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OK-1035, A SELECTIVE INHIBITOR OF DNA-DEPENDENT PROTEIN KINASE

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SUMMARY Screening for inhibitors of DNA-dependent protein kinase (DNA-PK) revealed 3-cyano-5-(4-pyridyl)-6-hydrazonomethyl-2-pyridone, designated OK-1035, to be a potent and selective inhibitor. When a synthetic peptide was used as a substrate, OK-1035 caused 50% inhibition of DNA-PK activity at 8 μM, a concentration more than 50 times lower than those required against seven other protein kinases tested. OK-1035 inhibited the phosphorylation by DNA-PK of consensus peptide as well as that of recombinant human wild type-p53. Kinetic studies indicated that OK-1035 inhibited DNA-PK activity in an ATP-competitive manner.

DNA-PK is a nuclear serine/threonine protein kinase the activity of which is regulated by DNA (1,2). DNA-PK forms a complex with Ku antigen (p70/p80) and DNA to express its kinase activity (3). A consensus amino acid sequence for protein phosphorylation by DNA-PK is -S/T-Q- and -P-S/T- (4). Many of the transcription factors and DNA-binding elements, for example, Sp1, Fos, Jun, Myc, Ku antigen and the tumor suppressor protein p53, are reported to be phosphorylated by DNA-PK *in vitro* (5). Wild-type p53 (wt-p53) is a phosphoprotein with an anti-oncogenic property and is

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Abbreviations used:
DNA-PK, DNA-dependent protein kinase; PKA, catalytic subunit for cAMP kinase; CK I, casein kinase I; CKII, casein kinase II; PKC, protein kinase C; cdk2, cyclin-dependent kinase 2-cyclin A; MAPK, mitogen-activated protein kinase; EGFRK, epidermal growth factor receptor tyrosine kinase; IC_{50}, median inhibitory concentration.

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reported to be phosphorylated at both amino- and carboxyl- terminal residues by several kinases *in vitro* and *in vivo*. The carboxyl-terminal serine 392 and 315 of human wt-p53 are phosphorylated *in vitro* by CK II and cdk2, respectively (5). In contrast, Wang and Eckhart (6) concluded that amino-terminal serine 7, 9, 18 and 37 in mouse wt-p53 are sites of phosphorylation *in vivo* using site-directed mutagenesis. Serine 15, which is equivalent to 18 in mouse, and 37 of human wt-p53 are reported to be sites for DNA-PK phosphorylation *in vitro* (8). Fiscella et al. reported that mutation of the serine 15 phosphorylation site of human wt-p53 reduces the ability of p53 to inhibit cell cycle progression (7). These findings suggest that DNA-PK may phosphorylate the amino-terminal domain of wt-p53 *in vivo* (8) and plays a critical role in p53-dependent cell growth suppression. The nuclear location, DNA-binding properties, and the above properties of DNA-PK as mentioned suggested that DNA-PK is involved in regulating the transcription and cell growth *in vivo*. To elucidate the function of DNA-PK in these processes, an inhibitor of DNA-PK must be useful material. This report describes the properties of OK-1035, a new potent and selective inhibitor of DNA-PK.

MATERIALS AND METHODS

Preparation of DNA-PK DNA-PK from HeLa cells (1 x 10^9 cells) was purified from the nuclear extract by three-step column chromatography, as previously described (4). The purified DNA-PK contained a 350-kd catalytic subunit and Ku (p70/p80) regulatory protein.

***In vitro* phosphorylation reaction** The *in vitro* standard assay screening for DNA-PK inhibitors was carried out with HPLC-purified synthetic peptide #15 (EPPLSQEAFADLWKK) (8) as a substrate. The reaction was carried out at 37°C for 10 min in a 20 μl reaction mixture that contained 0.6 x solution A (20 mM Hepes-KOH pH 7.9, 1 mM EGTA, 1 mM DTT, 10% glycerol, and 0.02% Tween-20), 7.5 mM $MgCl_2$, 50 μM [γ-^{32}P] ATP (0.25 μCi/nmol), 0.4 μg of sonicated calf thymus DNA, and 7 μg of peptide #15. After the reaction was terminated, the radioactivity bound to P81 paper (Whatman) was counted in a liquid scintillation counter. For determination of *in vitro* wt-p53 phosphorylation, the reaction was carried out in the same manner as the standard assay except that the peptide substrate was replaced by *E coli.*-produced recombinant human wt-p53 (2), with or without OK-1035, peptide #15, or peptide#15' (EPPLSEEAFADLWKK). After the reaction was terminated, p53 was separated by the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and radio activity was detected by autoradiography. The assay conditions for PKA, PKC, cdk2, CKI, CKII, MAPK and EGFRK have been described previously (9).

RESULTS

Screening for selective inhibitors of DNA-PK was performed with purified HeLa cell DNA-PK and DNA-PK selective peptide substrate (peptide #15), consisting of 15 amino acids (8). Among more than 10,000 microbial extracts or synthetic compounds, 3-cyano-5-(4-pyridyl)-6-hydrazonomethyl-2-pyridone, designated OK-1035 (Fig. 1), was

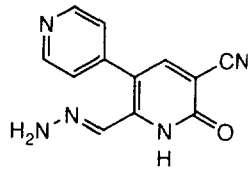


Figure 1. The structure of OK-1035 ($C_{12}H_9N_5O$; MW. 239).

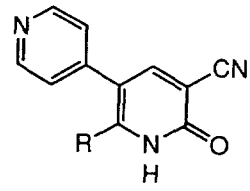
shown to be a potent and selective inhibitor of DNA-PK (Table 1). OK-1035 inhibited DNA-PK activity by 50% at the concentration of 8 μM under the standard assay conditions described in Experimental Procedures. When casein was used as the substrate, the IC_{50} value was 40 μM (data not shown). To evaluate the inhibitory effect of OK-1035 on various protein kinases *in vitro*, the IC_{50} value of OK-1035 against DNA-PK was compared with the IC_{50} values against seven other protein kinases (Table 1). IC_{50} values of OK-1035 against PKA and CK II were 390 and 420 μM, respectively, concentrations about 50 times higher than that against DNA-PK. OK-1035 did not inhibit PKC, cdk2, CK I, MAPK, or EGFRK: the IC_{50}s against these kinases exceeded more than 500 μM. Table 1 also shows the IC_{50} values of K252a and staurosporine, which are reportedly PKC inhibitors, against these kinases. These reported inhibitors of PKC did not inhibit DNA-PK activity, and their spectra of kinase inhibition differed from that of OK-1035.

The structure-activity relationships were examined for compounds having various side chains at the R position in the 2-pyridone ring (Table 2). Of the 14 compounds tested, OK-1035 and OK-1034, the side chain of which resembles that of OK-1035, showed 90% and 48% inhibition of enzyme activity at a concentration of 100 μM, respectively, whereas the other compounds did not cause significant inhibition of DNA-PK activity, even at the concentration of 500 μM. These findings suggested that the 6-hydrazonomethyl group on the 2-pyridone ring is important in inhibiting DNA-PK activity.

Table 1 Inhibition of DNA-PK and various protein kinases by OK-1035.
IC_{50} values of OK-1035, K252a, and staurosporine against eight protein kinases were determined. The assay conditions for each kinase were described previously (9). ND, not determined.

	$IC_{50}(\mu M)$							
inhibitor	*DNA-PK*	*PKA*	*PKC*	*cdk2*	*CKI*	*CKII*	*MAPK*	*EGFRK*
OK-1035	8	390	>500	>500	>500	420	>500	>500
K252a	61	1.3	1.2	ND	59	9.0	0.8	21
staurosporine	>100	1.1	0.0088	ND	50	15	>1.0	0.43

Table 2 Structure-activity relationship. OK-1035 and fourteen related compounds were tested for their ability to inhibit DNA-PK activity in the presence of each compound at a concentration of 100 or 500 μM.



		%inhibition	
OK-#	*R*	*500*	*100 (μM)*
1035	CH=N-NH2	99	100
1018	CH3	17	0
1042	CH2F	49	2
1025	CH2OCH3	30	1
1019	CH(OCH3)2	23	5
1015	CH2OH	37	0
1071	CH(OH)CH3	32	3
1017	COOH	24	0
1040	CONH2	23	0
1043	cyclohexyl	9	0
1026	phenyl	4	0
1034	CH=N-OH	79	48
1066	CH2NH(C2H5)2	22	0
1069	CH2N=C5H10	19	0

The kinetics were analyzed by a Lineweaver-Burk plot. As shown in Fig. 2, OK-1035 inhibited DNA-PK activity in an ATP-competitive manner, and in a non-competitive manner with peptide substrate or DNA.

Lees-Miller et al. (8) suggested that serine 15 and 37, each containing the -SQ- consensus sequence, are the phosphorylation sites of human wt-p53 by DNA-PK *in vitro*. To measure the inhibition by OK-1035 of the phosphorylation of wt-p53 by DNA-PK *in vitro*, *E. coli.*-produced recombinant human wt-p53 was used as the substrate in the standard assay instead of peptide #15. Human wt-p53 was well phosphorylated by DNA-PK in the presence of sonicated calf thymus DNA (Fig.3a). When 10, 40, or 160 μM of OK-1035 was added to this reaction, phosphorylation of wt-p53 by DNA-PK was decreased in a concentration-dependent manner (Fig. 3a). In the same way, the presence of peptide #15, 1, 2, or 3 mg/ml, led to the suppression of phosphorylation in a concentration-dependent manner (Fig. 3b). In contrast, the same

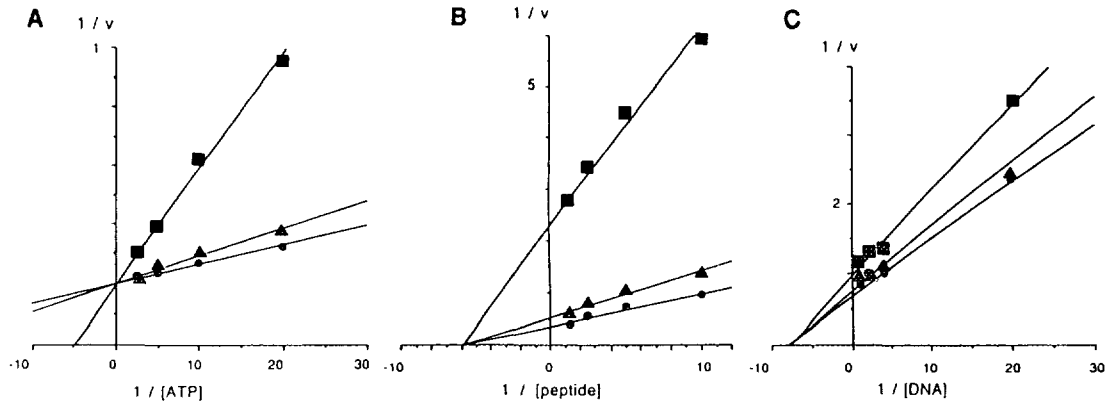


Figure 2. Kinetic study of inhibition of phosphorylation of peptide #15 by DNA-PK. Inhibition of the phosphorylation of peptide #15 by purified DNA-PK in the presence of 0 μM (●), 5 μM (▲), or 40 μM (■) OK-1035 was analyzed by Lineweaver-Burk plots; the standard assay condition was as described in Experimental Procedures, except that various concentrations of peptide (A), various concentrations of ATP (B), or various concentrations of DNA (C) were used.

concentration of peptide #15', which has a sequence similar to that of peptide #15 but different from the DNA-PK consensus sequence, produced no decrease in phosphorylation (Fig. 3b). These findings suggested that phosphorylation of wt-p53 by DNA-PK originated at the SQ-position.

DISCUSSION

The protein kinases shown in Table 1 play essential roles in signal transduction, cell cycle regulation, or cell transformation. Since DNA-PK works at the same stage as these kinases, an inhibitor of DNA-PK must have enough specificity for these kinases if it is to be used as an inhibitor in cell experiments. We found that OK-1035 had an IC_{50} value more than 50 times lower than the other kinases tested and selectivity was ensured.

Based on the structure-activity relationship of OK-1035, conversion of the side chain at the 6-position of the 2-pyridone ring should create compounds with various inhibitory potencies. Furthermore, the finding that only OK-1034 with a side chain at this position resembling the structure of OK-1035 among the 14 compounds tested, showed moderate inhibitory activity indicating the importance of the 6-hydrazonomethyl group on the 2-pyridone ring of OK-1035 for the inhibition of DNA-PK activity.

OK-1035 at 8 μM inhibited DNA-PK activity by 50% and did so in an ATP-competitive manner. Generally, the ATP concentration in a cell is presumed to be several mM, which is about 100 times higher than that used in the standard DNA-PK assay system *in vitro* (50 μM ATP). Thus, OK-1035 would be expected to have an

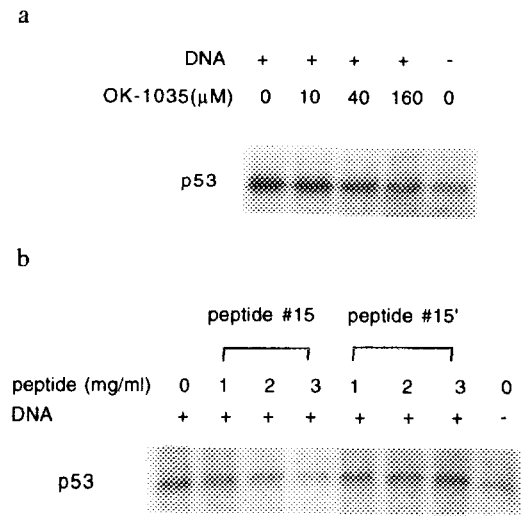


Figure 3. Inhibition *in vitro* of DNA-PK phosphorylation of wt-p53 by OK-1035.
(a) *E.coli*.-produced recombinant human wt-p53 was phosphorylated by DNA-PK in the presence or absence of OK-1035, with or without sonicated DNA. After termination of the phosphorylation reaction, as described in Experimental Procedures, the reaction mixture was loaded on 10% SDS-PAGE, and phosphorylated p53 was detected by autoradiography.
(b) DNA-PK consensus peptide #15 (EPPLSQEAFADLWPP) or nonconsensus peptide #15' (EPPLSEEAFADLWPP) was added to the phosphorylation reaction of recombinant wt-p53 by DNA-PK. The reaction and detection were carried out as described in (a). Inhibition of DNA-PK phosphorylation of wt-p53 by OK-1035 was competitive with peptide #15, but not with peptide #15'.

IC_{50} for DNA-PK activity of several mM in cell experiments. The inhibition of phosphorylation of wt-p53 by OK-1035 was determined in intact cells as follows. After exposing the OK-1035 and [^{32}P]-phosphorus to HCT116 cell culture for 3h, the cells were lysed, and wt-p53 was immunoprecipitated with anti-human wt-p53 antibody and separated by SDS-PAGE. The radioactivity on wt-p53 was determined by use of the BAS2000 image analyzer. Wt-p53 treated with more than 1 mM OK-1035 showed a 20 % lower radioactivity as compared with the untreated control (data not shown). Since wt-p53 is reportedly phosphorylated by at least five protein kinases *in vitro*, this observed result may be reasonable when OK-1035 completely and selectively inhibits DNA-PK activity.

Fritsche et al. (10) reported that when DNA cleavage caused by the addition of a DNA-damaging agent occurred in certain kinds of cells, it induced up-regulation of wt-p53 and suppressed of cell growth. During this process, DNA-PK may be activated by the formation of DNA ends, to phosphorylate the over-produced wt-p53 which participates in the cell growth regulation. Ku p80, one of a subunit of a regulatory component of DNA-PK, is a product of the human XRCC5 DNA repair gene (11).

Also recently, Blunt et al. reported that defective DNA-PK activity is linked to V(D)J recombination and DNA repair defects associated with the murine *scid* mutation (12). These findings suggest the importance of the role of DNA-PK and Ku complex, the property of which was influenced or stimulated by the DNA ends, in cell growth suppression and DNA repair mechanism. To elucidate the function of DNA-PK and its complex with Ku among these mechanism, OK-1035 must be a useful and important material.

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