDM2 as potential drug targets, we found previously that targeted inhibition of the MDM2-interacting protein CK1 using the ATP-competitive inhibitor of CK1 (D4476) or targeted CK1α siRNA can, like Nutlin-3, lead to co-ordinated p53 stabilisation and E2F-1 degradation. Indeed the acidic domain of MDM2 has been shown to be phosphorylated in unstressed conditions by CK1 among others,[6,7] which contributes to p53 destabilisation through increase of MDM2 ability to trigger p53 degradation.[8,9] Phosphorylation of MDM2 by CK1 would drive the binding of MDM2 to E2F-1 and therefore inhibit the ubiquitination of E2F-1 by displacing the E3 ubiquitin ligase SCF^{Skp2} from E2F-1.[10] This provided evidence that CK1 might form a compelling drug targeting for coupled activation of p53 and inhibition of E2F-1 that can lead to cell growth inhibition or death.[4]

Protein kinases such as CK1 have been described to contribute directly or indirectly to the inhibition of p53 and surprisingly some tumour-suppressing p53-activating kinases like Checkpoint kinase 1 (CHK1) and CHK2 can possess oncogenic functions under certain conditions.[11] Therefore, the p53-inhibiting kinases represent novel targets for anti-cancer therapies. In a similar manner to CK1,

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Glycogen synthase kinase 3 (GSK-3) and Casein Kinase 2 (CK2) were shown to phosphorylate the central acidic MDM2 domain in vitro and in vivo and therefore their inhibition rescued p53 from degradation.[12,13] Moreover the C-terminal phosphorylation of p53 by GSK-3 was shown to trigger the export of p53 from the nucleus and its subsequent degradation by the proteasome.[14] Aurora A/B, mitotic checkpoint kinases that plays a pivotal role in the cell cycle, were shown to inactivate p53 through direct phosphorylation of p53 at Ser^{269}/Ser^{215} resulting in unfolding of the p53 core DNA-binding domain.[15–17] CHK1 inhibition has been linked to p38 Mitogen-activated protein kinase (p38 MAPK) activation, which in turn caused p53 phosphorylation and subsequent p53-dependent apoptosis.[18]

In further exploring the development of reagents to study the role of CK1 in the p53 and E2F-1 response, we recently reported on the effects of a peptide inhibitor of MDM2 derived from the CK1α binding site of MDM2 that inhibits the ubiquitin ligase activity of MDM2 and has potent anti-proliferative activity in cells.[19] In order to further challenge the concept that CK1-signalling forms an inhibitory signal to p53, we sought to identify chemical moieties with CK1-inhibitory activity. Here, we report on the identification of a pyrazolo-pyridine analogue (MRT00033659) with novel bioactivity towards CHK1 predominantly in vitro and CK1 (along with 6 other kinases) and with sub-micromolar bioactivity in cells with respect to p53 protein stabilisation and E2F-1 de-stabilisation. Thus, MRT00033659 has pharmacological similarities to the CK1-inhibitor D4476 and the MDM2-inhibitor Nutlin-3. Another pyrazolo-pyridine analogue used as a control that was shown to inhibit in vitro GSK-3 (MRT00033680) was pharmacologically distinct from MRT00033659 and Nutlin-3 as it stabilised p53 protein, de-stabilised MDM2, and stabilised E2F-1. These data highlight the value in characterising and defining small molecule kinase inhibitors with novel properties in cell systems and identifies small molecule tool compounds that can be used to uncouple p53 and E2F-1 pathways from the biological outcome of growth inhibition.

Kinase profiling[20] of a small molecule library using a large panel of protein kinases identified two distinct CK1 inhibitory molecules (MRT00033659 and MRT00055778) that exhibited activity towards CK1 (synthesis route outline in Fig. S1A; Table 1). MRT00055778 (N1-[4-(5-methyl-3-phenylisoxazol-4-yl)pyrimidin-2-yl]acetamide) is commercially available from Maybridge (SPB-05257) and MRT00033659 (5-(3-acetamidophenyl)-3-methyl-1*H*-pyrazolo[3,4-*b*]pyridine) was synthesised in house (MRC-T). Compound MRT00033680 was synthesised at MRCT using the route published by Witherington et al. as shown in Figure S1B.[21] All final compounds were fully characterised by NMR and mass spectrometric analysis.

MRT00033659 was shown to inhibit CK1 and CHK1 among other kinases (32.74% and 31.48% activity versus control at 1 μM, respectively) with confirmatory inhibition of CK1 and CHK1 at 10 μM of 10.02% and 4.34% activity versus control. By contrast, MRT00055778 at 1 μM concentration inhibited CK1 by 44.42% and confirmatory inhibition based on remaining activity versus control at 10 μM of 7.20% (Table 1). Kinase profiling data on previously validated CK1 inhibitors—D4476 and IC261—which were screened at 10 and 25 μM, respectively, were from published studies and were included for comparison (Table 1). Both inhibitors showed some selectivity for CK1 within the protein kinase panel with inhibition shown by activity versus control of 4% and 9%, respectively. D4476, originally identified as an inhibitor of ALK5 (data not included in Table 1), also showed some bioactivity at

Table 1
Identification of small molecules that inhibit CK1 activity

Activity (% of control) at	MRT00033659		MRT00055778		D4476	IC261	MRT00033680	
	10 μM	1 μM	10 μM	1 μM	10 μM	25 μM	10 μM	1 μM
Number of compounds <20%	8	0	1	0	1	1	3	3
BTK	16.50	93.20	99.69	103.13	#N/A		69	85
CDK2	40.33	76.71	103.84	92.20	113 ± 8	83 ± 4	5	9
CHK1	4.34	31.48	104.72	80.59	84 ± 8	99 ± 2	82	92
CHK2	28.92	68.50	78.79	89.45	92 ± 5	76 ± 4	96	96
CK1δ	10.02	32.74	7.20	44.42	4 ± 0	9 ± 2	84	73
CSK	47.98	95.26	103.93	93.38	81 ± 6	81 ± 4	80	91
DYRK1α	39.90	76.17	101.55	91.90	86 ± 7	73 ± 1	2	5
ERK8	15.76	45.29	89.45	91.14	80 ± 4	68 ± 2	#N/A	#N/A
GSK3β	61.19	79.38	59.99	100.57	88 ± 5	74 ± 6	3	2
HIPK2	42.42	71.96	94.19	98.87	80 ± 4	96 ± 6	31	72
IGF-1R	12.54	70.82	92.48	82.68	#N/A		92	88
IKKB	71.95	96.95	80.40	44.37	63 ± 3	90 ± 5	90	70
IRR	48.82	73.86	81.42	83.18	#N/A		58	61
JNK2	70.88	98.46	23.44	85.19	77 ± 7	101 ± 3	88	99
MARK3	17.89	62.58	79.23	94.61	82 ± 4	88 ± 5	62	84
MELK	38.93	76.11	105.87	103.14	83 ± 1	75 ± 8	64	115
MKK1	45.89	75.69	66.79	95.13	93 ± 2	86 ± 2	103	90
MNK1	19.30	69.97	101.89	101.16	81 ± 1	79 ± 3	91	79
NEK2α	10.52	53.49	82.68	85.83	89 ± 7	75 ± 6	90	97
PIM1	28.18	73.14	88.87	104.71	93 ± 8	43 ± 1	53	89
PKD1	44.32	107.98	75.26	80.66	43 ± 4	62 ± 3	99	76
PRK2	42.17	99.92	96.46	77.58	107 ± 4	87 ± 5	76	81
p38α MAPK	#N/A	#N/A	#N/A	#N/A	38 ± 1	92 ± 4	91	84
SGK1	61.85	104.65	21.37	57.49	73 ± 2	79 ± 4	82	108
smMLCK	45.65	72.91	79.33	80.36	81 ± 5	70 ± 2	62	87
SRC	23.75	65.01	91.94	98.27	82 ± 0	70 ± 4	72	75
TBK1	48.24	88.90	104.56	106.21	#N/A		70	91
VEGFR	20.34	54.47	50.84	79.53	#N/A		#N/A	#N/A

The indicated protein kinase activities are presented as a percentage of control incubations. All protein kinases were of human origin, apart from MKK1 and PHK (rabbit), ERK2 (mouse), PKA (cow), and AMPK, CK1δ, DYRK1a, ROCK-II and RSK1 (rat).[30] ATP was present at approximate Km in all assays. D4476 and IC261 screens (at 10 μM and 25 μM, respectively) were taken for comparison as controls.[31] #N/A stands for non available. Activities were determined at least twice on 2 separate test experiments. Only affected kinases are shown here, others are listed in Table S1.