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The HSP90 and DNA topoisomerase VI inhibitor radicicol also inhibits human type II DNA topoisomerase

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ABSTRACT

Radicicol derivatives are currently investigated as promising antitumoral drugs because they inhibit the activity of the molecular chaperone heat shock protein (HSP90), causing the destabilization and eventual degradation of HSP90 client proteins that are often associated with tumor cells. These drugs interact with the ATP-binding site of HSP90 which is characterized by a structural element known as the Bergerat fold, also present in type II DNA topoisomerases (Topo II). We have previously shown that radicicol inhibits archaeal DNA topoisomerase VI, the prototype of Topo II of the B family (present in archaea, some bacteria and all the plants sequenced so far). We show here that radicicol also inhibits the human Topo II, a member of the A family (comprising the eukaryotic Topo II, bacterial gyrase, Topo IV and viral Topo II), which is a major target for antitumoral drugs. In addition, radicicol prevents in vitro induction of DNA cleavage by human Topo II in the presence of the antitumoral drug etoposide. The finding that radicicol can inhibit at least two different antitumoral drug targets in human, and interferes with drugs currently used in cancer treatment, could have implications in cancer therapy.

1. Introduction

"The rapid discovery of new drugs is greatly facilitated when a family of related proteins is targeted with a similar approach in chemistry" [1]. The new superfamily of ATP-dependent proteins comprising Gyrase (type II DNA topoisomerase, Topo II), HSP90, Histidine Kinases, and MutL, which have been described by Dutta and Inouye as the GHKL superfamily, could be suitable for such approach [2]. Although each subfamily of GHKL proteins does not share any significant primary sequence similarity, they all share a unique structural ATP-binding motif, called the Bergerat fold [2]. The proteins of the GHKL superfamily (thereafter called Bergerat fold proteins) are functionally diverse, as they include proteins involved in DNA mismatch repair, control of DNA topology, maintenance of protein stability, or else signal transduction.

Radicicol, a macrocyclic antibiotic isolated from the fungus *Monosporium bonorden* (Fig. 1a [3]), has been shown to inhibit the chaperone activity of HSP90 through direct interaction with its Bergerat fold [4]. HSP90 is an important new potential anticancer drug target because of its role in maintaining the conformation, stability and function of key oncogenic client proteins involved in signal transduction pathways (for recent reviews about Hsp90 inhibitors see [5–12]). Disruption by radicicol of the interaction between HSP90 and its client proteins leads to their destabilization and rapid degradation. It has been shown that radicicol or derivatives suppress cellular transformation in vivo by a variety of oncogens that are

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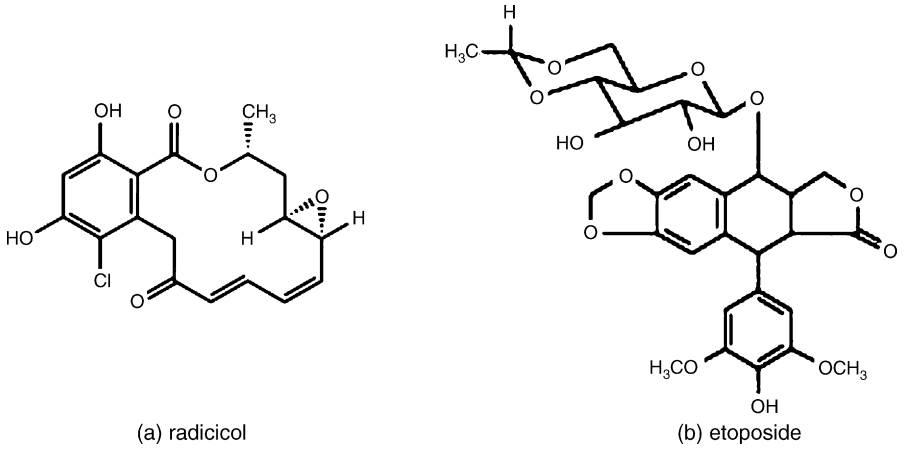

(a) radicicol (b) etoposide

Fig. 1 – Structural formula of radicicol and etoposide.

normally stabilized by HSP90, such as v-Src, K-ras, raf1 or mutated p53 protein [13–18]. Although radicicol itself cannot be used as a therapeutic agent, presumably due to metabolic instability in animals, different derivatives of radicicol or chimeric molecules are currently tested to find new HSP90 inhibitors that could be useful in cancer therapy [19–23].

Given the structural relationship between their ATPase domains, it is logical to assume that some molecules acting on the Bergerat fold would inhibit GHKL proteins from different subfamilies. As a confirmation of this hypothesis, it has been shown that radicicol inhibits two Bergerat fold proteins with histidine kinase activities [24]. This emphasizes that radicicol could have several different intracellular targets.

Recently, we have shown that radicicol inhibits the DNA topoisomerase VI (Topo VI) of the archaeon *Sulfolobus shibatae*, the prototype of the Topo IIB family [25]. Type II DNA topoisomerases (Topo II) are ubiquitous enzymes that catalyse the ATP-dependent crossing of two DNA duplexes through each other via transient double-strand breaks (DSBs) (for reviews see [26,27]). They are implicated in major biological processes, such as replication, recombination and transcription. In particular, the decatenation activity of Topo II is essential in all organisms to separate daughter chromosomes at the end of each replication round. Topo II has been classified into two evolutionarily distinct protein families: Topo IIA and Topo IIB ([28], for a review see [29]). The A family includes classical eukaryotic Topo II, bacterial DNA gyrase (also present in some Archaea), bacterial DNA topoisomerase IV (Topo IV) and several viral Topo II, whilst the B family presently only contains archaeal Topo VI and relatives from plants. Both Topo IIA and IIB are Bergerat fold proteins with homologous ATP-binding domain [30,31], but their nicking-closing modules are structurally dissimilar [32].

The inhibition of an archaeal Topo VI by radicicol suggests that Topo IIA could also be sensitive to this drug since they both contain a Bergerat fold. However, we have previously shown that radicicol has no effect on *Escherichia coli* DNA gyrase [25]. Nevertheless, since the spectrum of action of various drugs can be different for various Topo II of the A family (for instance, quinolones are very effective against DNA gyrase but not against eukaryotic Topo IIA), we decided to investigate a possible effect of radicicol on human Topo II. We show here that, unlike *E. coli* DNA gyrase, human Topo II is inhibited in vitro by radicicol. In addition, we report that radicicol prevents the formation of double-strand breaks by the antitumoral drug etoposide (an epipodophyllotoxin) in the presence of human Topo II. These results are significant, since human Topo II is the target of several important drugs currently used in cancer therapy.

2. Materials and methods

2.1. Enzymes and chemicals

Drugs were purchased from Sigma–Aldrich. Concentrated stock solutions (100 mM) were prepared in DMSO, except for novobiocin (H_2O). Stock solutions were aliquoted and stored at −20 °C in the dark. Just before tests, the drugs were diluted at the required concentration in DMSO. Human Topo II was purchased from TopoGEN (Columbus, OH). Wheat germ DNA topoisomerase I was purchased from Sigma. The enzymes were tested using negatively supercoiled pBR322 plasmids as substrate for relaxation and cleavage assays; kDNA for decatenation assay and relaxed pBR322 for unwinding assay. kDNA and plasmids were purchased from Promega, TopoGEN or invitrogen. AMP-PNP and ATP were purchased from Sigma.

2.2. Inhibition of topoisomerase II-mediated DNA relaxation

DNA relaxation was performed by incubating 2 units of human Topo II and 250 ng of supercoiled pBR322 DNA in 20 μl (total volume) of reaction mixture containing 50 mM Tris–HCl, pH 8.0; 0.5 mM dithiothreitol; 30 mg/ml BSA; 120 mM KCl; 10 mM $MgCl_2$; 1–3 mM ATP, and 0.3 μl of either DMSO or different drug concentrations. The reaction mixtures were incubated for 30 min at 37 °C and stopped by the addition of 2 μl of loading buffer. Samples were subjected to electrophoresis in a 1% agarose gel in TAE 0.5X buffer. Gels were stained after with buffer containing 1 μg/ml ethidium bromide. DNA bands were visualized under UV light at 254 nm.

2.3. Inhibition of topoisomerase II-mediated kDNA decatenation

kDNA decatenation was performed by incubating 2 units of human Topo II and 250 ng of kDNA in 20 μl (total volume) of reaction mixture containing 50 mM Tris–HCl, pH 8.0; 120 mM KCl; 0.5 mM dithiothreitol; 10 mM $MgCl_2$; 1–3 mM ATP, with 0.3 μl of different drug concentrations. Reaction mixtures were incubated for 30 min at 37 °C and stopped by addition of 2 μl loading buffer. Samples were subjected to electrophoresis and DNA was visualized as described in Section 2.2.

2.4. DNA unwinding assays

DNA unwinding assays were performed by incubating 2 units of human Topo II and 250 ng of relaxed pBR322 plasmid in 20 μl of reaction mixture (total volume) containing 50 mM Tris–HCl, pH 8.0; 0.5 mM dithiothreitol; 30 mg/ml BSA; 120 mM KCl; 10 mM $MgCl_2$, with 0.3 μl of differents drugs. The reaction mixture was incubated for 30 min at 37 °C before addition of 2 μl of 10% SDS followed by another incubation of 10 min at 37 °C. The reaction mixtures were then incubated for 30 min at 45 °C after addition of 2 μl proteinase K (1 mg/ml). The reaction was stopped by addition of 2 μl loading buffer. Samples were subjected to electrophoresis and DNA was visualized as described in Section 2.2.

2.5. Inhibition of topoisomerase II-mediated DNA cleavage

Topo II-mediated DNA cleavage was performed by incubating 2 units of human Topo II and 250 ng of negatively supercoiled pBR322 in 20 μl of the reaction mixture for relaxation as previously described (except for ATP) and 0.3 μl of different drug concentrations. Samples were incubated at 37 °C for 30 min, and cleavage products were trapped by addition of SDS and proteinase K as described in Section 2.4. The reaction was stopped by addition of 2 μl loading buffer and samples were analyzed on agarose gels as previously described. When Topo II-mediated cleavage was carried out in the presence of ATP or AMP-PNP, the concentration of the nucleotide was 3 mM. DNA cleavage assays with drugs were carried out with addition of drugs in the reaction mixture before topoisomerase addition. When radicicol or etoposide were added sequentially, etoposide was added to the reaction mixture 10 min after radicicol and the two drugs were incubated together for 20 more min at 37 °C. A control with a volume of DMSO corresponding to the addition of the two drugs was done.

2.6. Molecular modelling

The putative interaction between radicicol and *H. sapiens* DNA topoisomerase II has been modelled by superimposing the coordinates of the Bergerat fold domain from the radicicol-bound *S. cerevisiae* HSP90 structure [4]; PDB code 1BGQ, onto the corresponding domain from *H. sapiens* DNA topoisomerase IIA [75]; PDB code 1ZXM, using the program ALIGN [76]. The resulting root mean square deviation is 2.73 Å over the Ca from 143 residues.

3. Results

3.1. Radicicol inhibits human type II DNA topoisomerase catalytic activities

In order to determine the possible effect(s) of radicicol on human Topo II, we used the p170 (alpha) isoform of the human Topo II that is commercially available (TopoGEN). This isoenzyme is essential for life due to its role in chromosome condensation and segregation [33,34]. We tested the ability of radicicol to inhibit the catalytic activity of human Topo II using two different DNA substrates, negatively supercoiled pBR322 plasmid for relaxation assays and kinetoplast (kDNA) for decatenation assays. As shown in Fig. 2a, relaxation of pBR322 by human topo II was fully inhibited by concentrations of radicicol 100 μM (lane 6) or higher (lanes 3–5). This is similar to the concentrations required, in the same conditions, to completely inhibit the relaxation activity of *S. shibatae* Topo VI [25]. The relaxation activity of human Topo II was slightly

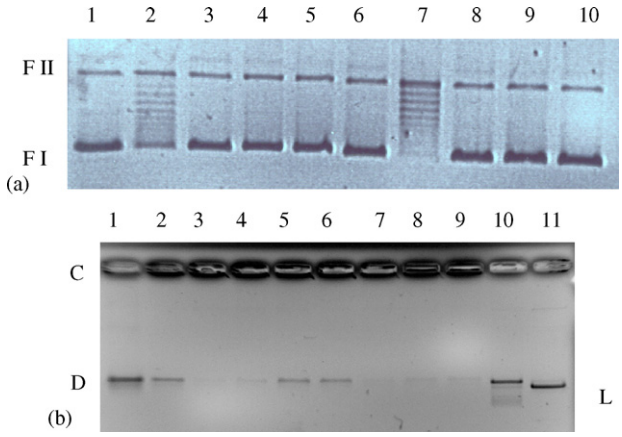

Fig. 2 – Effect of radicicol and others drugs on the relaxation (a) and decatenation activities (b) of human Topo II. (a) Relaxation of pBR322 in the presence of human Topo II and various drugs concentrations. Lane 1, negatively supercoiled plasmid control; lane 2, relaxation assay control with 2 units of Topo II enzyme and 1.5% DMSO; lanes 3–7, relaxation reactions with 2 units of Topo II and 1000, 500, 250,100 and 25 μM of radicicol respectively; lane 8, relaxation reaction with 2 units of Topo II and 100 μM etoposide; lane 9, relaxation reaction with 2 units of Topo II and 1 mM ICRF-193; lane 10, relaxation reaction with 2 units of Topo II and 1 mM novobiocin. (b) Decatenation of kDNA in the presence of human Topo II and various drugs concentrations. Lane 1, decatenation assay control with 2 units of Topo II; lane 2, the same as lane 1 with 1.5% DMSO; lanes 3–6, decatenation reaction with 2 units of Topo II and 1000, 500, 250, 100 μM of radicicol respectively; lane 7, decatenation with 2 units of Topo II and 100 μM etoposide; lane 8, decatenation reaction with 2 units of Topo II and 1 mM novobiocin; lane 9, control kDNA; lane 10, decatenate kDNA marker; lane 11, linear DNA marker. The position of negatively supercoiled plasmid (form I, F I), nicked circular DNA molecules (form II, F II), catenated kDNA (c), and decatenate kDNA (d) are indicated.

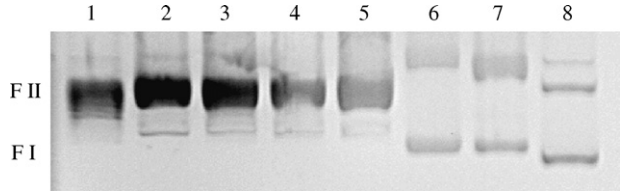

Fig. 3 – DNA topoisomerase I unwinding assay of relaxed pBR322 in the presence of radicicol and control intercalating (doxorubicin, adryamicin) and no intercalating (novobiocin) drugs. Lane 1, relaxed pBR322 plasmid control; lane 2, relaxed pBR322 incubated with 2 units of Topo I; lane 3, relaxed pBR322 incubated with 2 units of Topo I and 1.5% DMSO; lanes 4–7, the same as lane 2 but with 1 mM radicicol (lane 4), 1 mM novobiocin (lane 5), 100 μM doxorubicin (lane 6) or 100 μM adryamicin (lane 7); lane 8, negatively supercoiled pBR322 control. The position of negatively supercoiled plasmid (form I, F I), and nicked circular DNA molecules (form II, F II) are indicated.

inhibited by 1.5% DMSO, corresponding to the dose present in the highest concentration of radicicol tested (1 mM) (compare lanes 2 and 7). Lanes 8, 9 correspond to the inhibition of DNA relaxation by two known type IIA DNA topoisomerase inhibitors, ICRF 193 and etoposide. As shown in Fig. 2a, lane 3, the pattern of DNA migration was not affected by the highest concentration of radicicol tested (1 mM), suggesting that radicicol either does not bind to DNA or is only bound with low affinity and is removed during the migration of DNA into the gel.

Decatenation of kDNA was also inhibited by radicicol (Fig. 2b). The minimum radicicol concentration required for complete inhibition of decatenation by human Topo II (500 μM, lane 5) was higher than those required to inhibit relaxation or to completely inhibit decatenation by Topo VI (250 μM). This could be explained by previous results showing that human Topo II has a three-fold higher binding rate to kDNA than with plasmid DNA [35]. Lanes 10 (Fig. 2a) and 8 (Fig. 2b) show that at high concentration (1 mM), the well-known gyrase inhibitor novobiocin inhibited relaxation and decatenation by the human Topo II, as previously described by Hammonds and co-workers with ATPase assays [36].

3.2. *Radicicol does not intercalate into DNA*

Eukaryotic Topo II is especially sensitive to DNA intercalating agents. We have thus tested the possibility that radicicol intercalates into DNA (with low affinity) using the Topo I-catalysed unwinding assay first described by Pommier et al. [37]. As a control, we performed the same experiment using the non-intercalating drug novobiocin and the intercalating drugs doxorubicin and adryamicin. In this method, a relaxed plasmid is incubated in the presence of a eukaryotic Topo I and the drug to be tested. Compensatory positive supercoiling introduced in the relaxed plasmid by an intercalating drug that unwinds DNA will be removed by the activity of the enzyme. Removal of the drug after completion of the reaction thus produced negative supercoiling in the case of an intercalating drug. As shown in Fig. 3, incubation of relaxed pBR322 with wheat germ Topo I produced some modification of the pattern of relaxed topoisomers and the appearence of a low amount of linear DNA (compare lanes 1 and 2). The same result was obtained in the presence of 1 mM radicicol, indicating that this drug does not inhibit eukaryotic Topo I. As expected, the relaxed plasmids incubated with the Topo I in the presence of the two intercalating drugs were negatively supercoiled (Fig. 3, lanes 6 and 7). The two forms of the plasmid (nicked and supercoiled) migrated more slowly than control plasmids after treatment with Topo I and intercalating drug (compare lanes 6, 7 with lane 8), suggesting that intercalating drugs remained bound to the plasmids during the electrophoresis and modified slightly on migration in the gel. In contrast to those incubated with intercalating drugs, the relaxed plasmids that were incubated in the presence of radicicol or novobiocin remained fully relaxed (lanes 4 and 5), indicating that radicicol, as novobiocin, does not induce DNA unwinding. Radicicol also does not induce DNA overwinding (as it is the case for some drugs that interact with the DNA minor groove) since this would have produced positive supercoiling.

3.3. *Radicicol does not stabilize the cleavable complex in the presence of human Topo II*

Many drugs active against Topo IIA (either intercalators or non intercalators) stabilize the transient covalent complexes formed between DNA and the enzyme linked to DNA double-strand breaks (known as cleavable complexes), converting the enzyme into a physiological poison. We have shown previously that radicicol does not stabilize the cleavable complex in the presence of *S. shibatae* Topo VI [25]. However, since the mechanism of cleavable complex formation is different for human Topo II and archaeal Topo VI (being ATP dependent in the case of Topo VI but not in the case of the Topo IIA [38]), we decided to test if radicicol could stabilize the cleavable complex in the presence of human Topo II (Fig. 4). As a control, we

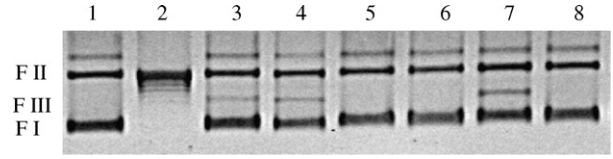

Fig. 4 – Radicicol does not induce cleavable complex formation. Negatively supercoiled pBR322 was incubated in the presence of the human Topo II and various drugs for 30 min at 37 °C and cleavage products were trapped by addition of SDS and proteinase K (see Section 2). Lane 1, negatively supercoiled pBR322 control; lane 2, relaxed assay control with 2 units of Topo II and 3 mM ATP; lane 3–4, the same as lane 2 with 2 units of Topo II and 100 μM etoposide in absence or presence of 3 mM ATP, respectively; lanes 5–6, assay reaction with 2 units of Topo II and 1 mM radicicol in absence or presence of 3 mM ATP; lane 7, assay reaction with 2 units of Topo II, 100 μM etoposide and 3 mM AMP-PNP; lane 8, assay reaction with 2 units of Topo II, 1 mM radicicol and 3 mM AMP-PNP. The position of negatively supercoiled plasmid (form I, F I), nicked circular DNA molecules (form II, F II), and linear DNA (form I, F I), are indicated.

incubated pBR322 with Topo II and etoposide, an antitumoral drug known to stabilize the cleavable complex in the presence of human Topo II. In this experiment, the human Topo II and the drugs were incubated for 30 min at 37 °C and cleavage products were trapped by addition of SDS and proteinase K (see methods). As shown in Fig. 4 lanes 3, 4, 7, one could observe the ATP independent formation of double-strand DNA breaks in the presence of 100 μM etoposide and human Topo II (as indicated by the appearence of a linear DNA form III). The same experiment with radicicol instead of etoposide (lanes 5,6,8) shows that 1 mM radicicol does not stabilize the cleavable complex (no double-strand break induction) either with or without ATP (lane 5 and 6), or in the presence of an ATP analogue (lane 8).

3.4. *Radicicol inhibits the etoposide-induced formation of double-strand DNA breaks by human Topo II*

Beside drugs that enhance DNA cleavage, Topo IIA are inhibited by compounds that do not interfere with the breakage-religation steps of the topoisomerization cycle but act at any other steps of the cycle (the so-called catalytic inhibitors). It has been shown that several catalytic inhibitors can prevent the stabilisation of the cleavable complex by cleavage enhancing drugs such as etoposide (Fig. 1b) or other poison [39–43]. We then tested the effect of radicicol on the stabilisation of the cleavable complex by etoposide (Fig. 5a and b). Control experiments confirmed that human Topo II is ATP-dependent (compare Fig. 5a, lanes 2 and 3) and that etoposide but not radicicol induces the formation of linear DNA (Fig. 5a, lanes 4 and 5, respectively; Fig. 5b, lanes 2 and 3, respectively). When radicicol (1 mM) and etoposide (100 μM) were added at the same time to the reaction mixture, we still observed the formation of cleavable complex (Fig. 5a, lane 6). However, when radicicol was added in the reaction mixture for 10 min at 37 °C before etoposide (at the same concentration than precedently), the induction of the cleavable complex was completely inhibited (Fig. 5b, lanes 5 and 6).

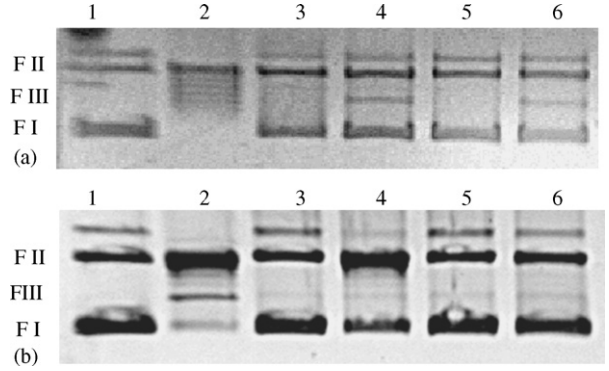

Fig. 5 – Radicicol inhibits the etoposide-induced formation of double-strand DNA breaks by human Topo II. Negatively supercoiled pBR322 was incubated in the presence of the human Topo II with various drugs concentrations for 30 min at 37 °C and cleavage products were trapped by addition of SDS and proteinase K (see Section 2). (a) Lane 1, negatively supercoiled pBR322 control; lane 2, relaxation assay with 2 units of human Topo II and 3 mM ATP; lane 3, the same as lane 2 but in absence of ATP; lanes 4 and 5, the same as lane 3 but with 100 μM of etoposide (4) or 1 mM of radicicol (5); lane 6, the same as lane 3 but with 100 μM of etoposide and 1 mM of radicicol added at the same time. (b) Lane 1, negatively supercoiled pBR322 control; lane 2, relaxation assay with 2 units of Topo II and 100 μM etoposide; lane 3, the same as lane 2 but with 1 mM radicicol; lane 4, the same as lane 2 but with 3% DMSO instead of etoposide; lanes 5 and 6, the same as lane 2 but with two differents concentrations of radicicol (100 μM and 1 mM) added before 100 μM etoposide. The position of negatively supercoiled plasmid (form I, F I), nicked circular DNA molecules (form II, F II), and linear DNA (form I, F I), are indicated.

4. Discussion

We have shown that radicicol, a well-known natural inhibitor of the eukaryotic chaperone HSP90, inhibits the alpha isoform of human Topo II, a member of the Topo IIA family, and a critical target for antitumoral drugs. Considering the close similarity between the alpha/beta isoforms, it is likely that radicicol inhibits also the beta isoform. The function of the beta isoform remains unclear, except for its involvement in neural development [44]. The beta Topo II is regulated differently than alpha and its level of expression does not change significantly during cell cycle [45]. The relative roles of the two isoenzymes as drug targets however have not been completely defined but the alpha isoform is probably the most important target of antitumoral drugs in vivo.

Radicicol does not induce supercoiling in a relaxed plasmid incubated in the presence of a eukaryotic Topo I (Fig. 3). This indicates that radicicol does not inhibit Topo II catalytic activities by altering DNA structure, as it is the case for intercalating drugs or for drugs binding to the DNA minor groove. Radicicol also does not stabilize the DNA cleavage in the presence of human Topo II (the same result was previously obtained with archaeal Topo VI). In that respect, radicicol behaves like Topo II inhibitors known as ''catalytic'' inhibitors, as opposed to drugs known as Topo II poisons.

We have previously reported that radicicol is also an inhibitor of the archaeal Topo VI, the prototype of the Topo IIB family [25], and it was reported earlier that radicicol inhibits two histidine kinases, the branched-chain alpha-keto acid dehydrogenase kinase and the yeast Sln1 protein [24]. HSP90, histidine kinases, human Topo II and archaeal Topo VI (all members of the GHKL protein superfamily) share an unusual ATP-binding module known as the Bergerat fold [2]. It has been shown by co-crystallization of radicicol and yeast HSP90 ATP-binding domain that radicicol prevents HSP90 activity by direct interaction with the ATP-binding site of its Bergerat fold. Since the Bergerat fold is the only structure common to Topo II, HSP90 and histidine kinases, it is reasonable to assume that radicicol also inhibits human Topo II by interacting with its Bergerat fold. The hypothesis of a direct interaction between radicicol and human Topo II is supported by in silico modelization showing that the ATP-binding sites of HSP90, Topo VI [25] and human Topo II (Fig. 6) can accommodate the presence of radicicol. The structure-based sequence

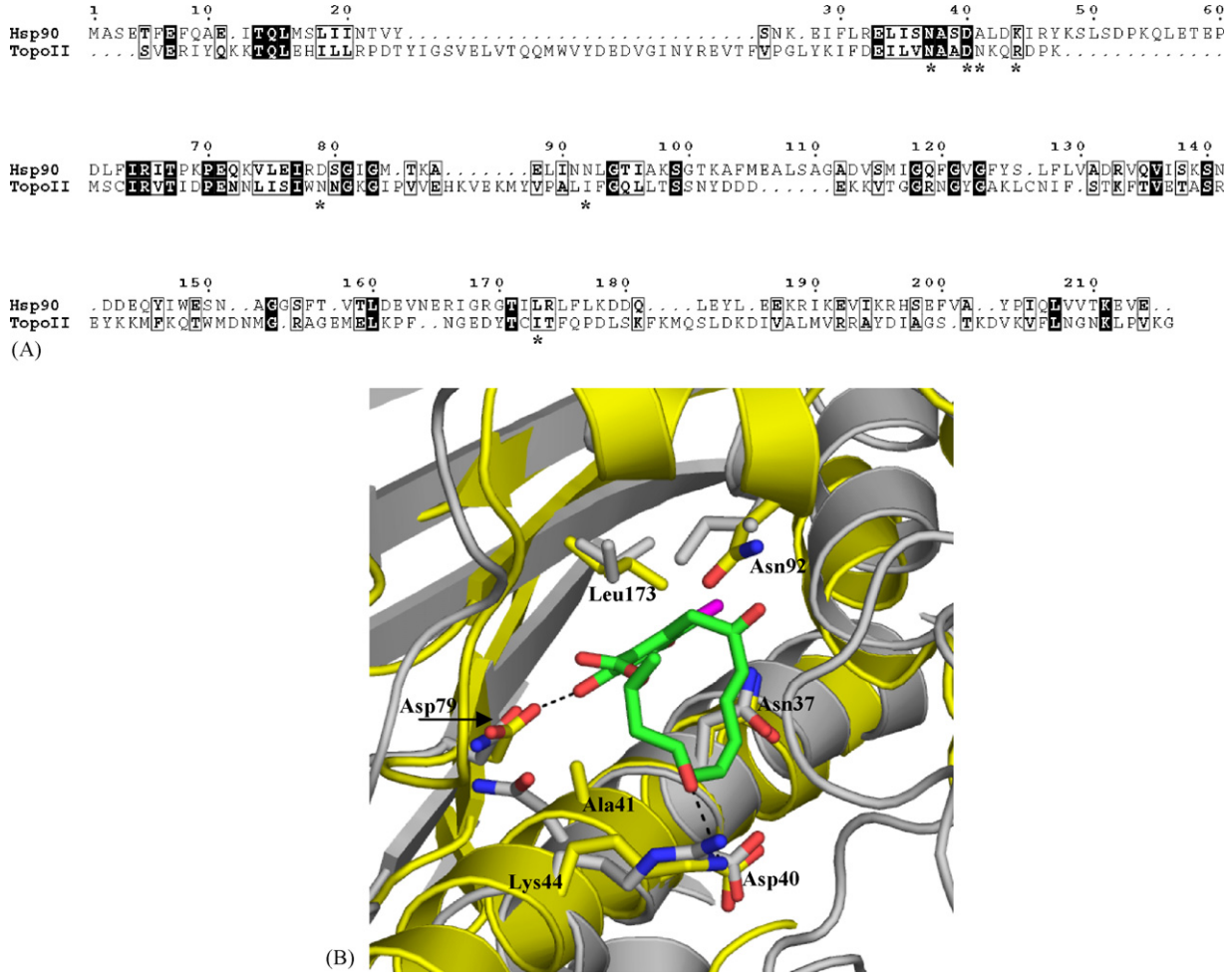

Fig. 6 – Comparison of the radicicol binding site from HSP90 with the homologous region in human DNA Topoisomerase II. (A) Structure-based amino-acid sequence alignment of the Bergerat fold regions of HSP90 from *Saccharomyces cerevisiae* and *Homo sapiens* DNA topoisomerase II. Strictly conserved amino-acid residues are in white on a black background. Partially conserved amino-acids are boxed. HSP90 amino-acids involved in radicicol binding are labelled with asterisks. (B) Superimposition of the radicicol binding site of *S. cerevisiae* HSP90 (yellow) (PDB code 1BGQ) with the homologous region in *H. sapiens* DNA topoisomerase II (grey) (PDB code 1ZXM) and bound radicicol. The radicicol molecule is shown as green sticks. HSP90 amino-acid residues contacting radicicol in the crystal structure are shown in sticks as well as their counterparts from *H. sapiens* DNA topoisomerase II. For clarity, only HSP90 amino-acid residues are indicated. The radicicol chlorine atom is colored violet. The two hydrogen bonds responsible for radicicol binding are depicted by black dashed lines.

alignment between radicicol-bound HSP90 and the ATP-binding domain from human Topo II shows that the seven HSP90 residues (Asn37, Asp40, Ala41, Lys44, Asp79, Asn92 and Leu173) involved in radicicol binding are homologues in the human Topo II (Fig. 6A). Furthermore, the two residues (Lys 44 and Asp79) responsible for hydrogen bonds with radicicol in HSP90 have similar physicochemical and steric properties in Topo II (Arg and Asn, respectively). Hence, these two interactions should still be involved in radicicol binding. Finally, from the superimposition of both structures, no residues from Topo II should preclude access to the putative radicicol binding site (Fig. 6B). We have performed a similar simulation to determine if radicicol could interact with the binding site of the bisdioxopiperazines, which are eukaryotic topoisomerase II drugs which partially inhibit ATP hydrolysis and convert these enzyme into an inactive salt-stable closed clamp around DNA [46–48]. The bisdioxopiperazine ICRF-187 binding site has been shown to partially overlap with the ATP-binding site [49]. In that case, we found that the ICRF binding pocket cannot accomodate radicicol (not shown). Although these observations strongly support the idea that the radicicol binding site on human Topo II is located in its ''Bergerat'' fold domain, as previously observed for HSP90, one cannot completely exclude that radicicol interacts with another site on human Topo II. For instance, although novobiocin binds to the Bergerat folds of bacterial Topo IIA [50], it inhibits HSP90 by interaction with another domain of the protein [51–54]. Direct proof that radicicol interacts with the Bergerat fold of human Topo II will require solving the structure of the human Topo II-radicicol complex.

If radicicol indeed binds to the Bergerat fold of human Topo II, it probably inhibits Topo II activity prior to DNA cleavage by interfering with ATP binding, as suggested for other Topo II catalytic inhibitors that interact with the ATP-binding module of Topo IIA [55,56]. Interestingly, we observed that radicicol not only fails to stabilize the DNA cleavage complex in the presence of human Topo II but also inhibits the stabilization of the induced-etoposide cleavable complex (Fig. 4b). It has been shown previously that several catalytic inhibitors similarly prevent the formation of cleavable complex in the presence of Topo II poisons. As in the case with some of these compounds, radicicol could act by interfering with the binding of the poisons, and/or by interfering with the cleavage activity of the Topo II [39–43,57,58]. We favor the former hypothesis since ATP does not seem to be required for the DNA cleavage activity of the Topo IIA family (since double-strand break activity is obtained with the A′–B′ domain [59] and since the Bergerat fold is located quite far away from the active tyrosine involved in DNA cleavage). A direct competition between radicicol and/or etoposide for overlapping binding site would be in agreement with data showing the existence of two potential binding sites for etoposide in human Topo II, one within the active site of the core enzyme, and the other within the N-terminal ATPase domain containing the Bergerat fold [60]. This explain why radicicol only prevent the stabilization of the DNA cleavage when added before etoposide.

The inhibition of human Topo II by radicicol contrasts with the absence of effect of this drug on *E. coli* DNA gyrase [25], since both enzymes contain a Bergerat fold and belong to the same family (A) of Topo II. This indicates that radicicol affinity can vary for different subfamilies of GHKL proteins and even between different proteins of the same subfamily. This is not surprising, since it has been known for a long time that DNA gyrase and eukaryotic Topo II exhibit different patterns of responses to various pharmaceutical compounds. For instance, most quinolones-based drugs, like nalidixic acid, are specific for DNA gyrase, while antitumor drugs, like etoposide, preferentially inhibit eukaryotic Topo II. The different sensitivity of DNA gyrase and Topo IIA to radicicol is also reminiscent of data previously obtained with another Bergerat fold interacting drug, novobiocin, since this classical inhibitor of Topo IIA has no effect on *Sulfolobus shibatae* Topo VI [25].

The common sensitivity of *S. shibatae* Topo VI and human Topo II to radicicol indicates that the archaeal enzyme could be a useful tool to screen for new inhibitors that could also target HSP90 and/or human Topo II (or possibly other Bergerat fold proteins, such as MutL or histidine kinases). In particular, the *S. shibatae* Topo VI should be a priori more stable and easier to handle than human HSP90 or Topo II, since it has been isolated from a hyperthermophilic microorganism. The crystal structures of the Bergerat folds of *S. shibatae* Topo VI, yeast Topo II and yeast HSP90 and are all available for modelling protein/drugs interactions [31,49,61,62].

The sensitivity of human Topo II to radicicol in vitro is similar to the sensitivity of the archaeal Topo VI (in the mM range) but much lower than that of HSP90 (in the nanomolar range). It is thus unlikely that the effect of radicicol on eukaryotic cells or organisms in vivo (assumed to be HSP90-specific) previously reported in the literature are due to a combined effect of this drug on both HSP90 and Topo II. However, one has to be cautious with such a conclusion since it is difficult to extrapolate the sensitivity of human Topo II to radicicol in vitro to the in vivo situation. The drug may be concentrated and/or metabolised into more active compounds in vivo.

Moreover, Barker and coworkers have recently shown by immunoprecipitation that Topo II and HSP90 physically interacts in human cell extracts and that radicicol enhances the growth inhibition and cell killing by etoposide in vivo [63,64]. Interestingly, the DNA topoisomerase activity detected in crude extract is enhanced in the presence of radicicol, probably because this drug destabilizes the Topo II/HSP90 complex, increasing the number of free Topo II molecules [64]. This suggests that the synergy observed between radicicol and etoposide is due to an increase in the number of free Topo II available to form cleavable complexes. The difference between those last results and the data reported here is easily explained by the fact that we worked with purified Topo II and higher concentrations of radicicol.

Although etoposide is a very effective drug for the treatment of several types of cancer, it induces DNA damages, such as chromosomal translocations, leading to therapy-related leukemia [65,66]. However it has been shown that combined treatment with etoposide and catalytic inhibitors of Topo II (resembling radicicol in their mode of action) could improve the antitumor selectivity of etoposide [67–71]. The combination of etoposide and low concentration of radicicol has already been tested by Barker and coworkers who found a synergic effect [63]. In contrast, our in vitro data suggest that high concentration of radicicol may reduce the etoposide effect, while inhibiting the Topo II DNA topoisomerase activities and HSP90 chaperone activities. The use of radicicol derivatives (if good ones could be obtained, see below) in cancer therapy could then have a complex pattern.

Radicicol and its derivatives, cannot be used presently in clinical trials because of their toxicity and metabolic instability in animals. In fact, they bear a resorcinol moiety that makes them prone to glucuronidation which leads to unsuitable pharmacokinetics. Presently the more promising Hsp90 inhibitor for anticancer treatment is geldanamycin and its derivatives. The inhibitor 17-allylamino, 17-demethoxygeldanamycin (17AAG) has been the first Hsp90 inhibitor to be tested in completed human phase I trials [72–74] and also in several phase II trials (see table in [74]). However, we have shown previously that, in contrast to radicicol, geldanamycin does not inhibit DNA topoisomerase VI [25] and modelling study have shown that geldanamycin, which is larger than radicicol, cannot be accomodated in the active site of Topo II ([25] and Marc Graille, data not shown).

As new Hsp90 inhibitors are currently under investigation, it will be interesting to test them also against human Topo II.

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