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Short Communication

Dnacin A1 AND dnacin B1 ARE ANTITUMOR ANTIBIOTICS THAT INHIBIT cdc25B PHOSPHATASE ACTIVITY

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Abstract—The $p80^{cdc25}$ protein is a protein phosphatase directly involved in $p34^{cdc2}$ protein kinase activation by dephosphorylation. The *cdc25B* gene is one of three human *cdc*25 homologs which can complement the temperature-sensitive *cdc*25 mutation of *Schizosaccharomyces pombe*, and is expressed at high levels in human cell lines, particularly in some cancer cells. A fusion protein of glutathione-*S*-transferase (GST) and the catalytic domain of cdc25B protein was constructed and found to retain phosphatase activity in the manner of a $p80^{cdc25}$ phosphatase by using a chromogenic substrate, *p*-nitrophenylphosphate. Two benzoquinoid antitumor compounds, dnacin A1 and dnacin B1, inhibited phosphatase activity in a non-competitive manner.

Key words: cdc25B, protein phosphatase, antitumor agents, dnacins, *p*-nitrophenylphosphate

A central and rate-limiting function in the transition from G2 into M is performed by $p34^{cdc2}$, a protein kinase conserved in M phase promoting activity throughout the eukaryotes [1]. The activity of $p34^{cdc2}$ is itself subject to regulation by phosphorylation and dephosphorylation. In the fission yeast, *Schizosaccharomyces pombe*, tyrosine-15 dephosphorylation of $p34^{cdc2}$ is directly executed by the $cdc25^{+}$ phosphatase, $p80^{cdc25}$ [2]. The activation of $p34^{cdc2}$ dependent on the activity of $p80^{cdc25}$ is thought to have a role in linking completion of DNA replication to the onset of subsequent M phase [3]. Such regulatory organization is highly conserved in other eukaryotic cells [4–6]. Mammalian counterparts of $p80^{cdc25}$ also seem to have important roles in determining the timing of the onset of mitosis through dephosphorylation.

From human cells, three *cdc*25 homologs (*cdc25A*, *cdc25B*, and *cdc25C*) have been cloned by complementation of a temperature-sensitive *cdc*25 mutant of *S. pombe* or on the basis of conserved amino acid sequences [7–9]. Among them, *cdc25B* is the most highly expressed, and its elevated expression is observed in some cancer cells such as SV40-transformed fibroblasts [8]. Recently, the cdc25B protein was found to dephosphorylate human $p34^{cdc2}$ kinase not only at tyrosine-15, corresponding to tyrosine-15 of yeast $p34^{cdc2}$, but also at threonine-14 [10]. These results suggest that chromosomal aberrations and changes in ploidy that frequently occur in cancer cells are concerned with abnormal cell cycle control through overexpression of *cdc25B*.

Based upon these observations, compounds which inhibit the function of cdc25B should prove useful in cancer therapeutics. Here we report that two antitumor antibiotics, dnacin A1 and dnacin B1 [11, 12], inhibit cdc25B phosphatase activity.

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‡ Abbreviations: pNPP, *p*-nitrophenylphosphate; GST, glutathione-*S*-transferase.

Materials and Methods

Standard molecular cloning procedures were followed as described by Sambrook *et al.* [13]. The scheme of expression plasmid construction for the production of GST-cdc25B fusion protein was as follows. The C-terminal region (arginine-355 to glutamine-566) of the cdc25B protein showed a high degree of sequence similarity to the corresponding regions of other $p80^{cdc25}$ homologs and is thought to be responsible for the phosphatase activity [8]. The DNA sequence encoding this region was prepared by the polymerase chain reaction as described below. Plasmid #9-5 [8] containing the entire open reading frame of *cdc25B* gene was used for the template DNA. Two oligonucleotides with the *Bam*HI linker, [5′-GCGGATCCCGCGTCC-TCCGCTCAAAA-3′] and [5′-GCGGATCCTCACTG-GTCCTGCAGCCG-3′] were designed for the 5′ and 3′ primers, respectively. One hundred microliters of the reaction mixture contained 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.5 U of AmpliTaq DNA polymerase (Perkin–Elmer Cetus, Norwalk, CT, U.S.A.), 0.1 ng of each primer and 1 ng of #9-5 DNA. Thirty cycles of reactions at 94° for 1 min, 55° for 2 min and 72° for 3 min were performed in a DNA thermal cycler (Perkin–Elmer Cetus). The reaction products were digested with *Bam*HI and separated on a 1.5% agarose gel. The 0.65 kb fragment was inserted into the *Bam*HI site of pGEX-2T (Pharmacia, Uppsala, Sweden), an expression vector encoding GST‡. The resultant plasmid was introduced into the *Escherichia coli* JM109 strain and the GST-cdc25B protein was produced and purified as described previously [2].

The standard GST-cdc25B phosphatase assay was performed in 100 μL of buffer containing 25 mM HEPES, pH 8.0, 10 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 10 mM pNPP and 25 μg/mL enzyme. After incubation at 37° for 100 min, the reaction was terminated by adding 25 μL of 1 N NaOH and the absorbance at 405 nm was measured by a microplate reader.

Dnacin A1 and dnacin B1 were prepared in our research

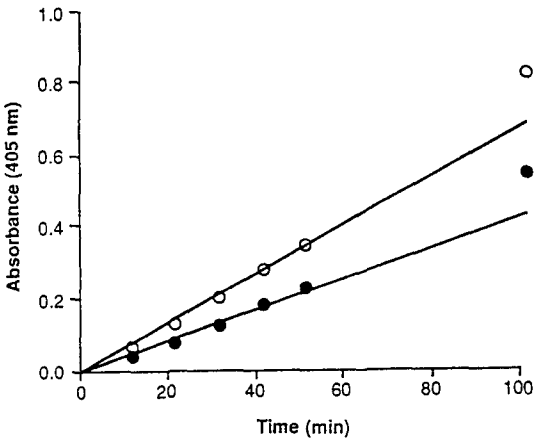


Fig. 1. Time course of dephosphorylation of pNPP induced by GST-cdc25B. GST-cdc25B was incubated in the presence of either 10 (●) or 20 mM (○) pNPP for various periods and the *p*-nitrophenol released from pNPP was measured as described in Materials and Methods.

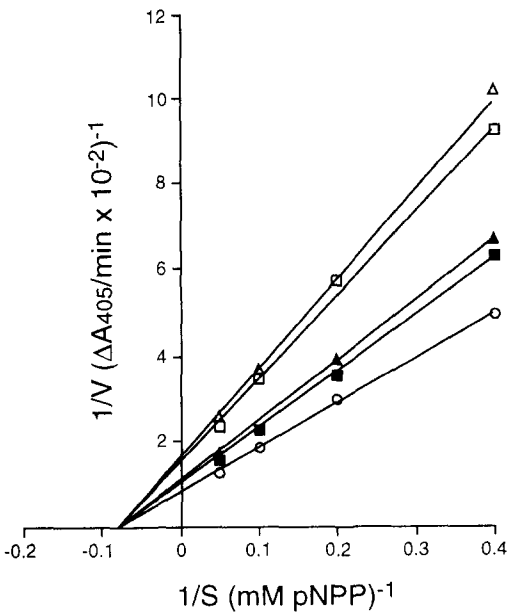


Fig. 2. Lineweaver–Burk analysis of the inhibition of GST-cdc25B phosphatase activity by dnacins. GST-cdc25B phosphatase activity against pNPP was assayed either in the absence of dnacins (○) or in the presence of 125 μM dnacin A1 (□), 62.5 μM dnacin A1 (■), 62.5 μM dnacin B1 (△), or 31.3 μM dnacin B1 (▲).

laboratories. All other chemicals were of the highest grade available.

Results and Discussion

The phosphatase assay revealed that the chromogenic *p*-nitrophenol was linearly released from pNPP during incubation with the purified GST-cdc25B protein within 100 min (Fig. 1) and that this fusion protein had phosphatase activity as did the almost full length of cdc25B protein [10]. According to further kinetic analysis, the apparent K_m value of the enzyme for pNPP was determined to be 16.6 mM (Fig. 2). Next we examined the effects of several phosphatase inhibitors on the GST-cdc25B phosphatase. As shown in Table 1, vanadate and molybdate inhibited phosphatase activity, while okadaic acid and sodium fluoride did not. In addition, phosphatase activity was maintained even in the presence of 5 mM EDTA. These characteristics agree with those of cdc25 phosphatases derived from other eukaryotes [2, 14]. Therefore, we concluded that the calalytic domain of GST-cdc25B functioned in the manner of a $p80^{cdc25}$ phosphatase.

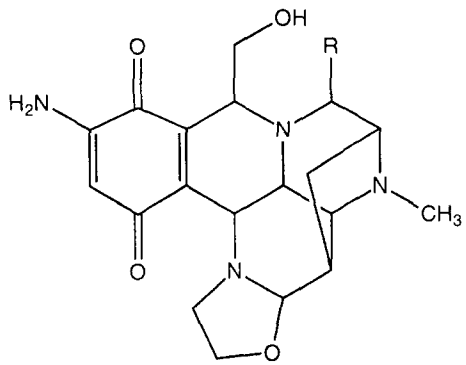
We subsequently tested whether or not various cytotoxic agents affected GST-cdc25B phosphatase, and found that dnacin A1 and dnacin B1, benzoquinoid antibiotics [11, 12], considerably reduced phosphatase activity. The IC_{50} values of dnacin A1 and dnacin B1 were 141 and 64.4 μM, respectively. Further analysis by Lineweaver–Burk plot indicated that dnacin A1 and dnacin B1 inhibited it in a non-competitive manner ($K_i = 0.16$ and 0.10 mM, respectively) (Fig. 2).

Dnacins are antitumor antibiotics which prolong the life-span of mice with leukemia P388 at doses correlated with

Table 1. Effects of phosphatase inhibitors on the activity of GST-cdc25B phosphatase

Drug	Activity
None	1.00
0.5 mM *o*-vanadate	0.11
0.1 mM ammonium molybdate	0.12
50 mM sodium fluoride	0.99
10 μM okadaic acid	0.97
5 mM EDTA	1.00

GST-cdc25B was incubated in reaction buffer in the presence or absence of the indicated drugs. Activities are expressed relative to a control incubation without the addition of these drugs.



dnacin A1 R=CN
dnacin B1 R=OH

Fig. 3. Structures of dnacin A1 and dnacin B1.

the potency of inhibition of cdc25B phosphatase*. These drugs were previously isolated as antibiotics having the ability to interact with DNA by a screening system using the Hfr strain of *E. coli* [15] and these chemical structures have been recently determined (Fig. 3) [16]. In fact, DNA-bound dnacin B1 has been shown to generate oxygen-free radicals resulting in DNA damage [17]. The effective dose (12.5 μM) at which DNA cleavage was observed *in vitro* seems to be slightly lower than that for inhibition of cdc25B phosphatase described here. Although the primary cellular target of dnacins is presumed to be DNA strands in susceptible cells, we could not exclude the possibility that inhibition of $p80^{cdc25}$ phosphatases by dnacins in tumor cells is partly involved in their antitumor properties.

The elevated expression of *cdc25B* in some cancer cells [8] is of some importance. Reduction of its expression by inhibitors might suppress abnormal phenotypes of cancer cells including chromosomal aberrations and changes in ploidy. However, all human cdc25 proteins seem to play important roles in all proliferating cells, whether they are normal or transformed. Unlike cdc25B, cdc25A phosphatase was shown to function at the start of the cell cycle, possibly through activation of cdc2-related kinases expressed early in the cycle [18]. Recently, Barette *et al.* [19] reported an assay system of cdc25A phosphatase, whereby inhibitors of this enzyme are likely to act as antimitotic compounds if they also affect other human cdc25 homologs. To clarify the relationship between inhibition of cdc25B phosphatase and antitumor activity, more specific inhibitors of cdc25B will be needed.

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* Ootsu K, unpublished data.