DNA binding properties of a new class of linked anthramycin analogs

J. Dean Farmer Jr, Gary R. Gustafson, Andrea Conti, Matthew B. Zimmt and J. William Suggs*
Department of Chemistry, Brown University, Providence, RI 02912, USA

Received October 2, 1990; Revised and Accepted January 14, 1991

ABSTRACT

We have investigated the DNA binding properties of the anthramycin analogues **4**, **5**, and **6** using fluorescence spectroscopy. A considerable fluorescence enhancement occurs when pyrrolo [1,4] benzodiazepines (P[1,4]Bs) are covalently attached to duplex DNA, which was used to show that neither the presence of RNA, single-stranded DNA, or protein had any effect on the degree of fluorescence enhancement resulting from the incubation of **5** and **6** with DNA. The enhancement was found to be dependent on the presence of the imine functionality in each of the compounds. A wavelength of 320 nm was used to excite the chromophore and its emission wavelength maximum was 420 nm. Additionally, we have discovered that the P[1,4]B ring system exhibits exceptionally favorable fluorescence polarization anisotropy (FPA) decay characteristics. For these more detailed fluorescence measurements, we used the structurally simpler analogue **4**. The time resolved maximum FPA for **4** in glycerol at 25°C is 0.28. This result indicates that the P[1,4]B family of antibiotics could serve as sensitive probes of DNA dynamics in the 0.1 to 35 ns time scale.

INTRODUCTION

The pyrrolo[1,4]benzodiazepine class of anti-tumor antibiotics are among the most selective DNA alkylating agents known. The naturally-occurring members of this class, which include anthramycin(**1**)¹, tomaymycin(**2**)² and neothramycin(**3**)³, bind only to duplex DNA and only in the minor groove at the guanine NH₂^{4,5}. Recently, we have developed efficient synthetic methods for the preparation of simple analogs of this class of antibiotics, including the novel DNA crosslinkers **5** and **6**⁶ (Scheme 1).

Earlier studies, using tomaymycin and neothramycin, have demonstrated that fluorescence spectroscopy is a sensitive and rapid assay for the investigation of the kinetics, pH dependence and macromolecular selectivity of pyrrolo[1,4]benzodiazepine's reactions with nucleic acids^{4,7}. There is a marked fluorescence enhancement when this class of antibiotics binds covalently to DNA. Apparently, the snug fit of these molecules within the DNA minor groove leads to less efficient energy transfer from the excited state compared to the drug free in solution. This

Scheme 1

enhancement is retained with the analogs **4**, **5** and **6**, allowing for facile characterization of their DNA binding properties. Furthermore, we find the pyrrolo[1,4]benzodiazepine ring system exhibits exceptionally favorable fluorescence polarization anisotropy (FPA)^{8,9} decay characteristics. This observation suggests the anthramycin family of antibiotics could be used to explore DNA dynamics in the 0.1 to 35 ns time scale.

MATERIALS AND METHODS

The UV-Vis absorption measurements for determination of the calf thymus DNA concentration were taken on a Perkin and Elmer 552A UV/Vis spectrophotometer. All fluorescence measurements were made on a Gilford Fluoro IV spectrophotometer with irradiation of the sample occurring at 320 nm and emission observed at 420 nm. The synthesis of compounds **4**, **5**, and **6** are described elsewhere¹⁰. In the molecular selectivity studies, wheat germ and E. Coli rRNA, wheat germ tRNA, and RNase A were purchased from Sigma. The E. Coli tRNA was purchased from P.L. Biochemicals and single-stranded DNA from Pharmacia. The NaBH₄ was obtained from Aldrich. Anthramycin was a gift of Dr. A. Batcho, Hoffmann LaRoche.

* To whom correspondence should be addressed

Calf thymus DNA (solid calf thymus DNA provided by Miles Serevac) was sonicated in TE buffer at pH 7.4 to give material which was approximately 200 bp long. The concentration of the calf thymus solution was determined by its UV absorption at 260 nm. Unless otherwise noted, the standard conditions were a drug concentration of 10 μ M and a DNA : drug ratio of 100:1 (moles bp:moles drug) for each reaction. The reaction mixtures contained a total volume of 1.5 ml with 50 mM NaCl, 10 mM Tris (pH 7.4) and 10 μ M drug in each. The fluorimeter was calibrated at 20% for these reactions.

For pH studies compounds **5** and **6** were each reacted at the standard conditions. The reactions were heated to 65°C for 18 hours. The buffer solutions were Tris pH 7.5 and sodium citrate at pHs of 4.0, 5.0, and 6.0. In the DNA : drug ratio studies the drug concentration was held constant and the amount of calf thymus DNA varied.

Reduction of the Imine Bond by NaBH₄

The concentration of NaBH₄ was maintained at 1mM. The reducing agent reacted with water very rapidly. Immediately before each reaction a new solution was made by adding 0.378 g of solid NaBH₄ to 10 ml of water, vortexing and then using the solution quickly. In the first series of reactions, fluorescence readings were taken of the solution when it contained only the drug. The NaBH₄ was then added and allowed to react with the drug for 1.5 hours at 25°C, at which time fluorescence readings were taken again. Finally, the DNA was added and the mixture heated to 65°C and allowed to react for 1.5 hours followed by measurement of the solution's fluorescence. In the second series of reactions, fluorescence readings were taken of the solution containing only the drug. DNA was added, the mixture heated to 65°C and allowed to react for 1.5 hours and another fluorescence reading was taken. Lastly, the NaBH₄ was added and the solution left to react for 1.5 hours. At the end of this period the last fluorescence reading was taken.

Molecular Selectivity Studies

The standard conditions were used. The calibration of the fluorimeter was set to 20%. In each reaction, all reagents were mixed simultaneously and reacted at 65°C. The reaction times were 18 hours for the RNA studies, 2.5 hours for the single-stranded DNA and 2 hours for the protein studies.

Fluorescence Polarization Anisotropy

The frequency doubled output of a Coherent Antares 76-S mode-locked and Q-switched Nd-YAG laser was used to synchronously pump a dye laser (DCM in methanol) tuned with optical filters and a single 8- μ m etalon. The cavity dumped dye laser output (3 Hz, 20 μ J, 50 ps, 643 nm) was frequency doubled (ADP) and polarized (>5000:1). The excitation polarization was adjusted to horizontal (perpendicular) or vertical (parallel) with a zero order half wave plate, yielding a final polarization greater than 500:1. Excitation energies ranged from 0.4–2.0 μ J. Fluorescence polarization anisotropies were calculated as

$$\text{FPA}(t) = [I_{\text{parallel}}(t) - I_{\text{perpendicular}}(t)] / [I_{\text{parallel}}(t) + 2I_{\text{perpendicular}}(t)]$$

where $I_{\text{parallel}}(t)$ is the relative fluorescence intensity observed at time t with polarization parallel to the excitation light and $I_{\text{perpendicular}}(t)$ is the relative fluorescence intensity at time t with polarization perpendicular to the excitation light.

Samples were held in a 1mm pathlength quartz cuvette and the fluorescence was collected in a back scattering geometry with a 50mm focal length quartz lens. The collected light passed through a vertical polarizer, an ISA H-20 monochromator and was detected with a multichannel plate coupled to a Tektronix 7912-AD transient waveform recorder. The parallel and perpendicular signals were normalized for variations in excitation intensity using a pick off positioned after the ADP doubling crystal.

RESULTS

We have previously shown that these rationally designed DNA crosslinkers **5** and **6** bind reversibly to DNA and protect the DNA against the action of certain restriction enzymes⁶. In addition to demonstrating reversible crosslinking of plasmid DNA by **5** and **6** using alkaline agarose gel assays¹¹, we have demonstrated their ability to crosslink restriction fragments and short oligonucleotides using denaturing polyacrylamide gel assays¹². The fact that the conditions which lead to maximum fluorescence enhancement correlate with maximum protection from nuclease digestion, together with the close structural similarity between tomaymycin and these synthetic analogs, allow us to equate DNA binding with fluorescence enhancement. The fluorescence enhancement is due to covalently bound pyrrolo[1,4]benzodiazepine since ethanol precipitation and resuspension of DNA treated with **5** gives essentially the same fluorescence intensity as that measured prior to ethanol precipitation.

Initial experiments were designed to establish the reaction conditions for maximum fluorescence enhancement, using **5** and **6**. Most experiments were carried out with **5**, since the DNA crosslinking properties of **5** and **6** have been found to be similar. The temperature dependence of binding was the first variable measured by fluorescence enhancement. The standard conditions of pH 7.4 in a 10 mM Tris buffer with 50 mM NaCl and sonicated calf thymus DNA (base pair:drug ratio 100:1) at 10 μ M drug concentration were used. The greatest fluorescence enhancement with **5** occurred at 65°C (Figure 1). At this temperature, binding was complete after 5 hrs. Binding also took place at 25°C and 37°C, but was incomplete even after 48 hrs. Thus, covalent binding reaches equilibrium significantly faster

Figure 1. Temperature dependence studies of binding by **5** to DNA. DNA was incubated with **5** at 25°C, 37°C, and 65°C and fluorescence readings were taken at various time intervals. A drug concentration of 10 μ M and a DNA : drug ratio of 100:1 (moles bp:moles drug) were maintained for each reaction. The reaction mixtures contained a total volume of 1.5 ml with 50mM NaCl and 10mM Tris (pH 7.4) in each. The fluorimeter was calibrated at 20% for these reactions.

at 65°C. In order to show that the fluorescence enhancement differences were not due to temperature induced changes in **5**, control experiments were performed in which fluorescence intensity of **5** was monitored in the absence of DNA as a function of temperature. No changes were seen.

The pyrrolo[1,4]benzodiazepine class of antitumor antibiotics are known to bind slowly to DNA. However, there is a second possible explanation for the temperature dependence and slow increase in fluorescence intensity seen in the binding of **5** and **6** to calf thymus DNA. We know from previous work that the binding of **5** and **6** to DNA is reversed by higher temperature. This is also clearly shown in figure 2, where heating to 95°C for 15 minutes removes the fluorescence enhancement (this is fully reversible, since cooling to 65°C restores the fluorescence enhancement. Notice that anthramycin, which is known to stabilize the DNA helix against melting, is not reversed at 95°C). Thus, at 65°C **5** can bind either monofunctionally, with one pyrrolo[1,4]benzodiazepine unit exposed to solvent, or it can crosslink, burying both units in the DNA minor groove. At 65°C any **5** bound monofunctionally that does not have an adjacent dG with which it can crosslink can dissociate, until it loads onto a crosslinkable site, yielding the maximum fluorescence enhancement. The data suggest dissociation and rebinding can occur, slowly, even at 25°C.

The pH dependence in the binding of **5** and **6** to calf thymus DNA was next examined (Table 1 and Table 2). Both **5** and **6** showed fluorescence enhancement at pH 6 and 7.5, but little at lower pHs. Lack of binding at acidic pH is also seen with the rest of the members of the anthramycin family. For example, 64% of DNA-bound tomaymycin is released upon lowering the pH to 6.0 and 91% is released at pH 4. This pH sensitivity is

Figure 2. Thermal reversibility of **5**'s binding to DNA. DNA was added to a solution of **5** so as to make the solution 10μM in **5** and create a DNA : drug ratio of 100:1. The reaction mixtures contained a total volume of 1.5 ml with 50mM NaCl and 10mM Tris (pH 7.4). The solutions were heated at 65°C for 5 min and then heated to 95°C for the duration.

Table 1. pH Dependence of Fluorescence with **5**

pH	DNA ^a Rel. Fluorescence		No DNA Rel. Fluorescence	
	t = 0	t = 18h ^b	t = 0	t = 18h ^b
7.5	15.5	42.7	17.2	17.3
6.0	14.1	45.3	17.4	18.3
5.0	38.1	38.0	14.1	18.8
4.0	27.2	9.1	15.5	6.5

^a calf thymus at 100bp: drug; ^b Incubation at 65°C.

modulated by the functional groups on the pyrrolo[1,4]benzodiazepine nucleus, since only 15% of DNA-bound anthramycin is released at pH 4. This acid sensitivity is due to the fact that these compounds bind to DNA via an aminal linkage, a very acid labile functional group. Those pyrrolo[1,4]benzodiazepine antibiotics with an array of hydrogen bonding groups on the aromatic ring or the five-membered ring, such as anthramycin and siberimycin, form complexes with enhanced acid stability (stable below pH 3). Simpler molecules, such as tomaymycin, **5** and **6** bind to DNA only at or close to neutral pH.

The effect of DNA concentration on fluorescence enhancement was also investigated. Both **5** and **6** were incubated at bp:drug ratios of 50:1, 100:1 and 200:1 for 1.5 hrs (Table 3). In each case, the fluorescence increased as the DNA concentration increased. With a greater variety of DNA sequences per drug, there is a higher probability that a high affinity crosslinkable binding site will be present.

The naturally occurring pyrrolo[1,4]benzodiazepines show extraordinary selectivity for binding to duplex DNA. Two types of fluorescence experiments were carried out which show that the synthetic analogs **5** and **6** retain this specificity. First, the analogs were incubated with samples of tRNA, rRNA, the proteins RNase A and BSA, each of the four deoxynucleotides and each of the four single stranded polydeoxynucleotides. Incubations were carried out at 37°C and 65°C with large molar excesses (>100:1) of the proteins and nucleic acids. In all of these experiments there was no significant increase in the

Table 2. pH Dependence of Fluorescence with **6**

pH	DNA ^a Rel. Fluorescence		No DNA Rel. Fluorescence	
	t = 0	t = 18h ^b	t = 0	t = 18h ^b
7.5	27.6	37.4	20.2	26.5
6.0	32.8	38.1	20.4	20.7
5.0	38.8	38.8	23.3	23.2
4.0	30.4	20.2	17.8	10.3

^a calf thymus at 100bp: drug; ^b Incubation at 65°C.

Table 3. Effect of Base Pair Ratio on Fluorescence Enhancement.

ratio (bp:drug)	Relative Fluorescence 5 ^a		Relative Fluorescence 6	
	time=0	1.5 hr	time=0	time=5.5 hr
no DNA	50	52	17	20
50:1	45	108	16	28
100:1	52	132	17	31
200:1	61	154	15	30

^aIncubation at 65°C

Table 4. DNA:RNA Binding Selectivity of **5**

ratio DNA:RNA ^a	Relative fluorescence T=0	Relative fluorescence T=2 hrs
no DNA or RNA	19.9	23.4
100:1	24.4	63.4
10:1	24.3	60.6
1:1	24.7	57.0
1:0	20.1	60.8

^a Ratio 4:DNA 1:200

Figure 3. Fluorescence polarization anisotropy of **4**. A solution of **4** in 8:1 glycerol:methanol at 21°C was excited at 317 nm and the fluorescence observed at 410 nm. The upper trace is excitation light polarized parallel to the detection polarizer and the bottom trace is for excitation light polarized perpendicular to the detection polarizer (arbitrary intensity units).

fluorescence intensity of **5** or **6**. Likewise, the duplex DNA poly (dA-dT)•poly (dA-dT), when incubated with **5** under standard conditions, produced no fluorescence enhancement. The linked anthramycin analogs **5** and **6**, like the naturally-occurring pyrrolo[1,4]benzodiazepine antitumor antibiotics, require a GC base pair for DNA binding.

Second, competition experiments were used to show that the selectivity for DNA exhibited by the natural products was retained by the analogs. Table 4 gives the fluorescence enhancement when **5** is incubated with calf thymus DNA in the presence of increasing amounts of commercial *E. Coli* rRNA. The rRNA generates no interference with binding of the dimeric anthramycin analog to DNA.

Covalent binding to DNA takes place when pyrrolo[1,4]benzodiazepines alkylate the NH₂ of guanine, reaction taking place at the imine carbon (Scheme 2). If formation of the covalent linkage is dependent upon the presence of the imine, then reduction of

Scheme 2

Table 5. Effect of NaBH₄ of the Fluorescence of the 5/DNA Adduct

bp/drug	Relative Fluorescence		
	t = 0	t = 1.5h ^a	After NaBH ₄ ^b
50:1	54.9	94.6	77.9
100:1	50.5	109.2	95.8
200:1	59.5	143.2	126.7
0	49.0	49.0	35.3

^a **5** incubated with DNA at 65°C, 50mM NaCl, 10mM Tris pH 7.4

^b DNA/**5** from 'a' treated with NaBH₄

Table 6. The effect of NaBH₄ on the binding of **5** to DNA

bp/drug	Relative Fluorescence		
	t = 0	after NaBH ₄	t = 1.5h
50:1	58.8	42.8	49.7
100:1	57.7	46.5	57.8
200:1	59.5	44.6	67.1

^a **5** mixed with DNA and fluorescence recorded; NaBH₄ added and mixed for 3h and the fluorescence recorded. Afterwards the reaction was incubated for 1.5h at 65°C and the fluorescence recorded.

the imine to an amine should prevent such binding. Once the drugs are bound to the DNA in the minor groove, they would be sterically protected from reduction. Failure to bind covalently to DNA due to imine reduction in the pyrrolo[1,4]benzodiazepines should therefore result in the elimination of any fluorescence enhancement. In order to test this prediction, NaBH₄ was chosen to serve as the imine reducing agent, since

it is selective for the imine functional group over the other functional groups present in **5** and in DNA.

The experiment was performed in two parts. In the first part, the NaBH₄ was added to a solution of **5** before the addition of DNA. Fluorescence readings were taken of the solution when it contained only the drug, after the drug and NaBH₄ had been allowed to react, and after the DNA had been added and allowed to react with any remaining drug for 1.5 hours. In the second part of the experiment, the NaBH₄ was added after the drug and DNA had been allowed to react. The results from the first part of the experiment (table 4) show that there is a decrease in fluorescence resulting from reaction of NaBH₄ with **5** (compare columns 1 and 2). This is due to the reduction of the imine functionality. Allowing the solution to react at 65°C for 1.5 hours did produce some enhancement, although it is very small when compared with the enhancements shown in figure 3. This may be due to incomplete reduction of the imine by the NaBH₄. NaBH₄ reacts rapidly with water, so complete reaction is difficult. In the results from the second part of the experiment, there appears to be a certain amount of fluorescence reduction that occurs when the NaBH₄ is reacted with the drug and DNA adduct. This reduction in fluorescence corresponds to that seen with the free drug in solution. Given the fact that these drugs can bisalkylate DNA, there will be a certain degree of monofunctional binding that takes place. This leaves one of the ends of the P1₄Bs free in solution and susceptible to reduction by the NaBH₄, leading to the corresponding decrease seen. Overall, NaBH₄ efficiently blocks DNA binding of **5** (as measured by fluorescence enhancement) and the DNA adduct is resistant to reduction. These results provide evidence that the imine functional group is necessary for DNA binding in the synthetic analog **5** as it is in the naturally-occurring pyrrolo[1,4]benzodiazepines.

Since the dimeric pyrrolo[1,4]benzodiazepines **5** and **6** are capable of binding to DNA (at least in principle) monofunctionally or as intrastrand or interstrand crosslinkers, we chose to use the simpler pyrrolo[1,4]benzodiazepine **4** for more detailed fluorescence measurements. The structurally related natural product tomaymycin has been found to exist as two fluorescent ground-state species in protic solution and when bound to DNA¹³. These two compounds are the 11R and 11S diastereomers. When bound to DNA, they have fluorescence lifetimes of 3.3 and 5.7 ns (pH 7.5, 25°C) respectively. Similarly, **4** exhibits a fluorescence first half life of 8.5 ns in 7:1 MeOH-EtOH and the decay is non-exponential (presumably due to diastereomers at C-11 formed by methanol addition across the imine bond).

A related fluorescence property of the anthramycin family of antibiotics which has not been studied heretofore is their fluorescence polarization anisotropy (FPA). In this technique, one measures the difference in fluorescence using excitation light polarized parallel or perpendicular to the detection polarizer. Several groups have studied the FPA decays of intercalative dyes with DNA^{14,15}. These measurements provide sensitive probes of DNA dynamics in the 0.1–100 ns time scale. The maximum theoretical FPA (observed when the the absorption and emission dipoles are parallel) is 0.40. Many fluorescent DNA intercalators (ethidium bromide for example) have FPAs near zero (although other kinds of fluorescence-based measurements can produce valuable dynamical information¹⁶). Most studies have been carried out on dyes with FPAs in the vicinity of 0.20¹⁵. The measured FPA of **4** in glycerol at 25°C is 0.29 ± .02 (figure 3). Thus, pyrrolo[1,4]benzodiazepines should be excellent dyes for the study of DNA dynamics by FPA decay. Unlike intercalators,

they offer the potential of being inserted into specific sequences since they form covalent bonds to DNA. Studies along these lines are presently underway and will be reported in due course.

The linked pyrrolo[1,4]benzodiazepines **5** and **6** are unique among DNA crosslinkers in their specificity for dG-containing duplex DNA. Most crosslinking agents are reactive electrophiles which bond indiscriminately to cellular macromolecules.^{17,18,19} The more selective of these agents, such as psoralens, need to be activated by some process (such as light absorption) prior to DNA alkylation²⁰. Even in the case of psoralens, RNA as well as DNA is reactive²¹. Selectivity is a desirable design goal for DNA crosslinkers. Many clinically useful antitumor agents have as their mode of action DNA crosslinking²². However, side reactions with other classes of molecules are thought to result in unwanted cellular damage.

The use of fluorescence enhancement permits a very rapid and convenient assay for the effects of linker length, charge and stiffness on DNA alkaline binding. For example, it is seen from figure 2 that a 12 atom linker with two tertiary nitrogens produces a linked pyrrolo[1,4]benzodiazepine (**6**) that binds more stably at higher temperatures than a linked anthramycin analog with 7 atoms and only one tertiary amine (**5**). Since it is now relatively simple to prepare linked pyrrolo[1,4]benzodiazepines, it is possible to systematically investigate the effects of linker structure on their DNA binding and crosslinking properties.

ACKNOWLEDGEMENT

This work was aided by grant CH-475 from the American Cancer Society and RR0-4735 from the NIH (JWS). The time resolved fluorescence studies were aided by Brown University BRSG and a Dreyfus Young Investigator Grant to MBZ.

REFERENCES

1. Tendler, M.D., Korman, S. (1963) *Nature* **199**, 501.
2. Arima, K., Kohsaka, M., Tamura, G., Imanaka, H., Sakai, H. (1972) *J. Antibiotics* **25**, 437–444.
3. Takeuchi, T., Miyamoto, M., Ishizuka, M., Naganawa, H., Kondo, S., Hamada, M., Umezawa, H. (1976) *J. Antibiotics* **29**, 93–96.
4. Kohn, K.W., Glaubiger, D., Spears, C.L. (1974) *Biochim. Biophys. Acta* **361**, 288–302.
5. Hurley, L.H., Petrusek, R. (1979) *Nature* **282**, 529–531.
6. Farmer, J.D., Rudnicki, S.M., Suggs, J.W. (1988) *Tetrahedron Lett.* **29**, 5105–5108.
7. Maruyama, I., Tanaka, N. (1981) *J. Antibiotics* **34**, 427–435.
8. Barkley, M.D., Maskos, K. (1986) *Polymer Preprints* **27**, 322–323.
9. Thomas, J.C., Allison, S.A., Appellof, C.J., Schurr, J.M. (1980) *Biophys. Chem.* **12**, 177–188.
10. Synthesis of **4** and **5** can be found in ref. 6. Synthesis of **6** is a part of manuscript in preparation and is analogous to **5**.
11. Cech, T. R. (1981) *Biochemistry* **20** 1431–1436.
12. Farmer, J. D. Jr., (1989) Ph.D. thesis, Brown University.
13. Barkley, M.D., Cheatham, S., Thurston, D.E., Hurley, L.H. (1986) *Biochemistry* **25**, 3021–3031.
14. Hard, T., Kearns, D.R. (1986) *J. Chem. Phys.* **90**, 3437–3444.
15. Fujimoto, B.S., Schurr, J.M. (1987) *J. Chem. Phys.* **91**, 1947–1951.
16. Magde, D., Zappala, M., Knox, W.H., Nordlund, T.M. (1983) *J. Phys. Chem.* **87**, 3286–3288.
17. Summerton, J., Bartlett, P. A. (1978) *J. Mol. Biol.* **122**, 145–162.
18. Brooks, P., Lawley, P. D. (1961) *Biochemical J.* **80**, 495–503.
19. Kohn, K. W. *Molecular Mechanisms of Crosslinking by Alkylating Agents and Platinum Complexes in Sartorelli, A. C., Luzo, J. S., Bertino, J. R. (eds) Molecular Actions and Targets for Chemotherapeutic Agents*, pp 3–16 New York, Academic Press 1981.
20. Hearst, J. E., Isaacs, S. T., Kanne, D., Rapoport, H., Straub, K. (1984) *Quart. Rev. Biophysics* **17**, 1–44.
21. Hui, C.-F., Cantor, C. R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1381–1385.
22. Kohn, K. W., in *Development of Target-Directed Anti-Cancer Drugs*, Chang, Y. C., Goz, B. G., Minkoff, M. (eds), pp181–195, Raven Press 1983.