Published in final edited form as:

Free Radic Biol Med. 2007 October 15; 43(8): 1132–1144. doi:10.1016/j.freeradbiomed.2007.07.005.

Mechanism of the cytotoxicity of the diazoparaquinone antitumor antibiotic kinamycin F

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Abstract

The bacterial metabolite kinamycin F, which is being investigated as a potent antitumor agent, contains an unusual and potentially reactive diazo group as well as a paraquinone and a phenol functional group. Kinamycin F reacted with glutathione (GSH) in a complex series of reactions which suggested that kinamycin F may have its cytotoxicity modulated by GSH. Consistent with this idea 2-oxo-4-thiazolidinecarboxylic acid treatment to increase cellular GSH levels and buthionine sulfoximine treatment to decrease GSH levels resulted in decreased and increased kinamycin F cytotoxicity, respectively, in K562 leukemia cells. Kinamycin F weakly bound to DNA and induced DNA damage in K562 cells that was independent of GSH levels. The GSH-promoted DNA nicking induced by kinamycin F in vitro was attenuated by deferoxamine, dimethyl sulfoxide, and by catalase, which indicated that DNA damage initiated by this agent occurred in an iron-, hydrogen peroxide- and hydroxyl radical-dependent manner. Electron paramagnetic resonance spectroscopy experiments showed that the GSH/kinamycin F system produced a semiquinone free radical and that the hydrogen peroxide/peroxidase/kinamycin F system generated a phenoxyl free radical. In conclusion, the results indicated that kinamycin F cytotoxicity may be due to reductive and/or peroxidative activation to produce DNA- and protein-damaging species.

Keywords

kinamycin F; anticancer drug; quinone; semiquinone radical; topoisomerase II; cytotoxicity; phenoxyl radical; diazo; electron paramagnetic resonance spectroscopy; cytotoxicity

Introduction

The bacterial metabolites kinamycins A, B, C and D were isolated in 1970 from *Streptomyces murayamaensis* and were shown to exhibit good activity against Gram positive, but not Gram negative bacteria [1]. The deacetylated form of the kinamycins, kinamycin F (Fig. 1), though it was first prepared by chemical deacetylation of kinamycin C [2], has been detected more

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recently as a metabolite in culture broths of *S. murayamaensis* [3] and is the subject of this study. Kinamycin C has been subjected to the 60-cancer cell NCI panel and generally displayed submicromolar GI_{50} values (<http://dtp.nci.nih.gov>). Kinamycin C has also displayed antitumor activity in an animal model [2]. The kinamycins contain a diazo group, which is unusual for a natural product. This diazo group likely plays a key role in their antitumor activity. The kinamycins were initially assigned an N-cyanocarbazole structure. However, we [4] and another group [5] independently determined that the structures assigned to the kinamycins were incorrect and that the kinamycins are in fact derivatives of diazobenzo[b]fluorene, rather than of N-cyanobenzo[b]carbazole. The novel antitumor antibiotic lomaiviticin A isolated from a marine actinomycete named *Micromonospora lomaivitiensis* has also recently been found to be extraordinarily potent (low picomolar IC_{50} values) against the modified NCI 24-cancer cell line panel [6]. The major structural difference between lomaiviticin A and the kinamycin structure is that the former is dimeric, with two kinamycin-like systems joined via a carbon-carbon bond linking ring D of each monomer. The total synthesis of kinamycin C has just been reported [7] which should lead to the development of more active congeners.

Recent studies on prekinamycin, a precursor analog of kinamycin, showed that in aromatic solvents containing a radical initiator and an organic tin reducing agent, radical-trapping arene products were formed that involved the loss of dinitrogen to form a postulated reactive radical intermediate and an orthoquinonemethide electrophile [8,9]. A role for reductive activation of kinamycin D by DTT in its DNA-cleaving ability has also recently been suggested [10]. In the presence of a cupric acetate oxidant the kinamycin F analog 9-diazo fluorene was able to nick DNA [11] which suggests possible oxidative activation. In other prior chemical studies we showed that the diazo group of isoprekinamycin, an analog of kinamycin A, reacted with the nucleophile 2-naphthol, which is known to have good reactivity towards aryldiazonium salts, to yield the azo-coupled product [12]. Collectively these observations led us to the hypothesis that the kinamycins represent variants of the same pharmacophore in which the diazo group, with its diazonium-like character, has unusual biological reactivity and plays a key role in their action as cytotoxic agents.

Our recent biological studies that evaluated kinamycin A and kinamycin C as potential new anticancer agents showed that both compounds potently inhibited cancer cell growth and that kinamycin C induced a rapid apoptotic response in K562 cells [13]. Kinamycin A also displayed an unusual G_1/S cell cycle block in synchronized CHO (Chinese hamster ovary) cells only upon entry to a second cell cycle, an effect that may have arisen due to slow deacetylation of kinamycin A to kinamycin F by intracellular esterases. From the cell cycle and cluster analysis data we concluded that the kinamycins potentially have a cellular target or targets different than anticancer drugs currently in clinical use. Pretreatment of both kinamycins with DTT partially protected against their ability to inhibit DNA topoisomerase II α (which contains protein sulfhydryl groups), suggesting that reaction with critical sulfhydryl groups may play a role in their mechanism of action [13]. In the present study, to better elucidate the mechanism(s) by which the kinamycins exert their biological activity, we examined reductive and oxidative activation mechanisms of the more water soluble deacetylated form, kinamycin F, which may be the active metabolite. The results presented here show that kinamycin F can be reductively activated to a semiquinone free radical and peroxidatively activated to a phenoxyl free radical. In addition, kinamycin F can damage DNA in intact cells and is capable of binding and nicking DNA in vitro in the presence of GSH. Reductive activation of kinamycin F by a reducing enzyme or some other reducing species may enable the diazo group to target DNA or critical proteins causing inhibition of cell growth.

Materials and methods

Materials

Thioglo-1 was from EMD Biosciences (Mississauga, Canada), kDNA was obtained from TopoGEN (Columbus, OH) and pBR322 plasmid DNA was obtained from MBI Fermentas (Burlington, ON, Canada). Unless otherwise indicated, all other chemicals were from Sigma-Aldrich (Oakville, ON, Canada). Errors quoted from non-linear-least-squares analyses (SigmaPlot, SyStat, Point Richmond, CA) are standard errors. The level of significance used was < 0.05 .

Preparation of kinamycin F

The producing strain, *S. murayamaensis*, was obtained from the American Type Culture Collection (Manassas, VA) and kinamycin D was isolated as described [3,14]. Sodium methoxide (0.1 mM) in methanol was added to a solution of kinamycin D in methanol and stirred for 1 h at room temperature. The reaction mixture was then added dropwise to a suspension of H^+ -ion exchange resin (Baker CGC-271 weak acid resin, J.T. Baker, North York, Canada) in methanol. After stirring for 5 min, the ion exchange resin was removed by vacuum filtration and washed with a small amount of methanol. Removal of the solvent from the filtrate gave a solid residue which was chromatographed on silica gel with elution by acetone to provide kinamycin F. Stock kinamycin F solutions were prepared in either DMSO or methanol, as indicated. When methanol was used it was evaporated with a flow of nitrogen after it was dispensed.

Cell culture and growth inhibition assays

Human leukemia K562 cells, obtained from the American Type Culture Collection, and K/VP.5 cells (a 26-fold etoposide-resistant K562-derived cell line with decreased levels of topoisomerase II α protein and mRNA) [15,16] were maintained as suspension cultures in Dulbecco's modified Eagle's medium (Invitrogen, Burlington, Canada) containing 4 mM L-glutamine and supplemented with 20 mM HEPES, 10% fetal calf serum (Invitrogen), 100 units/ml penicillin G, and 100 μ g/ml streptomycin in an atmosphere of 5% CO_2 and 95% air at 37°C (pH 7.4).

For the measurement of growth inhibition, K562 and K/VP.5 cells in exponential growth were harvested and seeded at 6000 cells/well in 96-well plates (100 μ l/well). Twenty-four h later cells were treated with vehicle or various concentrations of kinamycin F and allowed to grow an additional 72 h. For assessment of the effects of increasing or lowering GSH levels on kinamycin F cytotoxicity, K562 cells in exponential growth were seeded to yield when kinamycin F-treated approximately 12,000 cells/well in 96-well plates (100 μ l/well). Forty-eight or twenty-four h after seeding, cells were treated in the presence or absence of either OTC (5 mM) or BSO (100 μ M), respectively, and were then allowed to grow an additional 24 or 48 h, respectively. The cells were then treated with vehicle or various concentrations of kinamycin F and allowed to grow an additional 72 h. Kinamycin F was dissolved in DMSO and the final concentration of DMSO did not exceed 0.5% (v/v), which was an amount that was shown not to affect cytotoxicity. Neither the OTC nor the BSO treatment caused any measurable cytotoxicity. After treatment cells were assayed with the MTS CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega, Madison, WI). The spectrophotometric 96-well plate cell growth inhibition assay measures the ability of the cells to enzymatically reduce MTS. Three replicates were measured at each drug concentration and the IC_{50} -values for growth inhibition were measured by fitting the absorbance-drug concentration data to a three- or four-parameter logistic equation as described [17].

Kinetics of the reaction of kinamycin F with GSH and DTT

The reaction of GSH or DTT with kinamycin F was followed spectrophotometrically by repeated spectral scanning on a Cary 1 double beam spectrometer (Varian, Mississauga, Canada) with a thermostated cell compartment. The reaction was initiated by adding a small volume of stock GSH or DTT to 700 μ l of freshly prepared 20 μ M kinamycin F and 20 mM Tris, pH 7.6, in a semi-micro quartz cell at 37°C. The reaction mixtures also contained 5% (v/v) DMSO, part of which was from the stock kinamycin F.

Thermal denaturation of DNA assay and spectrophotometric DNA titration assay

Ligand binding to DNA stabilizes the DNA double helix, causing an elevation in the DNA melting temperature (T_m). The T_m of sonicated calf thymus DNA (5 μ g/ml) was measured in 10 mM Tris buffer (pH 7.5) in a Cary 1 (Varian) double beam spectrophotometer by measuring the absorbance increase at 260 nm upon the application of a temperature ramp of 1°C/min as previously described [18]. The maximum of the first derivative of the absorbance-temperature curve was used to obtain the T_m . Doxorubicin (2 μ M), which increases T_m by 13.2°C [18] and is a strong DNA intercalator [19], was used as a positive control.

The changes in the visible spectrum of kinamycin F upon the addition of small amounts of calf thymus DNA was recorded to determine whether the kinamycin F bound to DNA. The semi-micro spectrophotometer cell contained 40 μ M of kinamycin F and 0.5% DMSO in buffer (10 mM Tris, pH 7.5) at 20°C.

Ethidium bromide displacement assay

The ethidium bromide displacement assay was carried out essentially as described [20]. In a 96-well plate, calf thymus DNA (16 μ g/ml), water and kinamycin F (pH 6.5) were incubated for 5 min in the dark at room temperature, after which time ethidium bromide (1 μ M) was added. The fluorescence of the solutions was immediately measured in a Fluostar Galaxy (BMG, Durham, NC) fluorescence plate reader using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The C_{50} , which is the concentration of the drug which results in a 50% decrease in the fluorescence intensity of the ethidium bromide-DNA complex [20], was determined from a plot of fluorescence vs. kinamycin F concentration.

pBR322 DNA nicking assay

The nicking of double-stranded closed circular plasmid pBR322 DNA by a mixture of kinamycin F and GSH was followed after incubation by separating the reaction products using ethidium bromide gel electrophoresis. The 10 μ l reaction mixture contained 80 ng of pBR322 plasmid DNA, kinamycin F, and 5 mM GSH that had been titrated to pH 7.6. The order of addition was kinamycin F, DNA and then GSH. The reaction mixture was incubated at 37°C for 5 h after which time loading buffer (10 mM Tris pH 8, 60% sucrose, and 0.5% bromophenol blue) was added. Either deferoxamine (100 μ M; to complex adventitious iron), or DMSO (0.5% (v/v); to trap hydroxyl radicals) was pre-incubated 30 min at 37°C with the DNA and the water used to make it up to volume. In the experiments in which catalase (50 μ g/ml) was used to test for H₂O₂-dependent DNA nicking, either untreated active catalase or catalase that had been boiled for 5 min to inactivate it, was added immediately prior to drug addition. The test solutions were added to the kinamycin F which had been deposited at the bottom of the reaction tube from a methanol stock solution and then dried. GSH (5 mM) was then added to start the reaction. The nicked pBR322 DNA was separated by electrophoresis (2 h at 8 V/cm) on a Tris-acetate-EDTA agarose gel (1.2%, wt/v) containing ethidium bromide (0.5 μ g/ml). The linear pBR322 DNA control was prepared enzymatically as described previously [21]. The DNA in the gel was imaged by its fluorescence on an Alpha Innotech (San Leandro, CA) Fluorochem 8900 imaging system equipped with a 365 nm UV illuminator and a CCD camera.

Fast Micromethod DNA Single-Strand-Break Assay

The Fast Micromethod DNA Single-Strand-Break assay, which is based upon the Fluorometric Analysis of DNA Unwinding assay (FADU assay), in which damaged DNA with alkaline-labile sites or single strand breaks undergoes an increased rate of unwinding under alkaline conditions, was performed essentially as described [22]. The effect of increasing or lowering cellular GSH levels on kinamycin F-induced DNA strand breaks was also determined. In these parallel experiments K562 cells were pre-incubated with either OTC (5 mM) or BSO (100 μ M) for either 24 or 48 h, respectively. The cells were centrifuged at 1000 g for 6 min and washed twice with HBSS containing either OTC (5 mM) or BSO (100 μ M) prior to the addition of kinamycin F. Exponentially growing cells (35,000) were incubated at 37°C for 2 h in HBSS supplemented with 1.25 mM CaCl_2 , 0.81 mM MgSO_4 , and 4.17 mM NaHCO_3 (pH 7.4) in the absence or presence of kinamycin F. Control cells were treated with an equal volume of the vehicle (2% (v/v) DMSO). Following incubation, cells were centrifuged at 1000 g for 6 min and the supernatant was discarded. Cells were resuspended in 125 μ l Tris-EDTA buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and 25 μ l of the suspension (280 cells/ μ l) was placed into 4 wells of a 96-well plate. A set of blanks containing 25 μ l of Tris-EDTA buffer were also analyzed in the same manner. Lysing buffer (25 μ l of 9 M urea, 0.2 M EDTA, 0.1% SDS, (pH 10.0)) supplemented with PicoGreen (Invitrogen) (20 μ l of original stock dye/ml of lysing solution)) was gently added to each well. Lysing occurred in the dark at room temperature for 1 h. To initiate DNA unwinding, 250 μ l of working 0.1 M NaOH solution was added to give a final pH of 12.4. Measurements were immediately started in the fluorescence plate reader using an excitation wavelength of 485 nm and an emission wavelength of 520 nm at 25°C and continued for 40 min. All values were corrected with blank readings on wells that only contained the buffer.

Fluorometric assay of DCF oxidation

DCFH-DA is a non-fluorescent compound that, when taken up by cells, is hydrolyzed by esterases to yield non-permeable DCFH [23]. Cellular or exogenous oxidants are then able to oxidize reduced DCFH to the fluorescent DCF. 3-Amino-1,2,4-triazole (AT) can be used to inhibit cellular catalase to measure H_2O_2 -dependent oxidation of DCF [24]. In the present study, K562 cells were first incubated with either AT (20 mM) or an appropriate amount of HBSS in complete media for 30 min at 37°C. The cells were centrifuged (1000 g, 6 min) and rinsed twice in either HBSS or HBSS containing AT (20 mM). Cells were resuspended in HBSS, with or without AT, and incubated with 50 μ M of DCFH-DA for 20 min at 37°C. Cells were washed twice (10 min incubation with each wash) in either HBSS or HBSS containing AT (20 mM). Following washes, cells were allowed to rest for 20 min at 37°C in HBSS or HBSS containing AT (20 mM). Cells (50,000) were placed into each well of a 96-well plate and the volume was adjusted to 50 μ l with HBSS or HBSS containing AT (20 mM). The fluorescence was then recorded on a fluorescence plate reader for 10 min to obtain baseline data using an excitation wavelength of 485 nm and an emission wavelength of 520 nm at 30°C, after which, either the positive control H_2O_2 (200 μ M) or kinamycin F (5 μ M) was added.

Topoisomerase II α kDNA decatenation inhibition assay

The ability of kinamycin F to inhibit topoisomerase II α was determined via a spectrofluorometric decatenation assay as we have described [13]. Topoisomerase II α is able to decatenate the highly knotted circular kDNA resulting in smaller circles of DNA in an ATP-dependent reaction. Each 20 μ l reaction contained 0.5 mM ATP, 50 mM Tris (pH 8.0), 120 mM KCl, 10 mM MgCl_2 , 30 μ g/ml bovine serum albumin, 40 ng kDNA, kinamycin F (0.5 μ l in DMSO), 15 ng of topoisomerase II α protein (the amount that gave approximately 80% decatenation) and 0.12 μ M DTT. Full length human topoisomerase II α was obtained as previously described [21]. Control reactions containing 2.5% (v/v) DMSO were shown not to

affect the activity of topoisomerase II α . The reactions were incubated at 37°C for 20 min, after which time the reactions were terminated by the addition of 12 μ l of 250 mM Na₂EDTA. Samples were centrifuged at 8000 g, 25°C for 15 min, and 20 μ l of supernatant was mixed with 180 μ l of 600-fold diluted PicoGreen dye in a 96-well plate. Fluorescence, which was proportional to the amount of kDNA present, was measured in a fluorescence plate reader using an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Electron paramagnetic resonance spectroscopy

A freshly prepared 15 μ l aliquot of kinamycin F in the reaction systems indicated was injected into an 8-cm length of gas-permeable Teflon tubing (Zeus Industrial Products, Raritan, NJ) which was then folded at both ends and inserted into a quartz EPR tube open at both ends, and placed in the EPR cavity as described [25]. The EPR spectra were recorded with a Bruker (Milton, Canada) EMX EPR spectrometer. Pre-purified grade thermostated (25°C) air or argon (400 l/h) was flowed continuously over the sample while the spectra were recorded. Recording of the first derivative EPR spectra was started approximately 2 min after the sample was prepared. A total of 20 spectra (42 s/scan) were recorded over 14 min and their signals were averaged. The instrument settings were microwave power 20 mW, modulation frequency 100 kHz, microwave frequency 9.25 GHz, modulation amplitude 0.5 or 2.0 G as indicated, time constant 0.02 s, 1024 data points/scan and a 10 or 50 G scan range as indicated. The kinamycin F (0.5 mM) was dissolved in DMSO, which was present in the final reaction mixture at 5% (v/v). For the GSH treatment the final GSH concentration was 2 mM (pH 7.6). For the HRP/H₂O₂ experiments the HRP concentration was 100 μ g/ml and the H₂O₂ concentration was 25 μ M.

Computational methodologies for the docking of kinamycin F in an x-ray crystal structure of DNA

All molecular modeling was done using SYBYL 7.2 on a Hewlett-Packard XW4100 PC workstation with a Redhat Enterprise 3 Linux operating system. All molecules except the DNA were built using SYBYL. Kinamycin F was docked into the doxorubicin binding site of a 6 base-pair x-ray crystal structure of two molecules of doxorubicin bound to double-stranded DNA, d(TGGCCA)/doxorubicin (<http://www.rcsb.org/pdb/>; PDB ID: 1DA9) [26], using the genetic algorithm docking program GOLD version 3.1 [27] and the default GOLD parameters [28] as we have described [18]. GOLDScore was used as the fitness function with flipping options of amide bonds, planar and pyramidal nitrogens, and internal hydrogen bonds being allowed. No early termination was allowed. The 1DA9 x-ray structure shows that the first and second base pairs buckle out to accommodate bound doxorubicin [26]. Thus, the 1DA9 X-ray structure of the doxorubicin-DNA complex was used for the docking experiments, rather than constructing DNA in SYBYL, because it was reasoned that this DNA structure would be a more realistic model for the binding of kinamycin F because of its structural similarity to doxorubicin. Kinamycin F was first geometry optimized with the Tripos force field using a conjugate gradient with a convergence criterion of 0.01 kcal/mol and Gasteiger-Huckel charges and a distance-dependent dielectric constant. Two conformational isomers of kinamycin F that differed in their D-ring conformation were separately docked: one had an axial 3-methyl conformation, and the other had an equatorial 3-methyl conformation. The DNA structure was prepared by removing one of the bound doxorubicin molecules and removing all of the water molecules to avoid potential interference with the docking. Hydrogens were added to the DNA, and the SYBYL Biopolymer module was used to add Kollman-All charges to the DNA. Using atom H341 which is located around the middle of the DNA structure as the reference atom, the binding site was defined as being within 20 Å of the reference atom. Kinamycin F minimized structures were docked into the DNA structure to obtain the top 20 scoring GOLDScore structures for each conformer. As a test of the docking procedure, doxorubicin was docked back into the DNA structure with a heavy-atom root-mean-squared distance of 1.7

Å compared to the X-ray structure [26]. Values of 2.0 Å or less in the extensive GOLD test set are considered to be in good agreement [28].

Results

Kinamycin F reacts with GSH and DTT

It is well known that both diazonium groups [29] and quinones [30] react with thiol compounds. Therefore, the reaction of kinamycin F with both GSH and DTT was examined spectrophotometrically. The reaction with GSH (Fig. 2A) was spectrally complex. The reaction of kinamycin F with GSH was characterized by a small fast ($t_{1/2} < 1$ min) initial drop in absorbance at 464 nm (Fig. 2B), which was then followed by much slower increases in absorbance in both the UV and visible regions (Fig. 2A and 2B). While maximal changes in absorbance at 464 nm and 420 nm occurred 5 h after addition of GSH, at 330 nm a larger slow increase in absorbance was initially observed which was followed by a subsequent slow decrease in absorbance over the next 10 h. The shape of the absorbance-time plots and the lack of a single isosbestic point in Fig. 2A indicates that reaction with GSH produces more than one product in a complex series of reactions. The reaction with DTT (Fig. 2C) was spectrally less complex than with GSH (Fig. 2A). As shown by the spectral changes in Fig. 2C and 2D, kinamycin F reacted quickly ($t_{1/2} \sim 4.5$ min; calculated from a fit of the 339 nm data to a 3-parameter first-order exponential decay function) with DTT (pH 7.6). After this initial fast reaction the absorbance values remained nearly constant for the next 4 h (Fig. 2D).

Kinamycin F binds to DNA

Because kinamycin F has a planar structure like doxorubicin which intercalates into DNA [19,26], its binding to DNA was investigated. The ability of kinamycin F to bind to DNA was studied by measuring absorbance changes upon the addition of calf thymus DNA. Free kinamycin F has an absorbance maximum at 430 nm (Fig. 2A dashed line). As shown in Fig. 3A increasing concentrations (> 2 mM, base-pair concentration) of calf thymus DNA to kinamycin F (40 μ M) caused a decrease in absorbance at the peak maximum of 430 nm. This result and the shift in the peak maximum from 430 to 454 nm (data not shown) indicated kinamycin F weakly bound to DNA.

In separate experiments kinamycin F-induced changes in DNA melting temperature T_m were studied. While the changes were relatively small, suggesting weak binding, kinamycin F (20 μ M) increased the T_m of DNA from 68°C to 71°C. This value can be compared to our previously determined value for 2 μ M doxorubicin, which increases T_m by 13.2°C [18].

In order to further confirm that kinamycin F bound to DNA, an ethidium bromide displacement assay was used [20]. Ethidium bromide fluoresces strongly when bound to DNA, but only weakly fluoresces when free, and thus the displacement of ethidium bromide by kinamycin F was measured fluorometrically. As shown in Fig. 3B, kinamycin F displaced ethidium bromide from DNA and caused a 50% reduction in fluorescence at a concentration of 105 μ M (C_{50}). Using the formula $K_{app} = K_{EtBr}C_{EtBr}/C_{50}$ [20], the association constant for kinamycin F binding to DNA, K_{app} , was calculated to be $9 \times 10^4 \text{ M}^{-1}$. This is relatively weak binding as it is some 300-fold weaker than the value of $29.6 \times 10^6 \text{ M}^{-1}$ for the well known DNA intercalator doxorubicin [19]. The K_{app} value was calculated from the concentration of ethidium bromide (C_{EtBr} of 1 μ M) used in the assay; the concentration of kinamycin F that reduced the fluorescence of ethidium bromide by 50% (C_{50} of 105 μ M); and an association constant of ethidium bromide (K_{EtBr}) of $9.5 \times 10^6 \text{ M}^{-1}$ [20].

The molecular modeling docking program GOLD was used to determine whether kinamycin F could be docked into a doxorubicin-DNA x-ray structure [26], and how it interacted with

DNA. The structure of the equatorial conformation of kinamycin F docked into DNA is shown in Fig. 4. While both equatorial and axial conformations of kinamycin F docked well, they differed in their binding through an approximate 180° rotation about their long axis. As shown in Fig. 4 the interaction of equatorial kinamycin F with DNA is stabilized through hydrogen binding of the hydroxyl groups on C3 and C4 of the 6-membered D ring (Fig. 1) to DNA.

GSH promotes kinamycin F-induced nicking of pBR322 DNA

Because two other studies [6,10] indicated that the diazoparaquinones lomaiviticin A and kinamycin D may be able to cleave DNA under reducing conditions, it was decided to investigate whether kinamycin F could cleave DNA under much more physiological conditions. As shown in Fig. 5A, kinamycin F alone induced a small amount of nicking of pBR322 plasmid DNA after 5 h at 37°C, even in the absence of GSH (1.2- and 1.4-fold increase at 200 and 400 µM kinamycin F, respectively). DNA damage was greatly enhanced in the presence of physiologically relevant levels of GSH (5 mM) with 1.1-, 1.3-, 1.9-, 3.2-, 5.5-fold increases in nicked DNA observed at kinamycin F concentrations of 10, 50, 100, 200, 400 µM kinamycin F, respectively. At 400 µM kinamycin F in the presence of GSH, where the degree of nicking was very high, a small amount of linear DNA was observed (8.5% of the total)). This linear DNA produced from DNA double strand breaks, which appeared only at the highest kinamycin F concentration, likely occurred through an accumulation of a large number of DNA single strand breaks rather than through kinamycin F causing double strand breaks directly. To elucidate the mechanism by which kinamycin F induced nicking in DNA, experiments were carried out in which either DMSO, catalase or deferoxamine were added prior to the addition of either kinamycin F or GSH (Fig. 5B and 5C). Under these experimental conditions, kinamycin F-induced nicking was reduced from 3.2-fold over control when used alone to 1.0-, 0.9-, 1.2-fold over control with DMSO, catalase or deferoxamine pretreatments, respectively. Boiling catalase eliminated its ability to inhibit kinamycin F-induced DNA damage (Fig. 5C).

Kinamycin F promotes DNA single strand breaks in K562 cells

Having determined that the kinamycin F/GSH system could nick pBR322 DNA in vitro, it was investigated whether kinamycin F could also damage DNA in cells. To this end, the Fast Micromethod DNA Single Strand Break assay was used, which determines the frequency of single-strand breaks and alkaline-labile sites [22]. The unwinding of DNA strands under basic conditions was followed for 20 min, at which time the fluorescence of the remaining double stranded DNA was measured. As shown in Fig. 6 treatment of K562 cells for 2 h with kinamycin F resulted in a concentration-dependent reduction in remaining double-stranded DNA that achieved significance ($p < 0.05$; range 0.001 to 0.037) at all kinamycin F concentrations compared to cells not treated with kinamycin F.

To determine whether GSH levels were a determinant of kinamycin F-induced DNA strand breaks, parallel experiments were carried out in which cell GSH levels were increased (by OTC) or decreased (by BSO). OTC is a non-thiol precursor of cysteine which is converted by intracellular 5-oxoprolinase to cysteine, a precursor for the biosynthesis of GSH [31]. GSH levels in K562 cells were increased by preincubation with 5 mM OTC for 24 h. GSH levels were decreased in K562 cells by a 48 h preincubation with 100 µM BSO, an inhibitor of the rate limiting GSH-synthesizing enzyme γ -glutamylcysteine synthetase [31]. As determined using a fluorescent Thioglo-1 thiol assay we previously described [32], cellular levels of GSH levels in K562 cells were either increased by 87%, or reduced by 71% following OTC or BSO treatment, respectively. Because the Thioglo-1 assay measures thiols and is not specific for GSH, the OTC-induced increase in thiol levels likely reflects an increase in both cysteine and GSH levels. As shown in Fig. 6 neither OTC nor BSO treatment had any statistically significant

effect on kinamycin F-induced DNA strand breaks from 5 to 100 μM kinamycin F, compared to their respective kinamycin F-treated controls.

Kinamycin F inhibits topoisomerase II catalytic activity but does not act as a topoisomerase II poison

We previously showed that kinamycin A and kinamycin C inhibited the activity of topoisomerase II α [13]. As shown in Fig. 7A, kinamycin F inhibited the decatenation activity of human topoisomerase II α with an IC_{50} of $0.85 \pm 0.26 \mu\text{M}$ (Fig. 7A), which is 9- and 11-fold more potent than either kinamycin A or kinamycin C, respectively [13]. To determine whether kinamycin F acts as a topoisomerase II poison, a comparison of the growth inhibitory effects of kinamycin F on K562 and the K/VP.5 cell lines was used. The K/VP.5 cell line contains one-fifth the amount of topoisomerase II α compared to the parent K562 cell line [15,16], and can be a convenient test of whether a compound is a topoisomerase II poison [21]. Fewer DNA strand breaks will be produced in cells containing less topoisomerase II and thus, topoisomerase II poisons will be less potent towards the K/VP.5 cell line. As shown in Fig. 7B, the IC_{50} for growth inhibition of K562 cells was $0.33 \pm 0.02 \mu\text{M}$ while that of K/VP.5 cells was $0.42 \pm 0.10 \mu\text{M}$. Thus, the lack of cross resistance indicates that, while kinamycin F (like kinamycin A and kinamycin C [21]) can inhibit the catalytic activity of topoisomerase II α , it likely did not act as a topoisomerase II poison in a cellular context.

Reducing intracellular GSH levels increases kinamycin F cytotoxicity and increasing GSH levels decreases cytotoxicity

Because the results of Fig. 2 indicated that kinamycin F reacted with GSH, we investigated whether supplementation or depletion of intracellular GSH levels affected the cytotoxicity of kinamycin F. As before, to increase GSH levels K562 cells were preincubated with 5 mM OTC for 24 h and to lower GSH levels, K562 cells were preincubated with 100 μM BSO for 48 h. As shown by the data in Fig. 8 these concentrations of OTC or BSO did not cause any measurable cytotoxicity. This pretreatment was followed by a 72 h-exposure to various concentrations of kinamycin F, after which cell growth was measured with the MTS assay. As shown in Fig. 8, kinamycin F inhibited cell growth with an IC_{50} value of 1.7 μM for untreated cells. For the OTC- and BSO-treated cells this value was either increased 1.2-fold or reduced 4.3-fold, respectively. A *t*-test of three IC_{50} determinations showed that the small OTC-dependent increase in IC_{50} was barely significant ($p < 0.037$). However, the BSO-dependent decrease in IC_{50} was highly significant ($p < 0.001$). Taken together these results suggest that GSH partially protected these cells from the growth inhibitory effects of kinamycin F.

Kinamycin F induces oxidation of DCFH to DCF

In order to investigate whether kinamycin F directly or indirectly produced oxidative stress in K562 cells, the oxidation of DCFH-DA loaded into K562 cells was followed in a fluorescence plate reader. As shown in Fig. 9, treatment of K562 cells with kinamycin F (5 μM) significantly increased the rate of oxidation to DCF to nearly that produced by 200 μM H_2O_2 . In order to determine if this oxidation was H_2O_2 -dependent, experiments were also done in which cells were pretreated with the catalase inhibitor AT. While, as expected, AT treatment significantly increased the rate of oxidation to DCF of H_2O_2 -treated cells, there was no significant change in the rate of oxidation to DCF of kinamycin F-treated cells. This result indicates that kinamycin F did not measurably oxidize DCFH through an H_2O_2 -dependent pathway.

While DCFH oxidation to fluorescent DCF is often used as a measure of reactive oxygen and nitrogen species production, it has also been shown that some drugs can directly oxidize DCFH [23]. In order to determine if kinamycin F could directly oxidize DCF in vitro, it was determined that 20 μM kinamycin F in 20 mM Tris buffer (pH 8.0) increased the rate of oxidation of 0.5 μM DCFH (prepared from the DCFH-DA ester by base hydrolysis as described [33]) by 4.1-

fold in the presence of 100 μ M deferoxamine (to chelate trace iron in the buffer), and 5.1-fold in its absence (data not shown). This data indicates that kinamycin F oxidized DCFH through a direct reaction and, thus, it may have also increased the rate of oxidation to DCF through this mechanism in cells.

EPR spectroscopy of GSH- and HRP/H₂O₂-induced kinamycin F free radicals

Various reductants are well known to be able to form a semiquinone free radical through a one-electron reduction of quinones and then redox cycle to produce damaging ROS [30]. Because we showed in Fig. 5C that kinamycin F reacted rapidly with GSH, the ability of GSH to react with kinamycin F to produce a free radical was examined by EPR spectroscopy. As shown in Fig. 10A, kinamycin F reacted with GSH to produce an air-stable free radical EPR signal at $g \sim 2.0$ which was likely a semiquinone free radical. Upon making the reaction mixture hypoxic with argon the EPR signal quickly disappeared. Control experiments showed that kinamycin F alone did not produce an EPR free radical signal.

Kinamycin F also contains a phenol group that has the potential to be oxidized to a phenoxyl radical. Thus, we also investigated whether it could be oxidized by the HRP/H₂O₂ system to produce a phenoxyl radical as we have shown for the myeloperoxidase/H₂O₂ reaction with etoposide [34]. As shown in Fig. 10B the HRP/H₂O₂ system produced an EPR signal probably due to a phenoxyl radical with fine structure that likely arose from splitting due to Aring H-atoms (Fig. 1). The EPR signal slowly increased with time over 40 min, unlike the radical produced with the reaction with GSH, which slowly decreased with time. Control experiments showed that H₂O₂ and kinamycin F alone did not produce an EPR free radical signal. The free radical seen from the treatment of kinamycin F with HRP/H₂O₂ was different from that produced by treatment with GSH, because when the modulation amplitude in the GSH experiment was decreased from 2.0 G to 0.5 G no detectable fine structure in its EPR spectrum was observed (data not shown), unlike that seen in Fig. 10B.

Discussion

Kinamycin F contains a diazo, a paraquinone, and a phenolic functional group, all of which may contribute to its cytotoxicity either directly or after activation. Kinamycin F, the deacetylated form of kinamycin A and kinamycin C that we previously studied [13], had an *IC*₅₀-value for 72-h growth inhibition in K562 cells of 0.33 μ M (Fig. 7B), a value which was close to that found for kinamycin A and kinamycin C of 0.31 and 0.37 μ M, respectively [13]. We speculated at that time that the delayed entry of synchronized CHO cells into a G₁/S block may have been due to metabolic deacetylation of kinamycin A and kinamycin C to kinamycin F by intracellular esterases. The fact that kinamycin F was equally cytotoxic as kinamycin A and kinamycin C is consistent with this hypothesis.

We also previously showed that kinamycin A and kinamycin C both strongly inhibited the decatenation activity of human topoisomerase II α . Kinamycin F, with an *IC*₅₀ of 0.85 μ M (Fig. 7A) for inhibition of topoisomerase II α catalytic activity was an even more potent (9- and 11-fold, respectively) inhibitor than either kinamycin A or kinamycin C [13]. However, like kinamycin A and kinamycin C, the lack of kinamycin F cross resistance towards the K/VP.5 cell line (Fig. 7B), which contains one-fifth the amount of topoisomerase II α compared to the parent K562 cell line [15,16], suggested that kinamycin F, like kinamycin A and kinamycin C [13], did not function as a topoisomerase II poison in cells. We and others have shown that topoisomerase II α is highly sensitive to sulfhydryl-reactive drugs such as quinones [35,36] and cisplatin [21]. Similarly, kinamycin F may have inhibited topoisomerase II α by reacting with critical sulfhydryl groups on the protein. In support of this idea, we previously showed that the topoisomerase II α inhibitory effects of both kinamycin A and kinamycin C were attenuated by

pretreatment of these kinamycins with DTT [13]. Together these results suggest that kinamycin F did not exert its cytotoxicity primarily through the inhibition of topoisomerase II.

In Fig. 2 we showed spectrophotometrically that kinamycin F reacted with physiological levels of GSH and with DTT in a complex series of reactions indicating multiple products. While it is possible that some of these spectral changes may have been due to processes that did not involve bond making or breaking, the EPR results of Fig. 10 supports the conclusion that reduction of kinamycin F is occurring. Kinamycin F is a tetrasubstituted quinone. Substituted quinones are often relatively unreactive in nucleophilic Michael addition reactions due to their fully substituted vinylogous positions [37]. However, the tetra-substituted quinone mitomycin A, in its activation to a reactive electrophile, has been postulated to react with monothiol to produce multiple products, primarily through a reductive mechanism characterized by a transient addition of the thiol to mitomycin A in a Michael addition reaction [37]. Unlike the quinone system in the mitomycins, however, the kinamycin quinone system (Fig. 1) is fused on one side to a benzenoid aromatic ring (ring A) and on the other to a five-membered ring (ring C). Ring C would have aromatic character by virtue of a cyclopentadienyl anion contribution to the diazonium ion-like resonance structure. As such this system should be relatively resistant towards nucleophilic conjugate nucleophilic addition at carbon in the quinone.

It was previously reported that lomaiviticin A [6] and kinamycin D [10] could cleave DNA under reducing conditions. However, in the lomaiviticin A report [6] the conditions were not given. In the kinamycin D report [10] the incubation that yielded 27% nicked DNA was carried out for a long time (2 d) and at high kinamycin D (1 mM) and DTT concentrations (1 M). These conditions are very different from those used to generate the results shown in Fig. 5. As the results of Fig. 5A show, kinamycin F (> 200 μ M), even in the absence of GSH, induced a small amount of nicking of supercoiled pBR322 plasmid DNA after 5 h at 37°C, a result that suggests a direct reaction with DNA. Nicking of DNA (Fig. 5A) was greatly enhanced in the presence of cellular levels (5 mM) of GSH (1.9-fold the background level at a kinamycin F concentration of 100 μ M).

Deferoxamine, an iron chelator; DMSO, a scavenger of hydroxyl radicals; and catalase, an enzyme that decomposes H_2O_2 , all strongly inhibited kinamycin F/GSH-induced nicking (Fig. 5B and 5C) suggesting that kinamycin F, in the presence of GSH, nicks DNA in an iron-, H_2O_2 -, and hydroxyl radical-dependent manner. Hence, reductively activated (not necessarily by GSH) kinamycin F-induced ROS production may be, in part, responsible for DNA damage. Kinamycin F may not be directly producing hydroxyl radicals in a Fenton-type reaction but may be damaging DNA by high-valent iron-oxo species with hydroxyl radical-like reactivity [38]. It should be noted that the DNA damage observed in vitro (Fig. 5B and 5C) may not necessarily be occurring in the cell. The Fast Micromethod DNA Single Strand Break assay, which determines the frequency of single-strand breaks and alkaline-labile sites [22], showed that all concentrations of μ M kinamycin F (5 to 100 μ M) in a 2 h incubation also had the ability to significantly damage the DNA in K562 cells (Fig. 6). Supplementation of GSH levels with OTC treatment or depletion of GSH levels with BSO treatment had no significant effect on kinamycin F-induced cellular DNA damage. This result suggests that at the low micromolar concentrations at which kinamycin F is cytotoxic, GSH is neither activating or deactivating kinamycin F. These results would be consistent with the slow spectral changes seen in its reaction with GSH in Fig. 2B.

Kinamycin F-induced damage to DNA through production of ROS or reductively activated kinamycin F species would be greatly enhanced if kinamycin F or its activated intermediates were able to bind to DNA. The planar structure of kinamycin F is similar to doxorubicin which is a strong DNA intercalator [19,26]. Because millimolar concentrations of DNA in the

spectrophotometric titration (Fig. 3A) were required to cause absorbance changes in kinamycin F (40 μ M), this result indicates that kinamycin F binds only weakly to DNA. The small increase in the DNA melting temperature T_m effected by kinamycin F (20 μ M) likewise indicated only weak binding to DNA. The K_{app} value for binding of kinamycin F to DNA determined in the ethidium bromide displacement assay (Fig. 3B) was about 300-fold less than the value for doxorubicin of $29.6 \times 10^6 \text{ M}^{-1}$ and close to that for adriamycinone, the aglycone of doxorubicin without its amino sugar group [19]. However, a high degree of binding is not necessary to cause site-specific DNA damage if ROS or reductively activated kinamycin F species are produced while directly bound to DNA. Also kinamycin F-induced damage to DNA may be occurring in the absence of its binding to DNA. The results of the molecular modeling docking experiments support the conclusion that kinamycin F can intercalate into DNA (Fig. 4).

It was also shown that kinamycin F was able to strongly promote the oxidation of DCFH to fluorescent DCF in K562 cells (Fig. 9), which indicates that kinamycin F may be producing ROS. The kinamycin F-induced oxidation of DCFH in K562 cells was not H_2O_2 -dependent as the catalase inhibitor AT did not significantly change the rate of DCFH oxidation. Because in cell-free experiments kinamycin F was able to directly oxidize DCFH, it was not possible to determine if the kinamycin F-induced oxidation of DCFH in K562 cells observed was due to direct oxidation of DCFH or due to formation of some other activated kinamycin F-derived oxidizing species. It has been shown previously that other drugs can directly oxidize DCFH [23] which confounds the use of this widely used indicator of cellular oxidative stress. The lack of H_2O_2 dependence on kinamycin F-induced DCFH oxidation in K562 cells (Fig. 9) does not rule out H_2O_2 as a damaging cytotoxic species as H_2O_2 -dependent oxidation of DCFH may still be occurring, but at levels that are obscured by the direct oxidation of DCFH by kinamycin F.

EPR spectroscopy showed that kinamycin F reacted with GSH to produce an air-stable free radical EPR signal that was likely the kinamycin F semiquinone free radical (Fig. 10A). Because the EPR signal quickly disappeared when the reaction mixture was made hypoxic, this indicated that the excess GSH was able to directly reduce the kinamycin F semiquinone to the EPR-silent kinamycin F hydroquinone. The free radical semiquinone signal was seen under oxic conditions because the quinone Q and the hydroquinone QH_2 likely underwent an equilibrium disproportionation reaction ($\text{Q} + \text{QH}_2 \rightleftharpoons 2\text{Q}^{\bullet-} + 2\text{H}^+$) to yield low steady-state levels of the semiquinone $\text{Q}^{\bullet-}$ [39,40]. Semiquinones react very quickly with oxygen to produce the superoxide radical anion $\text{O}_2^{\bullet-}$, which in turn dismutates to produce H_2O_2 . Thus, the EPR results suggest that kinamycin F may, in part, also be growth inhibitory through the production of ROS. Enzymatic peroxidative activation of kinamycin F is another potential mechanism by which kinamycin F may exert its cytotoxicity as we also showed by EPR spectroscopy that the HRP/ H_2O_2 system produced what was likely a phenoxyl free radical (Fig. 10B). It should be noted that just because kinamycin F free radical species were seen in the EPR studies (Fig. 10) does not necessarily mean that ROS species are involved in the cytotoxicity of kinamycin F. We have also previously shown that the myeloperoxidase/ H_2O_2 system reacts with the anticancer agent etoposide to form an etoposide phenoxyl radical [34] that may oxidize and damage cellular thiols through H-atom abstraction [41]. A possible role for oxidative diazo activation has also been suggested for the diazo kinamycin F analog 9-diazo fluorene as it can promote DNA nicking in the presence of cupric acetate oxidant, possibly acting through a carbon-centered radical intermediate [11].

While the experiments carried out in this study showed that kinamycin F can be reductively activated by cellular levels of GSH to produce reactive radical species and can also be peroxidatively activated by a peroxidase/ H_2O_2 system, these results did not provide direct evidence that the diazo group of kinamycin F is involved in its cytotoxicity. While it has been shown that the kinamycin analog prekinamycin forms radical-trapping arene products that

involved the loss of dinitrogen in organic aromatic solvents containing a radical initiator and an organic tin reducing agent [8,9], the non-physiological conditions of these studies make direct comparisons with our study problematic.

At first glance, the data in Fig. 5 indicating that DNA nicking of pBR322 DNA in vitro was promoted by GSH would seem not to be consistent with the cytotoxicity data of Fig. 8 which showed that supplementation of GSH levels increased the IC_{50} value, while GSH depletion decreased the IC_{50} value. However, the nicking results of Fig. 5 show only that kinamycin F can be reductively activated by GSH to nick DNA, not that it is reductively activated by GSH in cells. In fact the cytotoxicity assay results of Fig. 8, where cellular GSH levels were either increased and decreased, suggest that GSH partially deactivated kinamycin F to inert species in the cytosol before it entered the nucleus and caused DNA damage. A reductase enzyme or some other reducing species may be responsible the activation of kinamycin F to a DNA damaging species. The amount of kinamycin F-induced single strand DNA damage in K562 cells (Fig. 6) was independent of GSH supplementation or depletion at all concentrations. This result also suggests no significant role for GSH in causing DNA damage. However, it should be noted that the strand break assay we used only measures significant levels of DNA damage, and thus may not detect minor, but important, changes in kinamycin F-induced DNA damage in the presence of OTC or BSO. The richness of the functionality in the kinamycins allows for a variety of hypothetical arrow-pushing mechanisms to be considered in predicting possible molecular mechanisms for bioactivation. Some of these potential mechanisms, perhaps involving loss of N_2 , lead to the creation of species which are potential more electrophilic than the natural product. It is clear that more study is needed to provide the definitive experimental evidence to fully understand the behavior of the kinamycins in a cellular context. The fact that kinamycin F bound to DNA in vitro and was able to damage DNA in cells would also implicate DNA as the biological target of kinamycin F. It is, however, not possible to rule out that kinamycin F has some other target or targets that contain critical protein sulfhydryl groups or some other nucleophilic protein group.

Acknowledgments

This work was supported by the Province of Manitoba, through the Manitoba Research and Innovation Fund, the Canadian Institutes of Health Research, the Canada Research Chairs Program and a Canada Research Chair in Drug Development for B.B.H.; by a grant to J.C.Y. from the NIH (grant CA90787); by a Discovery Grant to G.I.D. from the Natural Sciences and Engineering Research Council of Canada; and by a Manitoba Health Research Council Fellowship for K.A.O.

List of Abbreviations

AT	3-amino-1,2,4-triazole
BSO	buthionine sulfoximine
DCF	dichlorofluorescein, oxidized form
DCFH	2',7'-dichlorodihydrofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DMSO	dimethyl sulfoxide

EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance
GSH	glutathione
DTT	dithiothreitol
H₂O₂	hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HRP	horseradish peroxidase
IC₅₀	50% inhibitory concentration
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
OTC	(-)-2-oxo-4-thiazolidinecarboxylic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SE	standard error of the mean
T_m	DNA melting temperature

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Fig. 1.
Structure of kinamycin F.

Fig. 2.

Kinamycin F reacts with the thiols GSH and DTT. (A) The kinamycin spectrum was recorded prior to addition of 5 mM GSH (0 h, dashed line) and the spectral changes occurring in the 20 μ M kinamycin F solution are plotted at 1 h (dotted line), 2, 3, 4 h (solid lines) and 5 h (thick line) after the addition of 5 mM GSH. (B) Absorbance-time traces at 330, 420, and 464 nm obtained from the complete spectral data are plotted. (C) The kinamycin spectrum was recorded prior to addition of 40 μ M DTT (0 h, dashed line) and the spectral changes occurring in the 20 μ M kinamycin F solution are plotted 3 (solid line) and 9 (thick line) min after the addition of 40 μ M DTT. (D) Absorbance-time traces at 339, 456, and 559 nm obtained from the complete spectral data are plotted. The reactions were carried out in 20 mM Tris buffer (pH 7.6) at 37° C.

Fig. 3.

Kinamycin F binds to DNA as shown by absorbance changes upon the addition of DNA and by the fluorescence ethidium bromide displacement assay. (A) Changes in absorbance of kinamycin F (40 μM) at 430 nm were measured upon the addition of small volumes of calf thymus DNA. The assay mixture contained 10 mM Tris (pH 7.5) at 20°C. (B) Changes in the fluorescence at 590 nm of ethidium bromide bound to calf thymus DNA are plotted as a function of the kinamycin F concentration. The assay mixture contained calf thymus DNA (16 $\mu\text{g/ml}$) and ethidium bromide (1 μM) at a temperature of 25°C. The solid curved lines are from spline and quadratic fits, respectively, to the data.

Fig. 4.

Kinamycin F docks into DNA. The highest scoring structure of kinamycin F in the equatorial conformation (ball-and-stick structure) is shown docked into DNA (stick structure). The H-atoms are not shown for clarity. The complex forms both base stacking and H-bonding interactions (green dotted lines) with hydroxyl groups on C3 and C4 of the 6-membered D ring. The DNA structure is a doxorubicin/DNA x-ray structure (1DA9 from the Protein Data Bank) in which two doxorubicin molecules are bound to a 6-base pair piece of DNA. Only the first 3 base pairs of the DNA are shown in the figure for clarity. One doxorubicin was removed and kinamycin F was docked into its place with the genetic algorithm docking program GOLD.

Fig. 5.

GSH potentiates kinamycin F-induced DNA nicking. (A) This fluorescent image of the ethidium bromide-stained gel shows that when GSH (5 mM) was added to reaction mixtures containing various amounts of kinamycin F, the amount of DNA nicking was increased. In the absence of GSH a slight increase in nicking was seen at 400 μ M kinamycin F. (B) In this image it is shown that deferoxamine (100 μ M) and DMSO (0.5% (v/v)) pre-treatment of the DNA reduced the amount of DNA nicking induced by kinamycin F (200 μ M) and GSH (5 mM). (C) In this image it is shown that catalase (50 μ g/ml) decreased the amount of DNA nicking, but boiled catalase (b, 50 μ g/ml) did not. Catalase was added immediately prior to the kinamycin F (200 μ M) and GSH (5 mM). All lanes contained 80 ng of pBR322 plasmid DNA, except for those with linear DNA which contained 80 ng of linear pBR322 plasmid DNA. Linear DNA was present in lane 7 and lanes 4 and 11 in (A) and (B), respectively. NC, nicked circular DNA; LIN, linear DNA; SC, supercoiled DNA. All incubations for the DNA nicking experiments were for 5 h at 37°C.

Fig. 6.

Kinamycin F treatment produces DNA single strand breaks in K562 cells. The amount of DNA damage as a result of exposure to kinamycin F was determined using the Fast Micromethod DNA Single Strand Break assay. Unwinding of DNA in K562 cells was measured from the fluorescence of double-stranded DNA (dsDNA) remaining after exposure to various concentrations of kinamycin F. Error bars were from between four and 24 replicate measurements on experiments conducted on two different days. At all kinamycin F concentrations there was no significant difference between OTC- and the BSO-treated cells and untreated cells. All kinamycin F treatments (either with or without OTC and BSO treatments) were significantly different than the untreated control values (p range 0.037 to < 0.001).

Fig. 7.

Kinamycin F inhibits topoisomerase II α catalytic activity but is not a topoisomerase II poison. (A) Inhibitory effects of kinamycin F on the catalytic decatenation activity of topoisomerase II α . The solid line is a non-linear least squares fit to a 3-parameter logistic equation and yields an IC_{50} of $0.85 \pm 0.26 \mu\text{M}$. (B) Inhibition of growth of K562 ($\circ$) and K/VP.5 ($\bullet$) cells by kinamycin F. Cells were treated with kinamycin F for 72 h prior to the assessment of growth inhibition by an MTS assay. The curved lines are non-linear least squares fits to logistic equations and yield IC_{50} 's of 0.33 ± 0.02 and $0.42 \pm 0.10 \mu\text{M}$, respectively for K562 (solid line)

Fig. 8.

Reduction of intracellular GSH levels in K562 cells increases the potency of kinamycin F and increases in GSH levels decreases its potency. K562 cells were preincubated either without (○), or with 5 mM OTC (Δ), or 100 μM BSO (●) for 24 h or 48 h, respectively, and then treated for 72 h with kinamycin F. The data shown are an average of 3 replicates. Treatment of the cells with BSO performed on a different day yielded similar results. The curved lines are non-linear least squares fits to 4-parameter logistic equations and yield IC_{50} 's of 1.7 ± 0.1 , 2.0 ± 0.10 , and 0.39 ± 0.08 μM respectively for K562 cells not treated with BSO; or treated with OTC; or BSO, respectively. A *t*-test determined that the increase in IC_{50} with OTC treatment was significant ($p = 0.037$) and that the decrease in IC_{50} after BSO treatment was significant ($p < 0.001$). Error bars are standard errors.

Fig. 9.

Kinamycin F increases the oxidation of DCFH to DCF in K562 cells through a non-H₂O₂ dependent pathway. In the H₂O₂-treated cells inhibition of intracellular catalase with the catalase inhibitor AT significantly ($p < 0.001$) increased the rate of DCF production, but did not significantly affect the rate of DCF production by kinamycin F. Kinamycin F treatment in the absence ($p < 0.01$) or presence ($p < 0.05$) of AT significantly increased the rate of DCF production compared to untreated control cells as did the H₂O₂ treatment ($p < 0.001$ for both AT treated and untreated). K562 cells were loaded with DCFH-DA for 20 min with or without prior treatment with AT. After obtaining a fluorescence baseline measurement for 10 min, cells were treated with either 5 μ M kinamycin F or 200 μ M H₂O₂ and the increase in the rate of DCF fluorescence produced was measured. Error bars were from four replicate measurements. Significant differences between treated and untreated samples are designated by ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. Significant differences between treated samples and the corresponding treatment with AT are designated by †††, $p < 0.001$. NS is not significant. The results are averages from 4 wells.

Fig. 10.

EPR spectra of free radicals produced by treatment of kinamycin F with either GSH or HRP/H₂O₂. (A) EPR spectrum under oxic conditions of kinamycin F (0.5 mM) treated with GSH (5 mM). The modulation amplitude was 2 G and the sweep width was 50 G. (B) EPR spectrum under oxic conditions of kinamycin F (0.5 mM) treated with H₂O₂ (25 μ M) and HRP (100 μ g/ml) at pH 6.5. The modulation amplitude was 0.5 G and the sweep width was 10 G. For both spectra a total of 20 spectra were accumulated over 14 min and averaged. The spectra were recorded at 25°C.