Pergamon

Tetrahedron 58 (2002) 6367–6372

TETRAHEDRON

Separation of Cdc25 dual specificity phosphatase inhibition and DNA cleaving activities in a focused library of analogs of the antitumor antibiotic Dnacin

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Received 4 April 2002; accepted 1 June 2002

Abstract—Biological evaluation of 96 analogs and synthetic intermediates of the naphthyridinomycin-type antitumor antibiotic Dnacin led to the identification of several low-micromolar inhibitors of dual specificity phosphatases, specifically $Cdc25A_1$, $Cdc25B_2$, and VHR, as well as the tyrosine phosphatase PTP1B. While the parent Dnacins are potent DNA cleavage agents, most of the analog structures, even those that retained significant phosphatase inhibitory activities, did not lead to plasmid DNA cleavage. Thus, the DNA-targeting and the phosphatase-inhibitory activities of Dnacins can be assigned to different pharmacophores.

1. Introduction

Dual specificity phosphatases were initially discovered in viral strains and represent an exciting new field of research for the design of mechanism-based anticancer agents.[1] Both phosphoserine, phosphothreonine, and phosphotyrosine protein substrates are cleaved by DSPases. Mechanistically, they appear to be quite closely related to PTPases and are inhibited by vanadate but not by the standard PSTPase inhibitors.[2,3] In humans, a family of related cyclin-dependent kinases (CDKs) that regulate progression through each phase of the cell division cycle are activated by Cdc25 phosphatases that remove inhibitory phosphate from tyrosine and threonine residues.[4] There is a strong link between overexpression of Cdc25 DSPases and oncogenic transformation, particularly in human breast cancer.[5] In addition to synthetic compounds,[1] several natural products, including coscinosulfate,[6] nocardione,[7] and dysidiolide,[8] have been found to inhibit Cdc25 dual specificity phosphatases at micromolar levels (Fig. 1). The first natural products with documented Cdc25 inhibitory effects were Dnacin A_1 and B_1, two naphthyridinomycin-type antitumor antibiotics isolated from actinomycete, strain No. C-14482 (N-1001) by researchers from Takeda, Ltd. (Fig. 2).[9] Cdc25B DSPase activity was inhibited non-competitively with median inhibitory concentration (IC_{50}) values of 141 and 64 μM, respectively.[10] These alkaloids showed potent bactericidal activity against repair-deficient *E. coli* strains, such as recA, recB, and polA strains. In addition, DNA-cleaving activity in the presence of reducing agents was detected, and this activity was suppressed by scavengers for oxygen free radicals and an iron-specific chelator, desferrioxamine E. Therefore, the primary cellular target of Dnacin B_1 appeared to be DNA through the generation of superoxide radicals.[11,12]

Natural products continue to be major sources for the development of new drugs. Recent analyses of the number and sources of anticancer and antiinfective agents indicate that over 60% of the approved drugs and pre-NDA candidates are of natural origin.[13] While in standard discovery combinatorial libraries the compound-to-hit ratio is shifting from roughly 100,000:1 to 400,000 new compounds to 1 hit, estimates for natural product compound-to-hit ratios vary from 2000:1 to 11,000 extracts for a new drug.[14] Natural products therefore appear to have a clear evolutionary advantage in drug discovery, and in recent years, an expansion of the structural diversity pool by preparation of libraries of natural products or natural product-like molecules has become a major focus of combinatorial chemistry.[15]

2. Results and discussion

As part of our synthetic studies toward Dnacins and naphthyridinomycin-type alkaloids,[16,17] we have prepared and assayed a focused library of analogs which include intermediates of our synthetic approach toward these natural products. Our goal was to investigate if distinct structural

Keywords: Dnacin; dual specificity phosphatase inhibitors; Cdc25; VHR; tyrosine phosphatase; PTP1B; DNA cleavage; natural product library; SAR.

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PII: S0040-4020(02)00636-1

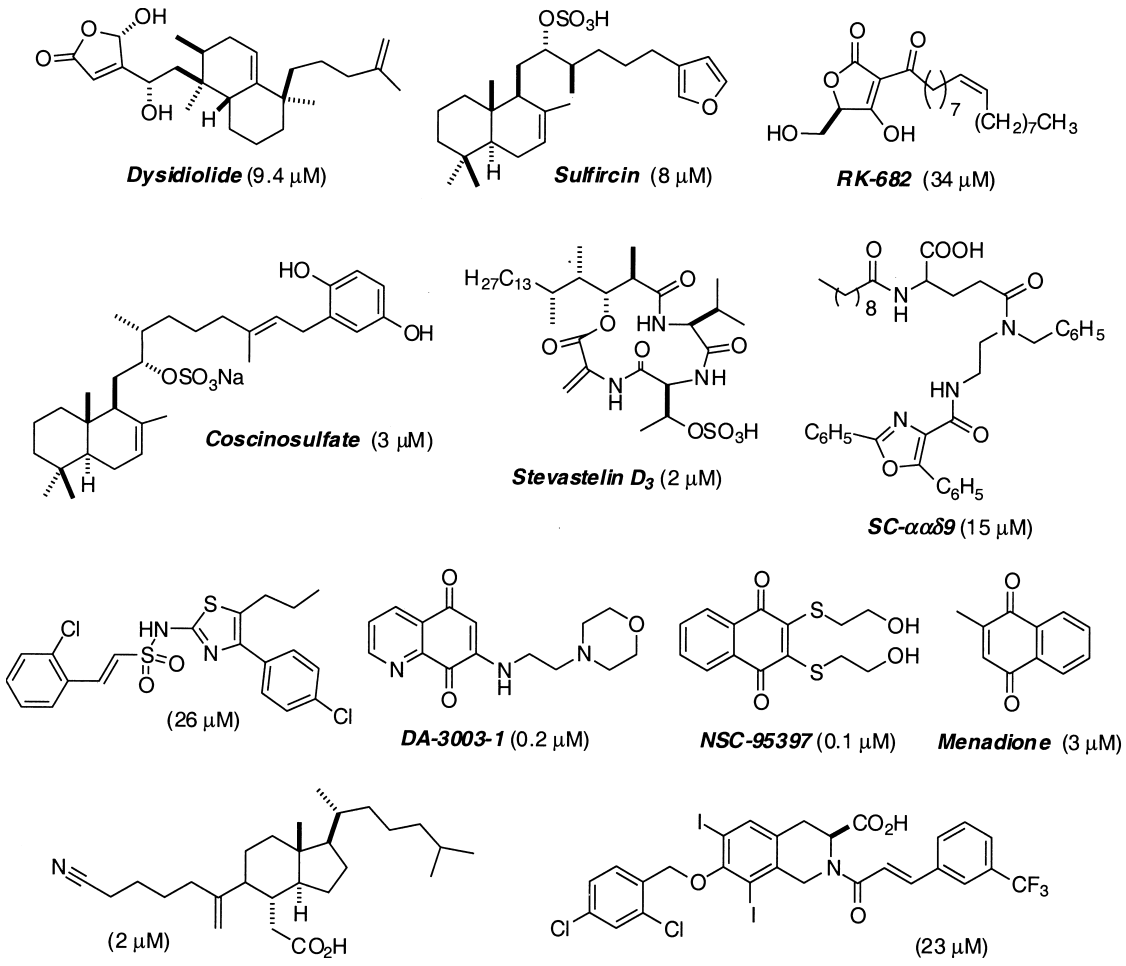

Figure 1. Selected natural products and synthetic inhibitors of DSPases (IC_{50} against Cdc25A or Cdc25B).

motifs responsible for the dual specificity phosphatase inhibitory activities of Dnacins could be identified, or if the DNA-damaging effects of Dnacins were intrinsically connected to the affinity of these agents toward the Cdc25 active site, presumably via the presence of the quinone nucleus.[1b,c,18]

Ninety-six Dnacin analogs were assayed at 100 μM for in vitro inhibition of recombinant full-length human $Cdc25B_2$ using *O*-methyl fluorescein phosphate as a substrate. Fig. 3 shows thirty representative samples from this structurally closely defined library, which included non-quinoid intermediates and squarate derivatives derived from our synthetic approach toward naphthyridinomycin-type natural products.[19] At 100 μM concentration, nine compounds caused >75% inhibition of $Cdc25B_2$: **1**–**8** and **11** (entries 1–8 and 11, Table 1).[20] A subsequent analysis at multiple concentrations revealed IC_{50} values for six of the most potent compounds that ranged between 12–44 μM (Fig. 4 and Table 1). In direct comparison, Dnacin A_1 and Dnacin B_1 had IC_{50} values of 5.2 and 1.4 μM, respectively (Fig. 4).[21] Thus, none of the analogs had inhibitory activities that exceeded those of the parent compounds. Because the IC_{50} of Dnacin A_1 and B_1 against the catalytic domain of human Cdc25B was previously reported to be 141 and 64.4 μM, respectively,[10] the lower IC_{50} values that we obtained in our assays were somewhat surprising. We believe the differences between the IC_{50}

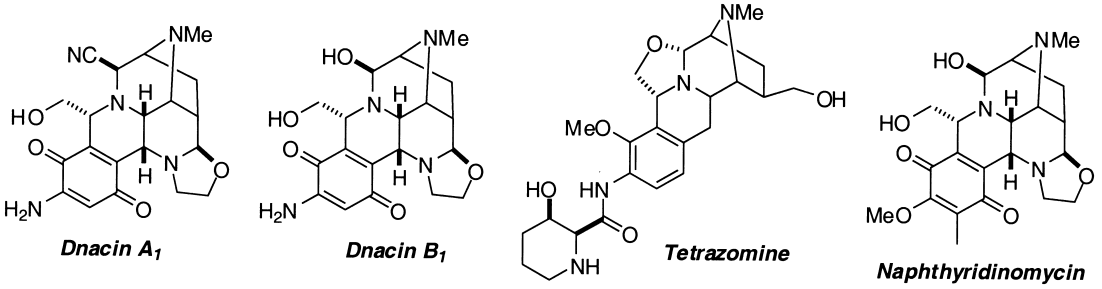

Figure 2. Naphthyridinomycin-type anticancer antibiotics.

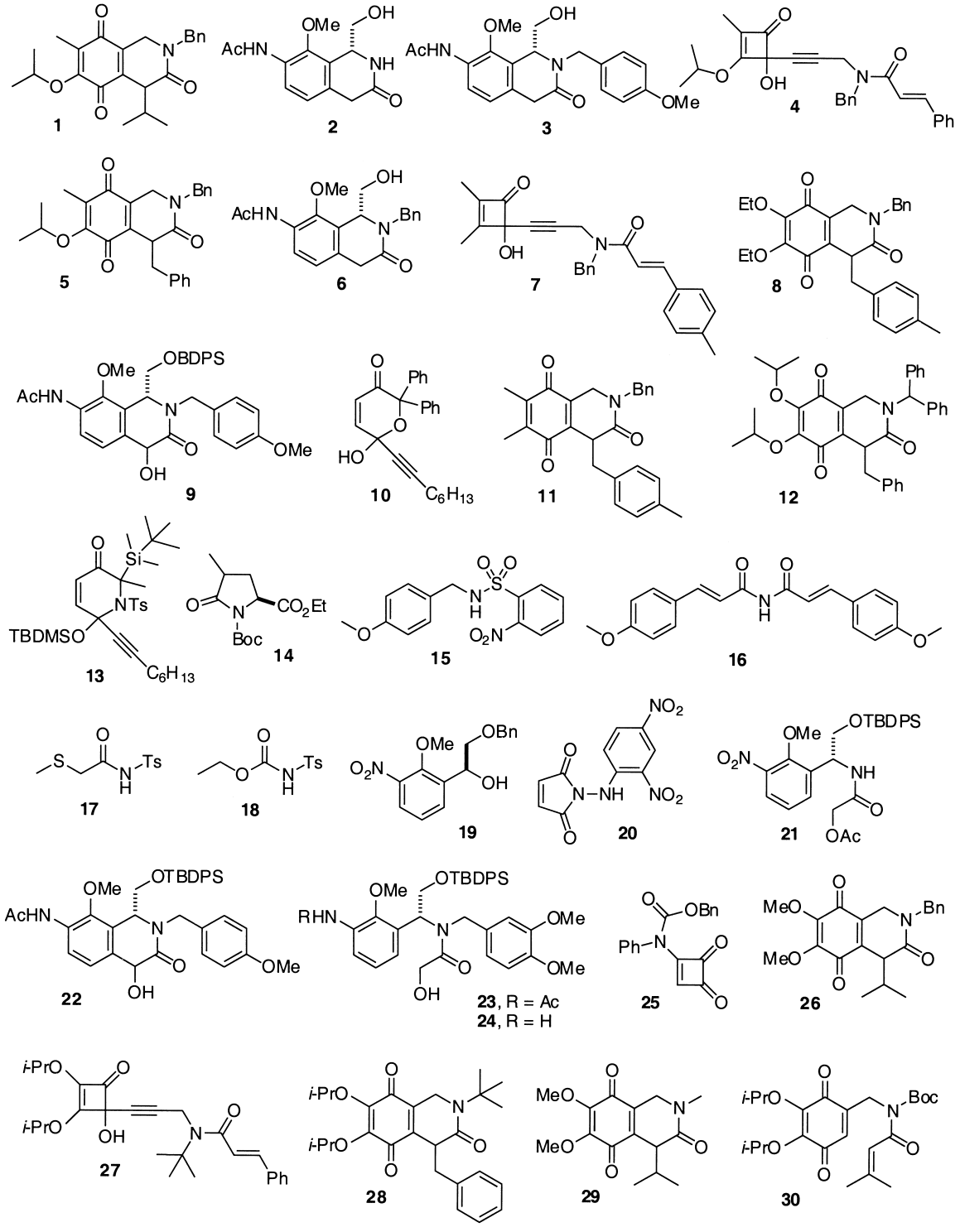

Figure 3. Representative structures of Dnacin analogues and synthetic intermediates assayed against DSPases.

values found in our study and that of Horiguchi et al.[10] are likely due to our use of the full-length enzyme, which is known to have different kinetic properties from the more enzymatically active but smaller catalytic domain,[22] and to our use of a substrate with much lower K_m[22] compared to *para*-nitrophenylphosphate used by Horiguchi et al.[10] It is conceivable that the Dnacins may interact with the amino terminus of Cdc25, which contains the regulatory domain.

In general, the nine active analogs showed the following enzyme inhibitory preference: $Cdc25B_2$> $Cdc25A_1$>VHR=PTP1B. Compounds **5** and **11** were the notable exceptions causing no inhibition of the dual specificity phosphatase VHR at 100 μM. To our knowledge, inhibition of PTP1B and Cdc25 in the absence of VHR inhibition has not been previously reported. Among the 96 analogs, the two most potent inhibitors of VHR were **1** and **7**, while the most potent inhibitors of PTP1B were **7**, **9**–**13**.

Table 1. Inhibition of protein phosphatases by analogs and synthetic intermediates of Dnacins

Entry	Compound	% Inhibition			
		$Cdc25A_1$	$Cdc25B_2$	VHR	PTP1B
1	**1**	91.4±4.3	99.5±1.4 (12)	67.2±6.3	48.0±4.1
2	**2**	80.2±3.5	96.8±1.2 (44)	30.1±1.7	26.5±3.8
3	**3**	68.0±1.9	92.4±0.7 (36)	38.6±1.7	37.6±4.5
4	**4**	47.7±4.0	92.4±2.2 (22)	35.7±4.8	36.1±3.9
5	**5**	57.1±4.3	88.0±3.2 (16)	0±0	42.6±4.2
6	**6**	49.5±3.6	88.0±1.9	22.9±6.8	30.7±9.4
7	**7**	67.1±4.6	89.3±5.0 (26)	58.2±14.6	62.8±1.7
8	**8**	64.0±6.2	76.2±7.9	17.4±6.2	31.8±3.7
9	**9**	15.2±15.6	27.5±5.6	37.6±10.0	61.5±3.4
10	**10**	46.0±20.8	62.8±8.0	40.3±7.0	60.4±5.6
11	**11**	47.6±20.8	77.1±7.1	0±0	56.1±2.6
12	**12**	51.2±16.0	46.3±18.0	48.5±4.3	51.3±2.5
13	**13**	23.9±11.3	55.7±7.0	14.3±3.0	49.9±1.6

All compounds were incubated at 100 μM for 60 min with either recombinant dual specificity phosphatase $Cdc25A_1$, $Cdc25B_2$ or VHR or the tyrosine phosphatase PTP1B. The inhibition of phosphatase activity relative to the DMSO vehicle control value from 3–6 separate determinations±standard deviation are listed for the most efficacious inhibitors among the 96 analogs tested. For several compounds, the IC_{50} values (μM) were determined for $Cdc25B_2$ and they are listed in parenthesis. All other members of the 96 compound library were essentially inactive.

None of these compounds caused >65% inhibition of PTP1B, and none showed significant specificity with the possible exception of **9**.

Dnacin is known to cause DNA damage.[11] Unlike the DNA damaging agent bleomycin, which causes prominent single and double strand DNA damage as revealed by the formation of both relaxed and linearized plasmid DNA, incubation of plasmid DNA with 400 μM Dnacin for 60 min produced considerable damage in the absence of any detectable relaxed or linearized plasmid DNA (Fig. 5).[23] Incubation with Dnacin B_1 simply led to the loss of the supercoiled plasmid DNA even when the incubation time was reduced (Fig. 6). The loss of DNA was clearly dependent on the time of incubation with the half maximal loss occurring at approximately 15 min (Fig. 6). We next examined the

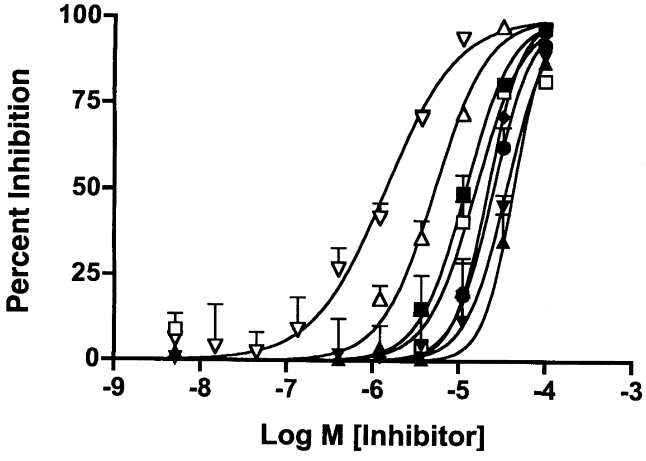

Figure 4. Inhibitory concentration response curve for Dnacin A_1, Dnacin B_1 and Dnacin analogs against $Cdc25B_2$. Samples were incubated for 60 min at various concentrations and $Cdc25B_2$ activity determined. **1**, ■; **2**, ▲; **3**, ▼; **4**, ◆; **7**, ●; **5**, □; Dnacin A_1, △ Dnacin B_1, ▽. *N*=3. Bars=standard deviation.

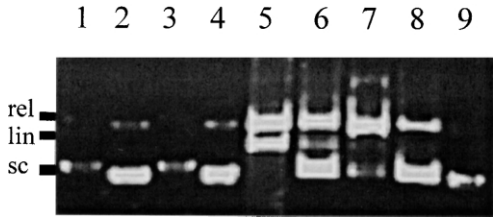

Figure 5. DNA cleavage profile of Dnacin B_1 and bleomycin. Supercoiled plasmid DNA (200 ng; pEGFP-C2-Cdc25 A)[24] was incubated for 60 min at 37°C (Dnacin samples) or ambient temperature (bleomycin samples), applied to a 0.75% agarose gel and after electrophoresis stained with ethidium bromide. Lane 1, 400 μM Dnacin B_1 and 500 μM DTT; lane 2, 500 μM DTT; lane 3, 400 μM Dnacin B_1; lane 4, 1.3 μM Dnacin B_1 and 500 μM DTT; lane 5, 1 μM bleomycin and 10 μM $FeSO_4$; lane 6, 1 μM bleomycin; lane 7, 10 μM $FeSO_4$; lane 8, 0.1 μM bleomycin and 1 μM $FeSO_4$, lane 9, control, plasmid only. These are representative of two independent experiments which produced identical results. rel, relaxed plasmid DNA; lin, linear plasmid DNA; sc, supercoiled plasmid DNA.

DNA cleaving properties of several Dnacin analogs including the most potent inhibitors of protein phosphatases. In contrast to Dnacin B_1, which cleaved almost all of the DNA substrate, in vitro incubation of plasmid DNA with most of the Dnacin analogs produced little cleavage (Fig. 7). Compounds **1**, **2** and **4** (lanes 1, 2, and 4) caused a slight reduction in the intensity of the plasmid DNA band but this was minimal when compared to the almost complete loss of DNA after Dnacin B_1 incubation (Fig. 7). Thus, it appears that truncated Dnacin analogs that lack the bridged piperidine moieties have reduced DNA cleaving activity while retaining significant Cdc25 inhibitory activity. These compounds may be useful platforms for the design of novel Cdc25 inhibitors.

In summary, we have screened a structurally focused library of 96 synthetic intermediates related to the Dnacin group of naphthyridinomycin antibiotics for some of the biological attributes of the parent natural products such as inhibition of the dual-specificity phosphatase Cdc25 and DNA cleavage. We discovered that the DNA cleaving attributes, which are a product of oxygen free radicals,[11,12] of the Dnacins are not required for Cdc25 inhibitory activity. Significantly, while the most potent $Cdc25B_2$ inhibitors **1** and **5** contain a fused *para*-quinone nucleus that is reminiscent of other potent DSPase inhibitors (Fig. 1),[1b,c] many other potent inhibitors lack this feature, and compounds **2**–**4**, and **7** provide unprecedented structural motifs and potential new lead structures for DSPase inhibition. In addition, the first

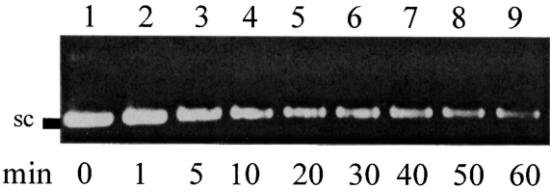

Figure 6. Time dependent DNA cleavage by Dnacin B_1. Reaction mixtures containing 200 ng plasmid DNA, 400 μM Dnacin B_1, and 500 μM DTT in 50 mM Tris–HCl were incubated for 0–60 min at 37°C. After agarose gel electrophoresis, plasmid DNA was stained. Lane 1, 0 min; lane 2, 1 min; lane 3, 5 min; lane 4, 10 min; lane 5, 20 min; lane 6, 30 min; lane 7, 40 min; lane 8, 50 min; lane 9, 60 min. These results were reproduced in a second independent experiment.

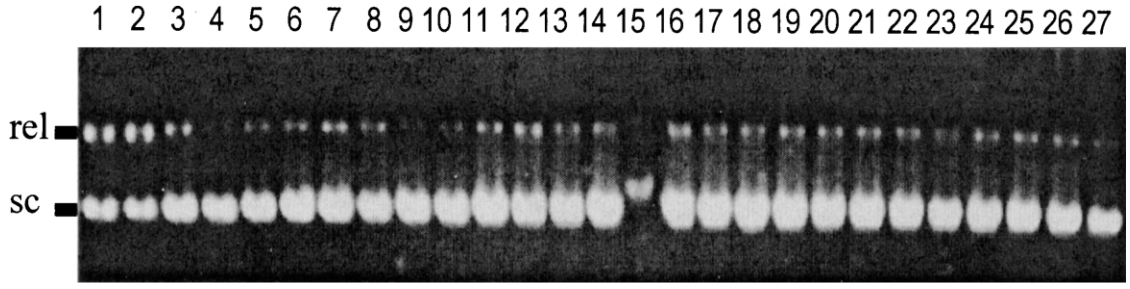

Figure 7. DNA cleavage by analogs. Reaction mixtures contained 200 ng plasmid DNA, 500 μM DTT, and 400 μM Dnacin analogs in 50 mM Tris–HCl. After incubation for 60 min at 37°C the samples were subjected to agarose gel electrophoresis and the plasmid DNA was stained with ethidium bromide. Lane 1, **1**; lane 2, **2**, lane 3, **3**; lane 4, **4**; lane 5, **7**; lane 6, **5**; lane 7, **6**, lane 8, **8**; lane 9, **13**; lane 10, **10**; lane 11, **9**; lane 12, **11**; lane 13, **12**; lane 14, H_2O; lane 15, Dnacin B_1; lane 16, **14**; lane 17, **15**; lane 18, **16**; lane 19, **17**; lane 20, **18**; lane 21, **19**, lane 22, **20**; lane 23, **21**; lane 24, **22**; lane, 25, **23**; lane 26, **24**; lane 27, **25**. These results were reproduced in a second independent experiment.

VHR-insensitive PTP1B and Cdc25 inhibitors were identified in this chemistry-driven approach. Since none of the tested compounds caused any noteworthy plasmid DNA cleavage, the basic nitrogen containing polycyclic substructure of Dnacins must be crucial for the effective generation of superoxide radicals but most likely insignificant for phosphatase inhibitory effects. Our results imply that the fundamental pharmacophore responsible for Cdc25 dual specificity phosphatase inhibition resides in the isoquinoline-5,8-dione functionality of the natural product.

3. Experimental

3.1. In vitro enzyme assays

The activities of the GST-fusion $Cdc25A_1$, $Cdc25B_2$, Cdc25C and VHR, as well as human recombinant PTP1B, were measured using *O*-methyl fluorescein phosphate as a substrate and a miniaturized, 96-well microtiter plate assay based on previously described methods.[1b] The final incubation mixtures (25 μL) were prepared using a Biomek 2000 laboratory automation workstation (Beckman Coulter, Inc., Fullerton, CA). Fluorescence emission from the product was measured after a 60 min incubation period at ambient temperature with a multiwell plate reader (PerSeptive Biosystems Cytofluor II; Framingham, MA; excitation filter, 485/20; emission filter, 530/30). Best curve fit for concentration-response plots were determined by using the Prism 3.0 curve fitting program (GraphPad Software, Inc., San Diego, CA).

3.2. Agarose gel electrophoresis

A slight modification of the method of Tanida et al.[11] was used to evaluate the DNA damaging potential of the Dnacin analogs. Briefly, supercoiled double stranded plasmid DNA (pEGFP-C2-Cdc25A) was generated after full-length human Cdc25A was cloned into pEGFP-C2 (BD Bioscience Clonetech, Palo Alto, CA) and used to transform DH5α *E. coli* as previously described.[24] Reactions contained 200 ng of plasmid DNA in 50 mM Tris–HCl, pH 7.5, with varying concentrations Dnacin B1 and 0 or 500 μM DTT. Samples were incubated at 37°C for 0–60 min. In some studies, bleomycin (1 or 0.1 μM) with or without 10 μM $FeSO_4$ was incubated at ambient temperature. DNA samples were applied to a 0.75% agarose gel and DNA separated by electrophoresis at 100 volts for 90 min. The DNA in the gel was stained with 0.5 μg/mL ethidium bromide and the gel photographed under UV illumination with a Stratagene Eagle Eye II (La Jolla, CA).

Acknowledgements

This work was supported by the National Institutes of Health (CA 78039) and the Fiske Drug Discovery Fund. We thank Ms Eileen C. Southwick for her technical support and for assaying the compounds for inhibitory activity against protein phosphatases.

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19. For the preparation of these compounds, see Refs. 16 and 17.
20. See Section 3 for in vitro enzyme assays.
21. Dnacin A1 and B1 were gifts from Takeda Chemical Industries, Ltd. (Osaka, Japan). Bleomycin (Blenoxane) was a gift from Bristol Myers Squibb (Wallingford, CT).
22. Chen, W.; Wilborn, M.; Rudolph, J. *Biochemistry* **2000**, *39*, 10781.
23. See Section 3 for agarose gel electrophoresis.
24. Rice, R. L.; Rusnak, J. M.; Yokokawa, F.; Yokokawa, S.; Messner, D. J.; Boynton, A. L.; Wipf, P.; Lazo, J. S. *Biochemistry* **1997**, *36*, 15965.