Published in final edited form as:

Biochem Pharmacol. 2012 December 15; 84(12): 1617–1626. doi:10.1016/j.bcp.2012.09.023.

The anticancer multi-kinase inhibitor dovitinib also targets topoisomerase I and topoisomerase II

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Abstract

Dovitinib (TKI258/CHIR258) is a multi-kinase inhibitor in phase III development for the treatment of several cancers. Dovitinib is a benzimidazole-quinolinone compound that structurally resembles the bisbenzimidazole minor groove binding dye Hoechst 33258. Dovitinib bound to DNA as shown by its ability to increase the DNA melting temperature and by increases in its fluorescence spectrum that occurred upon the addition of DNA. Molecular modeling studies of the docking of dovitinib into an X-ray structure of a Hoechst 33258-DNA complex showed that dovitinib could reasonably be accommodated in the DNA minor groove. Because DNA binders are often topoisomerase I (EC 5.99.1.2) and topoisomerase II (EC 5.99.1.3) inhibitors, the ability of dovitinib to inhibit these DNA processing enzymes was also investigated. Dovitinib inhibited the catalytic decatenation activity of topoisomerase II α . It also inhibited the DNA-independent ATPase activity of yeast topoisomerase II which suggested that it interacted with the ATP binding site. Using isolated human topoisomerase II α , dovitinib stabilized the enzyme-cleavage complex and acted as a topoisomerase II α poison. Dovitinib was also found to be a cellular topoisomerase II poison in human leukemia K562 cells and induced double-strand DNA breaks in K562 cells as evidenced by increased phosphorylation of H2AX. Finally, dovitinib inhibited the topoisomerase I-catalyzed relaxation of plasmid DNA and acted as a cellular topoisomerase I poison. In conclusion, the cell growth inhibitory activity and the anticancer activity of dovitinib may result not only from its ability to inhibit multiple kinases, but also, in part, from its ability to target topoisomerase I and topoisomerase II.

Keywords

Dovitinib; Anticancer; DNA; Topoisomerase II; Topoisomerase I

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

1. Introduction

Dovitinib (TKI258, CHIR258) is a multi-kinase inhibitor in phase III development for the treatment of renal cell carcinoma, and in phase II development in advanced breast cancer, relapsed multiple myeloma and urothelial cancer. Dovitinib has been shown in kinase profiling studies to strongly bind to many kinases at low nanomolar concentrations [1]. Dovitinib may exert its anticancer effects, in part, through its binding to, and inhibition of, the fibroblast growth factor receptor 3 (FGFR3) kinase as well as a number of other kinases that are members of the RTK superfamily, including the vascular endothelial growth factor receptor (VEGFR); fibroblast growth factor receptor 1 (FGFR1); platelet-derived growth factor receptor type 3; FMS-like tyrosine kinase 3 (FLT3); stem cell factor receptor (c-KIT) and colony-stimulating factor receptor 1 (CSF1R) [2], all of which it strongly inhibits. Dovitinib has also recently been shown to directly inhibit the recombinant SH2-domain-containing phosphatase SHP-1 [3] which may also contribute to its anticancer activity.

Dovitinib was designed to inhibit its target kinases by binding to the ATP binding site. The human genome, which contains approximately 518 kinases, also contains about 2000 nucleotide-dependent enzymes or other proteins [4]. This raises the possibility that kinase inhibitors such as dovitinib that are targeted to the ATP binding site may have off-target binding to enzymes with ATP binding sites such as topoisomerase II α [5]. A new class of topoisomerase II α inhibitors (EC 5.99.1.3) has, in fact, been designed to specifically target the ATP binding site [6]. In a previous study we related the lack of target specificity of dovitinib and 17 other small molecule anticancer kinase inhibitors with their ability to damage cardiac myocytes [7]. In preliminary experiments for this study we tested all 18 of these kinase inhibitors to see if they inhibited the decatenation activity of topoisomerase II α in order to determine if they exhibited off-target inhibition of this enzyme. Among these inhibitors, dovitinib was the most potent and was thus chosen for further study.

Dovitinib is a piperazine-linked benzimidazole-quinolinone compound that structurally resembles the piperazine-linked bisbenzimidazole minor groove binding dye Hoechst 33258 (Fig. 1A). Widely used anticancer drugs such as doxorubicin (and the other anthracyclines), mitoxantrone, amsacrine and camptothecin that bind to DNA and inhibit topoisomerase II α or topoisomerase I (EC 5.99.1.2) represent an important class of anticancer drugs [8–11]. Drugs that bind DNA might also be expected to inhibit cell growth through interference with topoisomerase I and topoisomerase II α and other DNA processing enzymes. Typically these DNA binding drugs inhibit the DNA topoisomerases and induce DNA single or double-strand breaks and cell death [9,10,12].

Both Hoechst 33258 and its congener Hoechst 33342 have been shown to inhibit topoisomerase I and topoisomerase II [13] and to induce topoisomerase I covalent cleavage complexes [14]. Even etoposide, which is a weak DNA intercalator (dissociation constant K_d of 11 μ M) [15]) has been shown in a recently published X-ray structure of etoposide bound to the covalent DNA-topoisomerase II β cleavable complex [16] to be intercalated into the cleaved and partially distorted duplex DNA. Camptothecin also intercalates between DNA base pairs in its topoisomerase I-DNA covalent complex [11]. A variety of kinase inhibitors including genistein, erastatin, tyrphostin, a bisindolylmaleimide, staurosporine [17], rebeccamycin derivatives [18,19], a DNA intercalating imidazoacridinone [20], and DNA intercalating ellipticine pyridocarbazole and benzopyridoindole analogs [21] have also been shown to inhibit topoisomerase II. Thus, dovitinib was evaluated to see if it exerted its cancer cell growth inhibitory effects in part through inhibition or poisoning of topoisomerase I or topoisomerase II α , through off-target binding to the ATP binding site and/or through its ability to bind to DNA.

2. Materials and methods

2.1 Materials, cell culture and growth inhibition assays

Dovitinib was from LC Laboratories (Woburn, MA). Unless specified, other reagents were obtained from Sigma-Aldrich (Oakville, Canada). The assay conditions and the expression, extraction and purification of recombinant full-length human topoisomerase II α were described previously [22]. Yeast (*Saccharomyces cerevisiae*) wild-type topoisomerase II was purified from protease-deficient strain JEL1t1. Yeast topoisomerase II was expressed for purification using the plasmid pGAL1TOP2 [23] and induction of topoisomerase II by galactose as described [24]. Human leukemia K562 cells, obtained from the American Type Culture Collection and K/VP.5 cells (a 26-fold etoposide-resistant K562-derived sub-line with decreased levels of topoisomerase II α mRNA and protein) [25] were maintained as suspension cultures in α MEM (Minimal Essential Medium Alpha) (Invitrogen, Burlington, Canada) containing 10% fetal calf serum. The spectrophotometric 96-well plate cell growth inhibition 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) CellTiter 96 AQueous One Solution Cell Proliferation assay (Promega, Madison, WI), which measures the ability of the cells to enzymatically reduce MTS after drug treatment, has been described [26]. Dovitinib was dissolved in DMSO and the final concentration of DMSO did not exceed an amount (typically 0.4 % or less) that had any detectable effect on cell growth. The cells were incubated with the drugs for 72 h and then assayed with MTS. The IC_{50} values for cell growth inhibition were measured by fitting the absorbance-drug concentration data to a four-parameter logistic equation as described [27]. The errors quoted are the S.E.M.s. The catenated kDNA and the primary anti-topoisomerase I and anti-topoisomerase II α antibodies were from TopoGEN (Port Orange, FL) and the secondary horseradish peroxidase-conjugated antibody was from Cell Signaling Technology (Danvers, MA). Where significance is indicated ($p < 0.05$), a Wilcoxon Signed Rank Test was used (SigmaPlot, San Rafael, CA).

2.2 Topoisomerase II α kDNA decatenation, pBR322 DNA relaxation and cleavage assays

A gel assay as previously described [27] was used to determine if dovitinib inhibited the catalytic decatenation activity of topoisomerase II α . kDNA, which consists of highly catenated networks of circular DNA, is decatenated by topoisomerase II α in an ATP-dependent reaction to yield individual minicircles of DNA. Topoisomerase II-cleaved DNA covalent complexes produced by anticancer drugs may be trapped by rapidly denaturing the complexed enzyme with sodium dodecyl sulfate (SDS) [27,28]. The drug-induced cleavage of double-strand closed circular plasmid pBR322 DNA to form linear DNA at 37 ° C was followed by separating the SDS-treated reaction products by ethidium bromide gel electrophoresis, essentially as described, except that all components of the assay mixture were assembled and mixed on ice prior to addition of the drug [27,28].

2.3 Topoisomerase I inhibition of pBR322 DNA relaxation assay

A gel assay as described [29] was used to determine if dovitinib inhibited topoisomerase I. The pBR322 DNA was from MBI Fermentas (Burlington, Canada). The topoisomerase I was from TopoGEN. The topoisomerase I inhibitor camptothecin (20 μ M) was used as a positive control. The percentage inhibition was obtained through densitometric analysis of the supercoiled bands relative to that obtained for pBR322 DNA alone (arbitrarily set to 100%) in the absence of enzyme.

2.4 Thermal denaturation of DNA assay

Compounds that either intercalate into or bind in the minor groove of DNA stabilize the DNA double helix and increase the temperature at which the DNA denatures or unwinds

[30]. The effect of 0.1, 0.2, 0.5, 1.0 and 2 μM of dovitinib and Hoechst 33258 on the increase in the DNA melting temperature, ΔT_m , of sonicated calf thymus DNA (5 $\mu\text{g}/\text{ml}$, 7.7 μM in DNA base pairs) was measured in 10 mM Tris-HCl buffer (pH 7.5) in a Cary 300 (Varian, Mississauga, Canada) double beam spectrophotometer by measuring the absorbance increase at 260 nm upon the application of a temperature ramp of 1 $^{\circ}\text{C}/\text{min}$ as we described [26].

2.5 γH2AX assay for DNA double-strand breaks in drug-treated K562 cells

The γH2AX assay was carried essentially out as described [31]. K562 cells in growth medium (0.5 ml in a 24-well plate, 1×10^6 cells/ml) were incubated with drug or with DMSO as a control for 5 h. Cell lysates (30 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis on a 14% gel. Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes and then treated overnight with rabbit anti- γH2AX primary antibody diluted 1:2000 (Upstate, Charlottesville, VA). This was followed by incubation for one h with peroxidase-conjugated goat-anti-rabbit secondary antibody (Cell Signaling Technology) diluted 1:2000. After incubation with luminol/enhancer/peroxide solution (Bio-Rad, Mississauga, Canada), chemiluminescence of the γH2AX band was imaged on a Cell Biosciences (Santa Clara, CA) FluorChem FC2 imaging system equipped with a charge-coupled-device camera.

2.6 Cellular assays for the detection of covalent DNA-topoisomerase II α and DNA-topoisomerase I protein complexes

The cellular ICE (immunodetection of complexes of enzyme-to-DNA), assays for topoisomerase II α or topoisomerase II β covalently bound to DNA were carried out as described [27]. The ICE assay used for the detection of covalent complexes of topoisomerase I, topoisomerase II α or topoisomerase II β bound to DNA was a modification of the original cesium chloride ultracentrifugation gradient assay used to isolate DNA [32]. The modification of this assay instead employed the selective precipitation of genomic DNA using DNAzol (Invitrogen).

2.7 Molecular modeling and docking of dovitinib into an X-ray structure of DNA and into the ATP binding site of topoisomerase II α

The molecular modeling and the docking were carried out as described [29]. The major protonated microspecies present at pH 7.4 was docked into the binding site of a 12-base pair X-ray structure (www.rcsb.org/pdb; PDB ID: 264D) [33] of a molecule of Hoechst 33258 bound in the minor groove of double-strand DNA, d(CGCAAATTTGCG)/Hoechst 33258, using the genetic algorithm docking program GOLD version 3.2 (CCDC Software, Cambridge, UK) with default GOLD parameters and atom types and with 500 starting runs as described [29]. The DNA structure was prepared by removing the Hoechst 33258 molecule and the water molecules to avoid potential interference with the docking, and hydrogens were added to the DNA. The binding site (6 $\text{\AA}$) was defined using Hoechst 33258 in the PDB ID: 264D structure. Hoechst 33258 docked back into its DNA structure with a heavy atom r.m.s. distance of 1.1 $\text{\AA}$, compared to the X-ray structure [33]. Values of 2.0 $\text{\AA}$ or less in the extensive GOLD test set are considered to be good [34]. For the docking of neutral dovitinib, the major species at pH 8, into the ATP binding sites of the X-ray structure of human topoisomerase II α (www.rcsb.org/pdb; PDB ID: 1ZXN) [5], 500 starts were used with the binding site defined by the bound non-hydrolyzable ATP analog, AMP-PNP (adenosine 5'-(β,γ -imido)triphosphate). Water and Mg^{2+} were removed from the structure before docking. The graphics were prepared with DS Visualizer 2.5 (Accelrys, San Diego, CA). MarvinSketch and its associated calculator plugins were used for displaying dovitinib and calculating its pK_a values and the major protonated microspecies present at either pH 7.4 or pH 8 (Marvin version 5.3.6, 2010, ChemAxon, <http://www.chemaxon.com>).

2.8 ATP/NADH-coupled ATPase assay

The determination of the ATPase activity of yeast topoisomerase II in the absence of DNA was based on the regeneration of hydrolyzed ATP coupled to the oxidation of NADH as described [31,35]. The ADP produced by the ATPase activity of topoisomerase II was rapidly converted back to ATP by pyruvate kinase/phosphoenolpyruvate, and the pyruvate produced was converted to lactate by L-lactate dehydrogenase (LDH) resulting in the oxidation of NADH. The assay measures the rate of loss of the NADH concentration from the fluorescence decrease (excitation and emission wavelengths, λ_{Ex} 340 nm, λ_{Em} 460 nm, respectively) which is proportional to the steady-state rate of ATP hydrolysis. The assay was performed at 30 °C in a 50 μ l volume (final concentrations: 50 mM Tris-HCl pH 8, 120 mM KCl, 10 mM magnesium acetate, 30 ng/ml bovine serum albumin (BSA), 0.5 mM dithiothreitol (DTT), 1 mM phosphoenolpyruvate, 20 units/ml pyruvate kinase, 20 units/ml lactate dehydrogenase, 0.5 mM NADH, 5 μ g yeast topoisomerase II) in a 96-well white plate. Initial velocities (v) were calculated from fluorescence-time data collected on a Synergy HT (BioTek, Winooski, VT) microplate reader. Dovitinib was shown not to inhibit either pyruvate kinase or lactate dehydrogenase as evidenced by the fact that NADH was rapidly oxidized in an experiment in which 0.5 mM ADP was added to assay mixtures containing up to 20 μ M dovitinib in the absence of topoisomerase II.

2.9 Fluorescence titration for the binding of dovitinib to DNA

The binding of dovitinib to sonicated calf thymus DNA was determined spectrofluorometrically on a Shimadzu 5000U spectrofluorimeter (Mandel Scientific, Guelph, Canada) at 22 °C in a 1 cm pathlength micro spectrofluorimeter cell in a volume of 1 ml. Successive emission spectra were recorded at the maximum of the dovitinib excitation wavelength of 360 nm by adding microliter amounts of DNA to a 5 μ M solution of dovitinib in Tris-HCl buffer (pH 6.8, 10 mM). The titration of dovitinib by DNA was likewise carried out at an excitation wavelength of 360 nm and the maximum of the emission wavelength of 510 nm by adding small aliquots of DNA to a 5 μ M solution of dovitinib.

3. Results

3.1 Binding of dovitinib to DNA

As shown in the fluorescence emission spectrum of Fig. 2A the fluorescence of dovitinib increased at 510 nm when successive equivalent amounts of DNA (5 μ M bp) were added to dovitinib which indicated that dovitinib bound to DNA. The DNA concentration dependence of the increase in fluorescence determined from a separate experiment is shown in Fig. 2B. A linear least squares fit of the concentration dependence of the fluorescence data to the McGhee-von Hippel excluded site model (a variation of the Scatchard equation) for binding of dovitinib to DNA [36] yielded a dissociation constant K_d of 7.0 ± 0.3 μ M (bp) and an n of 2.8 ± 0.1 where n is the number of DNA (bp) binding sites occupied by dovitinib. In Fig. 2C r is the concentration of the bound drug divided by the total concentration of DNA and C_f is the concentration of the free drug in the equation $r/C_f = (K_d)^{-1}(1 - nr)/[(1 - nr)/(1 - (n - 1)r)]^{n-1}$ [36]. The K_d value of 7.0 μ M for dovitinib compares to a value of 0.033 μ M (bp) for binding of Hoechst 33258 to calf thymus DNA [13]. The effect of dovitinib and Hoechst 33258 on ΔT_m are compared in Fig. 2D. The increase in ΔT_m indicated that dovitinib bound to DNA, though again, not as strongly as Hoechst 33258. The plot for Hoechst 33258 displayed curvature which indicated that at these concentrations it bound so strongly that it saturated the DNA binding sites.

3.2 Docking of dovitinib into an X-ray structure of DNA

The result from the docking of dovitinib into the minor groove of the PDB ID: 264D X-ray structure of DNA complexed to Hoechst 33258 [33] is shown in Fig. 1B. Dovitinib docked well into the DNA minor groove in place of Hoechst 33258. As shown in Fig. 1C there was a high degree of overlap of the benzimidazole and piperazine structures of the docked dovitinib with the corresponding benzimidazole and piperazine structures of the bound Hoechst 33258. Additionally, the aromatic quinolin-2-one structure of dovitinib overlapped well with the other benzimidazole aromatic ring of Hoechst 33258. These docking studies do not definitively demonstrate the binding mode of dovitinib to DNA, but only suggest a likely binding mode.

3.3 Dovitinib inhibits the decatenation activity, acts as a topoisomerase II α poison and induces DNA double-strand breaks in cells

The torsional stress that occurs in DNA during replication and transcription and daughter strand separation during mitosis can be relieved by topoisomerase II. Topoisomerase II alters DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-strand break made in a second helix [9,10,12]. As shown in Fig. 3A dovitinib inhibited the decatenation of concatenated kDNA by topoisomerase II α . Controls run in the absence of enzyme up to 100 μ M dovitinib had no effect on the high molecular weight kDNA that remained in the loading well and produced neither nicked circular DNA nor open circular DNA (not shown). Densitometric analysis of the decatenated DNA bands in the gel image of Fig. 3A yielded a non-linear least squares calculated IC_{50} value of 13 ± 4 μ M.

The decatenation assay is a measure of the ability of a dovitinib to inhibit the catalytic activity only and is not a measure of whether dovitinib acted as a topoisomerase II poison. The topoisomerase II poisons exert their cytotoxicity through their ability to stabilize a covalent topoisomerase II-DNA cleavable complex intermediate [9,10,12]. Stabilization of the covalent complex can lead to frank double-strand DNA breaks that are toxic to the cell. Thus, DNA cleavage assay experiments [27,28] were carried out to see whether dovitinib stabilized the cleavable complex. Etoposide was used as a positive control. As shown in Fig. 3B the addition of etoposide (lanes 1 and 2) to the reaction mixture containing topoisomerase II α and supercoiled pBR322 DNA induced formation of linear pBR322 DNA. Linear DNA was identified by comparison with linear pBR322 DNA produced by action of the restriction enzyme *HindIII* acting on a single site on pBR322 DNA (not shown). Dovitinib run in the absence of enzyme up to 100 μ M had no effect on the mobility of either nicked circular or supercoiled pBR322 DNA and did not by itself produce any linear DNA (not shown). As shown in Fig. 3B, dovitinib progressively induced formation of linear DNA which indicated that dovitinib acted as a topoisomerase II α poison. Based on integrated band intensities of linear DNA 3 μ M dovitinib produced about half as much linear DNA as 10 μ M etoposide. A cellular ICE assay was also used to determine if dovitinib could produce topoisomerase II-covalent complexes. As shown in Fig. 3C (upper image) and 3D both dovitinib and etoposide, in a concentration-dependent manner, progressively increased the amount of topoisomerase II α covalently bound to DNA. Dovitinib at 10 and 33 μ M significantly increased the amount of the topoisomerase II α -covalent complex compared to the untreated control, though not to the same degree as etoposide (Fig. 3D). At 100 μ M dovitinib there was a reproducible decrease in the amount of topoisomerase II α covalently bound to DNA compared to that found at 33 μ M. This “self-limiting” effect is similar to that observed for intercalating drugs such as doxorubicin and daunorubicin [37] and ethidium bromide [38] that abolishes drug-induced topoisomerase II-mediated DNA cleavage at higher drug concentrations. The mechanism for the inhibition is unknown, but it may be due to dovitinib saturating the DNA binding sites and altering DNA conformation. Both

dovitinib and etoposide also induced concentration dependent increases in the amount of the topoisomerase II β -covalent complex (Fig. 3C lower image) compared to the untreated control. As with topoisomerase II α , at the highest concentration of dovitinib (100 μ M) there was a decrease in the amount of topoisomerase II β bound to DNA compared to that at 33 μ M.

Phosphorylated H2AX (γ H2AX), which is a variant of an H2A core histone, rapidly localizes at the site of double-strand DNA breaks upon treatment of cells with drugs or ionizing radiation [39]. The thousands of γ H2AX molecules that are localized at the site of DNA double-strand breaks are thought to amplify the DNA damage signal and are a widely accepted marker of double-strand breaks [39]. Thus, in order to determine if dovitinib could induce double-strand breaks in intact K562 cells, the level of γ H2AX was determined by Western blotting. Experiments carried out as we previously described [31] and shown in Fig. 3E indicate that, in a concentration dependent manner, dovitinib increased levels of γ H2AX in K562 cells, though not to the same degree as with the etoposide positive control [28]. These results suggest that dovitinib acted as a topoisomerase II poison in a cellular context to produce damaging DNA double-strand breaks. Dovitinib may also be inhibiting kinases that phosphorylate H2AX or inhibit phosphatases that act on γ H2AX. These potential dovitinib activities would either limit or enhance, respectively, the experimentally observed phosphorylated γ H2AX signals shown in Fig. 3E.

3.4 Dovitinib inhibits the relaxation activity of topoisomerase I and induces the cleavage complex in K562 cells

Topoisomerase I is a DNA processing enzyme [40] that relieves torsional stress in DNA and is unlike topoisomerase II in that it does not require an ATP cofactor. Topoisomerase I relaxes DNA through a transient single strand break in DNA as compared to the transient double-strand break in DNA induced by topoisomerase II [40]. As shown by the gel image in Fig. 4A, dovitinib progressively inhibited the relaxation activity of topoisomerase I towards supercoiled pBR322 DNA. The well known topoisomerase I inhibitor camptothecin at 20 μ M was used as a positive control [40]. Controls run in the absence of enzyme up to 32 μ M dovitinib had no effect on the supercoiled DNA band that was used in the analysis (not shown). Densitometric analysis of the gel in Fig. 4A indicated that dovitinib inhibited topoisomerase I relaxation activity with a non-linear least squares calculated IC_{50} value of 6.2 ± 1.7 μ M.

The ability of dovitinib to produce a topoisomerase I-covalent complex in K562 cells was also carried out using a cellular ICE assay (Figs. 4B and 4C). Both dovitinib and the positive control camptothecin progressively increased the amount of the topoisomerase I-covalent complex. As shown in Fig. 4C, both dovitinib and camptothecin reproducibly increased the amount of the cleavage complex, though the amount produced by 100 μ M dovitinib was less than that produced by camptothecin.

3.5 Cell growth inhibitory effects of dovitinib on human leukemia K562 cells and K/VP.5 cells with a decreased level of topoisomerase II α

Cancer cells can acquire resistance to topoisomerase II poisons by lowering their level and/or activity of topoisomerase II [25]. We have previously used [27] a clonal K562 cell line selected for resistance to etoposide as a screen to determine the ability of compounds to act as topoisomerase II poisons. The etoposide-selected K/VP.5 cells are 26-fold resistant to etoposide and contain reduced levels of both topoisomerase II α (6-fold) and topoisomerase II β (3-fold) [41,42]. In addition, these cells are cross resistant to other known topoisomerase II poisons but are not cross resistant to camptothecin and other non-topoisomerase II α targeted drugs [42]. If there is less topoisomerase II in the cell, fewer DNA strand breaks are

produced by topoisomerase II poisons which results in reduced cytotoxicity. As shown in Fig. 5, K/VP.5 cells were resistant to dovitinib (IC_{50} 1.8 ± 0.1 μ M) compared to K562 cells (IC_{50} 0.66 ± 0.04 μ M). A propagation-of-errors analysis indicated that K/VP.5 cells were 2.7 ± 0.2 -fold cross resistant to dovitinib. These results are consistent with dovitinib acting, at least in part, as a topoisomerase II poison both in cells (Figs. 3C and 3D) and with recombinant topoisomerase II α (Fig. 3B). The growth inhibitory effects of Hoechst 33258 was determined for K562 and K/VP.5 cells for comparison since dovitinib is, in part, structurally similar to Hoechst 33258. Hoechst 33258 yielded IC_{50} values of 2.7 and 5.4 μ M, respectively for K562 and K/VP.5 cells, respectively. Thus, K/VP.5 cells were only 2-fold cross resistant compared to K562 cells. Given that Hoechst 33258 is such a strong DNA intercalator it is likely that it has a number of targets as well as topoisomerase II [13].

3.6 Dovitinib inhibited the ATPase activity of yeast topoisomerase II in the absence of DNA

Dovitinib was designed to exert its kinase inhibitory activity through binding to the ATP binding site of its kinase targets. To determine if dovitinib inhibited topoisomerase II either by binding competitively to the ATP binding site, or non-competitively at an adjacent site, we decided to investigate if dovitinib could inhibit the DNA-independent ATPase activity of yeast topoisomerase II. These experiments were carried out with yeast topoisomerase II due to the limited amounts of human topoisomerase II α available. The ATPase activity of yeast topoisomerase II is greatly enhanced in the presence of DNA [35]. However, because our results showed (Fig. 2) that dovitinib bound to DNA (Fig. 2), it was necessary to measure the enzyme kinetics of the 20-fold weaker [35] DNA-independent ATPase activity of yeast topoisomerase II (Fig. 6). Thus, in order to simplify the analysis, we chose to examine the effect of dovitinib on the DNA-independent ATPase activity.

Topoisomerase II is a homo-dimeric enzyme with 2 ATP binding sites. While the DNA-dependent ATPase activity displays cooperativity between the two ATPase sites, the DNA-independent ATPase activity shows no evidence of cooperativity [35]. The weighted linear-least squares calculated double reciprocal plot of Fig. 6A gave a DNA-independent K_m of 0.84 ± 0.04 mM for ATP and an apparent turnover number k_{cat} of 0.73 s $^{-1}$ (dimer basis). These values compare reasonably well to a previous determination under similar conditions with a K_m value of 0.3 mM and a k_{cat} value of 0.4 s $^{-1}$ [35]. Increasing dovitinib concentrations, as shown in the double reciprocal plot of Fig. 6A, progressively inhibited the ATPase activity of topoisomerase II in the low micromolar concentration range. For simple competitive inhibition each plot should have a common intercept on the y-axis [43], and for non-competitive inhibition each plot should have a common intercept on the negative x-axis. While the plots did not display a common y-axis intercept, they also did not display a common x-axis intercept. The lack of either good x- or y-intercepts may be due to the complex nature of the enzyme kinetics. Secondary plots (Fig. 6B) of the slope (K_m/V_{max}) from the plots in Fig. 6A vs. the dovitinib concentration yields, from the intercept divided by the slope, an apparent inhibition constant K_i of 2.4 ± 1.5 μ M, independent of whether the enzyme kinetics are competitive or non-competitive [43], where the error in K_i was calculated from a propagation-of-errors analysis. These results show that dovitinib potently inhibited the topoisomerase II ATPase activity independently of, and unrelated to its ability to bind to DNA.

3.7 Competition with ATP for the inhibition of the decatenation activity of topoisomerase II α by dovitinib and the non-hydrolyzable ATP analog AMP-PNP

Experiments were next performed to determine if dovitinib could compete with ATP in the topoisomerase II α -mediated decatenation of kDNA. This method has been previously used to test whether several drugs were ATP competitive inhibitors of *Drosophila melanogaster* topoisomerase II [17] or DNA gyrase [44]. As shown in Fig. 7A, ATP promoted the

topoisomerase II α -mediated decatenation of kDNA with an EC₅₀ of $15 \pm 7 \mu\text{M}$ and displayed no substrate inhibition up to 33 mM ATP. Both the ATP competitive inhibitor AMP-PNP (Fig. 7B) and dovitinib (Fig. 7C) inhibited decatenation activity at ATP concentrations ranging from 0.2 – 2 mM. A 10-fold increase in ATP concentration produced a 15-fold increase in the IC₅₀ for AMP-PNP (from 2.0 ± 0.4 to $30 \pm 8 \mu\text{M}$) (Fig. 7B). A 10-fold increase in ATP concentration produced a 3.6-fold increase in the IC₅₀ for dovitinib (Fig. 7C) (from 19 ± 3 to $69 \pm 5 \mu\text{M}$). This result is consistent with the conclusion that dovitinib, like AMP-PNP, is capable of inhibiting topoisomerase II α by interaction with the ATP binding site. In these experiments some of the inhibition caused by dovitinib may also be due to its binding to kDNA.

3.8 Docking of dovitinib into the ATP binding site of the X-ray structure of topoisomerase II α

To determine if the ATP binding site of human topoisomerase II α could accommodate dovitinib, docking was carried out on the X-ray structure of human topoisomerase II α (www.rcsb.org/pdb; PDB ID: 1ZXN) [5]. In this closed clamp form of the enzyme, the non-hydrolyzable ATP analog, AMP-PNP is bound in each ATP binding site. Dovitinib docked well into either of the ATP binding sites (Fig. 8). The results of docking dovitinib to the ATP-902 site (Fig. 8A) shows that dovitinib only partially overlaps with the planar adenosine moiety of the non-hydrolyzable ATP analog AMP-PNP and thus did not fully occupy the ATP binding site. The overlap of the quinolinone moiety of dovitinib with the adenine moiety of ATP was very good and the two ring structures were nearly parallel to each other. The docked structure was also examined for steric clashes using the “bump” monitor tool in DS Visualizer 2.5. Only a single bump was seen between Ser149 and the benzimidazole ring with a Ser149 oxygen-to-carbon atom distance of 2.7 Å, which was not severe. Hence, if dovitinib binds in this site it could still competitively inhibit topoisomerase II. These *in silico* findings were consistent with results above that dovitinib inhibited topoisomerase II ATPase activity (Fig. 6) and competed with ATP for the inhibition of the decatenation activity of topoisomerase II α (Fig. 7).

4. Discussion

The results of this study have shown that the piperazine-linked benzimidazole multi-kinase inhibitor dovitinib, which has a chemical structure similar to the piperazine-linked bisbenzimidazole minor groove DNA binding compound Hoechst 33258 (Fig. 1A), also bound to DNA, though not as strongly as Hoechst 33258 (Fig. 2). This conclusion was supported both by spectrofluorometric titrations and DNA melting point studies and was also consistent with the molecular modeling and docking studies that showed that dovitinib docked well into the minor groove of an X-ray structure of a complex of Hoechst 33258 with DNA (Fig. 1B). Additionally the dovitinib-docked structure (Fig. 1C) showed a high degree of spacial overlap with the bound Hoechst 33258.

Drugs that bind DNA are well represented among our most effective anticancer drugs and typically these drugs inhibit and/or poison topoisomerase I or topoisomerase II [9–12]. Poisoning of topoisomerase I or topoisomerase II can lead to frank single and double-strand breaks and cell death. Dovitinib inhibited both the topoisomerase II α catalytic decatenation activity (Fig. 3A) and poisoned recombinant topoisomerase II α in the low micromolar concentration range as evidenced by the production of linear DNA from pBR322 DNA (Fig. 3B). We also showed in a functional ICE assay that dovitinib induced formation of both covalent topoisomerase II α -DNA and topoisomerase II β -DNA complexes in dovitinib-treated K562 cells (Fig. 3C and 3D). Likewise, we also showed that dovitinib inhibited the catalytic relaxation activity of topoisomerase I (Fig. 4A) and acted as a cellular topoisomerase I poison (Fig. 4B). These results are consistent with previous studies that

showed that Hoechst 333258 inhibits both topoisomerase I and topoisomerase II [13,14]. Additionally, we showed that dovitinib treatment induced formation of γ H2AX in K562 cells (Fig. 3E), which was indicative of DNA double-strand breaks. An additional functional test of whether dovitinib acted as topoisomerase II poison showed that it exhibited cross resistance (Fig. 5) to the etoposide-resistant K562-derived K/VP.5 cell line that expresses reduced levels of both topoisomerase II α and topoisomerase II β . Taken together these results suggest that dovitinib-mediated inhibition and poisoning of topoisomerase I, topoisomerase II α and topoisomerase II β may have contributed to the cell growth inhibitory effects of dovitinib. The inhibitory effects of dovitinib on topoisomerase I and topoisomerase II were likely due, in part, to its ability to bind to DNA. Binding of dovitinib to DNA could potentially affect other DNA processing enzymes which may also contribute to its cell growth inhibitory activity.

Because dovitinib was designed to exert its kinase inhibitory activity through binding to the kinase ATP binding site, we investigated whether dovitinib bound to the ATP binding site of topoisomerase II. Enzyme kinetic experiments showed that dovitinib strongly inhibited (K_i of 2.4 μ M) the DNA-independent ATPase activity of topoisomerase II (Fig. 6). These experiments were carried out in the absence of DNA in order to simplify the analysis. The lack of either good x- or y-intercepts (indicative of non-competitive or competitive inhibition, respectively) in the double-reciprocal plots of Fig. 6A may be due to the complex nature of the enzyme kinetics. An additional test of whether dovitinib competed with ATP was carried out by comparing the relative changes in the dovitinib IC_{50} values for inhibition of the topoisomerase II α decatenation activity at a series of fixed ATP concentrations (Fig. 7C). These results suggested that dovitinib competed with ATP at the ATP binding site. Consistent with these latter results docking of dovitinib into the X-ray structure of human topoisomerase II α also showed that the ATP binding sites on topoisomerase II α could accommodate a dovitinib molecule at either site (Fig. 8B). It is also possible that dovitinib may inhibit topoisomerase II by binding non-specifically or by interacting allosterically with the ATP binding sites.

Dovitinib is a highly promiscuous kinase inhibitor as was shown in a profiling study in a 442 kinase panel, representing more than 80% of the human kinome [1]. In that study dovitinib strongly bound to 36 and 14% of all the kinases profiled with binding dissociation constant K_d 's of less than 3 and 0.3 μ M, respectively [1]. Thus, even at a concentration of 0.3 μ M dovitinib could bind to and inhibit as many as 62 different kinases. Thus, the kinase target, or combination of targets, that might be responsible for its anticancer activity cannot be known with certainty. Maximum plasma concentrations as high as 1.2 μ M are observed in clinical trials with 500 mg/day daily dosing of dovitinib [2]. This pharmacological concentration of dovitinib is within the concentration range that we observed dovitinib binding to DNA (Fig. 2D); dovitinib-induced formation of topoisomerase II α cleavage complexes (Fig. 3B); dovitinib inhibition of the topoisomerase I-mediated relaxation of pBR322 DNA (Fig. 4A); and dovitinib inhibition of the ATPase activity of topoisomerase II (Fig. 6B). Thus, all of these activities, in addition to its multi-kinase inhibitory activity could potentially contribute to the anticancer activity of dovitinib. It is also worth noting that inhibition of either topoisomerase I or topoisomerase II could contribute to the well known toxicities of these classes of inhibitors. In summary, dovitinib may not only exert its anticancer activity by inhibiting multiple kinases [1,2], but also, in part, by poisoning topoisomerase I and topoisomerase II. It is not possible, at present, to discern what portion of its cell growth inhibitory activity (and its anticancer efficacy) is due to each of its several activities.

Acknowledgments

Supported by grants from the Canadian Institutes of Health Research, the Canada Research Chairs Program, a Canada Research Chair in Drug Development to Brian Hasinoff, and NIH grants CA090787 to Jack Yalowich, and CA52814 and CA82313 to John Nitiss. The funding source(s) had no involvement in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

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Figure 1.

Dovitinib and Hoechst 33258. (A) Comparison of the structures of the benzimidazole dovitinib and the bisbenzimidazole DNA minor groove binding dye Hoechst 33258. (B) Docking of dovitinib into the X-ray structure of the DNA-Hoechst 33258 complex. The docked structure shown is the highest scoring structure of dovitinib docked into the PDB ID: 264D X-ray structure of a DNA-Hoechst 33258 complex in which a Hoechst 33258 molecule is bound to a 12-base pair piece of DNA. The H-atoms of the DNA are not shown for clarity. The Hoechst 33258 bound ligand was removed from the structure and dovitinib was docked into its place with the genetic algorithm docking program GOLD. (C) The docked structure of dovitinib (grey carbon, ball and stick structure) is compared to the bound

structure of Hoechst 33258 (green carbon, stick structure). For clarity the H-atoms are not shown.

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Figure 2.

Binding of dovitinib to DNA. (A) The increase in the fluorescence emission spectra (λ_{Ex} 360 nm) observed when successive amounts of sonicated calf thymus DNA (5 μM bp) were added to dovitinib (5 μM) in Tris buffer (10 mM, pH 6.8). (B) Increase in fluorescence at 510 nm (λ_{Ex} 360 nm) when successive amounts of sonicated DNA (5 μM bp) were added to dovitinib (5 μM). The solid line is a best fit line calculated from the fit to the McGhee-von Hippel model with the parameters derived from the plot in (C). (C) Plot and fitting of the data in (B) to the McGhee-von Hippel excluded site model for binding of dovitinib to DNA, where r is the concentration of bound dovitinib divided by the total concentration of DNA and C_f is the concentration of free dovitinib. The best-fit solid line is calculated with a K_d of 7.0 ± 0.3 μM (bp) and an n of 2.8 ± 0.1 where n is the number of DNA binding sites occupied by dovitinib. (D) Concentration dependence of the increase in the DNA melting temperature ΔT_m for dovitinib (○) and Hoechst 33258 (●) binding to DNA. The straight line is a linear least-squares calculated fit to the dovitinib data and the curved line is a spline fit to the Hoechst 33258 data. The Hoechst 33258 data shows saturation behavior, likely due to its much stronger binding to DNA. The dovitinib results were averages from 2 – 4 different experiments. RFU is relative fluorescence units.

Figure 3.

Effect of dovitinib on the decatenation activity and poisoning of topoisomerase II α and topoisomerase II β , and on the induction of DNA double-strand breaks in cells. (A) Effect of dovitinib on the inhibition of the topoisomerase II α -mediated decatenation activity of kDNA. This fluorescent image of the ethidium bromide-containing gel shows that topoisomerase II α decatenated kDNA to its open circular (OC) form (lane 11). Topoisomerase II α was present in the reaction mixture for all lanes but lane 10. ORI is the gel origin. Dovitinib progressively inhibited the decatenation activity of topoisomerase II α . An IC_{50} value of $13 \pm 4 \mu\text{M}$ was calculated from the data. The results were typical of experiments carried out on 4 different days. (B) Effect of dovitinib and etoposide on the topoisomerase II α -mediated relaxation and cleavage of supercoiled pBR322 plasmid DNA. This fluorescent image of the ethidium bromide-stained gel shows that topoisomerase II α (Topo II α) converted supercoiled (SC) pBR322 DNA (lane 3) to relaxed (RLX) DNA (lane 4). In this assay the relaxed DNA runs slightly ahead of the supercoiled DNA because the gel was run in the presence of ethidium bromide. Topoisomerase II α was present in the reaction mixture in all but lane 3. As shown in lanes 1 and 2, the etoposide positive control produced significant amounts of linear DNA (LIN). A small amount of nicked circular (NC), which may arise from strand breakage during isolation, is normally present in pBR322 DNA. The results were typical of experiments carried out on 4 different days. (C) Upper image, lanes 1 – 8: Chemiluminescent image of a Western slot blot determination of cellular covalent topoisomerase II α -DNA cleavage complexes produced in K562 cells determined

using an ICE (immunodetection of complexes of enzyme-to-DNA) assay. In these experiments K562 cells were either treated with DMSO vehicle (lane 2) or with the positive control etoposide (lanes 6 – 8) or dovitinib (lanes 3 – 5) for 1 h. Lane 1 contained 5 ng of purified topoisomerase II α (Topo II α). Lower image, lanes 9 – 14: Chemiluminescent image of a Western slot blot determination of cellular covalent topoisomerase II β -DNA cleavage complexes (Topo II β) produced in K562 cells treated as described above. In these experiments K562 cells were either treated with DMSO vehicle (lane 9) or with the positive control etoposide (lanes 13 and 14) or dovitinib (lanes 10 – 12). (D) Results of a densitometric analysis of dovitinib and etoposide-induced cellular covalent topoisomerase II α -DNA cleavage complexes produced in K562 cells determined using an ICE assay. Etoposide (100 μ M) and dovitinib (10 and 33 μ M) treatments significantly ($p < 0.05$, Wilcoxon Signed Rank Test) increased topoisomerase II α -DNA cleavage complexes. The results were from 6 determinations for dovitinib and from 4 – 7 determinations for etoposide. (E) Dovitinib and etoposide induced double-strand DNA breaks in K562 cells in a concentration dependent manner as indicated by formation of γ H2AX. K562 cells were treated with the concentrations of the drugs indicated for 5 h in growth medium, lysed and subjected to SDS-PAGE electrophoresis and Western blotting. The blots were probed with antibodies to γ H2AX and with GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as a loading control and a chemiluminescent-inducing horseradish peroxidase-conjugated secondary antibody. The results were typical of experiments carried out on 2 different days.

Figure 4.

Effect of dovitinib on the relaxation activity and cellular poisoning of topoisomerase I. (A) Effect of dovitinib and camptothecin on the ability of topoisomerase I to relax supercoiled pBR322 DNA. This fluorescent image of the ethidium bromide-stained gel shows that both compounds inhibited the relaxation activity of topoisomerase I. The topoisomerase I inhibitor camptothecin (20 μ M) was used as a positive control. All lanes except lane 2 contained topoisomerase I. In this gel, which was stained with ethidium bromide after it was run, the supercoiled DNA (SC) ran ahead of the relaxed DNA (RLX). Inhibition of topoisomerase I relaxation activity was essentially 100 % complete at 10 μ M dovitinib (lane 8). The band shifting seen in lane 9 at 32 μ M dovitinib was likely due to dovitinib binding to the DNA. Topoisomers due to partial inhibition of topoisomerase I by dovitinib can be seen in lanes 5 and 6. The results were typical of experiments carried out on 2 different days. (B) Chemiluminescent image of a Western slot blot determination of cellular covalent topoisomerase I-DNA cleavage complexes produced in K562 cells determined using an ICE (immunodetection of complexes of enzyme-to-DNA) assay. In these experiments K562 cells were either treated with DMSO vehicle (lanes 2 and 8) or with the positive control camptothecin (lanes 1 and 9) or dovitinib (lanes 3 – 7 and 10 – 11) for 1 h. The blots shown are from experiments on two different days. (C) Results of a densitometric analysis of dovitinib and camptothecin-induced cellular covalent topoisomerase I-DNA cleavage complexes produced in K562 cells determined using an ICE assay. Camptothecin (100 μ M) and dovitinib (100 μ M) treatments reproducibly increased topoisomerase I-DNA cleavage complexes. The results were from 4 – 5 determinations for dovitinib and from 3 – 5 determinations for camptothecin at 33 and 100 μ M.

Figure 5.

Comparison of the growth inhibitory effects of dovitinib on K562 and K/VP.5 cells with reduced levels of topoisomerase II α . K562 (○) and K/VP.5 (●) cells were treated with dovitinib for 72 h prior to the assessment of growth inhibition. The curved lines were calculated from non-linear least squares fits to 4-parameter logistic equations for K562 (solid line) and K/VP.5 (broken line) cells and yield IC_{50} values of 0.66 ± 0.04 and 1.8 ± 0.1 μ M, respectively and gives a relative resistance of 2.7 ± 0.2 . The results were from an average of 3 wells and the results were typical of determinations on two separate days.

Figure 6.

Inhibition of the DNA-independent ATPase activity of yeast topoisomerase II by dovitinib. (A) Double reciprocal initial velocity plots for the DNA-independent ATPase activity of yeast topoisomerase II in the presence of various concentrations of dovitinib. The straight lines were weighted (weight of $1/v$) linear least squares calculated fits to $1/v$. The slope and intercept in the absence of dovitinib gives a K_m of 0.84 ± 0.04 mM and a V_{max} of 12.9 ± 0.7 $\mu\text{M}/\text{min}$. The ATPase activity was measured in the absence of DNA in order to eliminate effects from the binding of dovitinib to DNA. (B) Secondary plot of the slope K_m/V_{max} from the plots in (A) vs. the dovitinib concentration. The straight line is least squares calculated and gives from the intercept divided by the slope an inhibition constant K_i of 2.4 ± 1.5 μM . The 50 μl reaction mixture (50 mM Tris-HCl pH 8) contained 5 μg of yeast topoisomerase I.

Figure 7.

Inhibition of decatenation activity of topoisomerase II α at various ATP concentrations by dovitinib and by the non-hydrolyzable ATP analog and ATP competitive inhibitor, AMP-PNP. (A) ATP dependence of the decatenation activity of topoisomerase II α . (B) AMP-PNP-mediated inhibition of the decatenation activity of topoisomerase II α at 0.2 (○), 1 (△) and 2 mM ATP (□). The curved lines were calculated from non-linear least squares fits to 2-parameter logistic equations for 0.2 mM (solid line), 1 mM (short broken line), and 2 mM ATP (long broken line) and yield IC_{50} values of 2.0 ± 0.4 , and 4.4 ± 1.4 μM and 30 ± 8 μM , respectively. (C) Dovitinib-mediated inhibition of the decatenation activity of topoisomerase II α at 0.2 (○), 0.6 (△) and 2 mM ATP (□). The curved lines were calculated from non-linear least squares fits to 2-parameter logistic equations for 0.2 mM (solid line), 0.6 mM (short broken line), and 2 mM ATP (long broken line) and yield IC_{50} values of 19 ± 3 , and 43 ± 11 μM and 69 ± 5 μM , respectively. The results are typical of two independent experiments on carried out on different days.

Figure 8.

Results of docking dovitinib into the N-terminal ATP binding structure of the X-ray structure of human topoisomerase II α (PDB ID: 1ZXN). Topoisomerase II α is shown in ribbon representation. The highest scoring docked space-filling structures of dovitinib are shown in each site. The closeup of the binding of dovitinib into the leftmost protomer is shown in (A). Dovitinib only partially overlaps with the planar adenosine moiety of the non-hydrolyzable ATP analog AMP-PNP (stick structure, no H-atoms). (B) The leftmost protomer is colored according to the secondary structure: helical structure (red), β -sheet (cyan), turns (green) and random coil (grey), whereas the rightmost protomer is in blue.