DNA Sequence Specificity for Topoisomerase II Poisoning by the Quinoxaline Anticancer Drugs XK469 and CQS

HANLIN GAO, EDITH F. YAMASAKI, KENNETH K. CHAN, LINUS L. SHEN, and ROBERT M. SNAPKA

Departments of Radiology (H.G., E.F.Y., R.M.S.) and Molecular Virology, Immunology, and Medical Genetics (H.G., R.M.S.), College of Medicine and Public Health and College of Pharmacy (K.K.C.), the Ohio State University, Columbus, Ohio; and Abbott Laboratories (L.L.S.), Abbott Park, Illinois

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ABSTRACT

The two known antineoplastic quinoxaline topoisomerase II poisons, XK469 (NSC 697887) and CQS (chloroquinoxaline sulfonamide, NSC 339004), were compared for DNA cleavage site specificity, using purified human topoisomerase IIα and human topoisomerase IIβ. The DNA cleavage intensity pattern for topoisomerase IIα poisoning by CQS closely resembled that of VM-26, despite the lack of any apparent common pharmacophore. In contrast, the topoisomerase IIα DNA cleavage intensity patterns of XK469 and CQS were very different from one another despite the similar overall structures of the two drugs. This suggests that the differences in DNA site specificity of topoisomerase II poisoning by XK469 and CQS may be caused by differences in their geometry, side chains, or electronic structure. The topoisomerase IIβ-mediated DNA cleavage sites of CQS and XK469 were also very different from one another, adding further support to this idea. Earlier work has demonstrated that a number of specific topoisomerase II poisons show very similar patterns of DNA cleavage with either topoisomerase IIα or topoisomerase IIβ, suggesting that the topoisomerase II isozymes play only a minor role in choices of DNA cleavage sites. However, both of the quinoxaline topoisomerase II poisons in this study showed distinctly different and unique DNA cleavage intensity patterns with each topoisomerase II isozyme. This indicates that topoisomerase II isozymes can play a major role in DNA cleavage site selection for some classes of topoisomerase II poisons.
quences (Capranico et al., 1994b; Capranico et al., 1997; Borgnetto et al., 1999).

The quinoxaline anticancer drugs CQS and XK469 (Fig. 1) were both found to have activity against solid tumors (Shoemaker, 1986; Valeriote et al., 1996; Corbett et al., 1998). In both cases, the molecular targets relevant to the anticancer activity remained elusive as the drugs progressed through animal model testing to human clinical trials. XK469 was found to be the first highly selective topoisomerase IIβ poison (Gao et al., 1999). Studies with topoisomerase IIβ knockout mouse cells confirmed the in vitro results and showed that topoisomerase IIβ is the cytotoxic target of XK469 in vivo (Snakpa et al., 2001). Based on its structural similarity to topoisomerase II isozymes, but the details of their structure and their electronic properties are clearly different, possibly accounting for the differences in topoisomerase II isozyme specificity and the requirement of chaotropic protein denaturants for detection of CQS stabilized topoisomerase II-DNA cleavage complexes. Our study tests the hypothesis that the structural and electronic differences in XK469 and CQS will strongly affect their patterns of topoisomerase II-mediated DNA cleavage.

Materials and Methods

Drugs and Reagents. XK469 (NSC 697887) was provided by the National Cancer Institute Drug Synthesis Branch, Bethesda, MD. CQS (chloroquinazoline sulfonamide, NSC 339004) was provided by Dr. R. Shoemaker (National Cancer Institute, Bethesda, MD). VM-26 (teniposide, NSC 122819) was obtained from the National Cancer Institute, Division of Cancer Treatment, Natural Products Branch (Bethesda, MD). Dimethyl sulfoxide was the solvent for all drug stocks. Recombinant human topoisomerase IIα was obtained from N. Osheroff (Vanderbilt University, Nashville, TN) (Kingma et al., 1997). Recombinant human topoisomerase IIβ was a generous gift of Dr. Caroline Austin, (University of Newcastle, Newcastle-upon-Tyne, UK) (Austin et al., 1995).

Mapping of Topoisomerase II-DNA Cleavage Sites. Sites of topoisomerase II mediated DNA cleavage stimulated by the drugs were mapped as described previously (Huang et al., 2001). Briefly, a DNA substrate consisting of a 516 base-pair EcoRI-StuI fragment of pBR322 (residues 3846–4362) was labeled with 32P by filling in the overhanging EcoRI end with Klenow fragment (USB Corp., Cleveland, OH) and a mix containing dCTP, dGTP, dTTP, and [γ-32P]dATP (3000 Ci/mmol; Amersham Biosciences, Piscataway, NJ). Topoisomerase II reaction mixes contained the end-labeled DNA fragment (1–2 × 105 dpm), 10 mM HEPES-HCl, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, 1 mM ATP, and the drug being tested. Reactions were started by adding the topoisomerase (0.8 or 1.2 μg of human topoisomerase IIα or IIβ, respectively) after a preincubation of the other components at 37°C for 5 min. These concentrations were chosen because they gave equal topoisomerase II poisoning with VM-26 (Huang et al., 2001). The epipodophyllotoxins VM-26 and VP-16 show little or no isozyme selectivity for topoisomerase II poisoning (Austin et al., 1995). The final reaction volume was 20 μl. After a 30-min incubation at 37°C, the reactions were terminated by addition of 2 μl of 4 M GuHCl. The DNA was ethanol-precipitated and then resuspended in proteinase K solution (0.2 mg/ml, 28 μl, 2 h, 45°C). The protein-free DNA was precipitated with ethanol and resuspended in gel loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue). The samples were heated to 70°C for 2 min, cooled to room temperature, and then loaded onto a polyacrylamide sequencing gel (8% acrylamide, 19.1% acrylamide/bisacrylamide, and 7 M urea in Tris-borate buffer). Electrophoresis was done at 1800 V for 2 or 6 h, and the gel was then transferred to Whatman 3MM paper and exposed to Hyperfilm for autoradiography. The 2- and 6-h electrophoresis times gave better resolution of the smaller and larger DNA fragments and allowed more complete mapping of topoisomerase II-mediated cleavages on the target DNA. Sanger dyeoxy DNA sequence ladders were made with the fmol cycle DNA sequencing system (Promega, Madison, WI). The primer, 5’-AAATTCCTGGA-GAGCGAAAGGGCC-3’, complementary to the EcoRI end of the 516-base pair pBR322 fragment, was labeled at the 5’-end by T4 polynucleotide kinase with [γ-32P]ATP and used without further purification. The polymerase chain reaction was carried out for 30 cycles with Taq DNA polymerase, using the appropriate dyeoxy-dideoxy-NTP mix for each reaction. The reactions were stopped by addition of fmol sequencing reaction stop solution, and the DNA was denatured at 70°C before gel loading. Because the sequenced strand
was labeled on the 5’ EcoRI end and was complementary to the strand on which the topoisomerase-mediated sequences were mapped, it was necessary to translate the sequence to determine the cutting sites. The significance of differences in the occurrence of specific bases within cleavage sites was determined by comparing the observed and expected values based on exact polynomial probabilities.

Results

Topoisomerase IIα. The results of this study are shown in Figs. 2 and 3. Figure 2 shows the patterns of topoisomerase IIα- and IIβ-mediated DNA cleavage on short (2-h electrophoresis) DNA sequencing gels, and the results are summed up in Fig. 3, which indicates all of the sites stimulated by the various drugs, including many weak DNA cleavage sites that are not clear from Fig. 2. Longer (6-h) electrophoresis experiments (not shown) were also done to refine and/or confirm the mapping of specific DNA cleavage sites as indicated in Fig. 3. The pattern of drug stimulated topoisomerase IIα-DNA cleavage was very similar for CQS and VM-26 (Fig. 2A). Both drugs tended to stimulate topoisomerase IIα-DNA cleavages at the same sites; in addition, the relative strengths of the cleavages tended to be similar for CQS and VM-26. Several of these sites of strong CQS and VM-26 stimulated cleavage represent enhanced cleavage of normal topoisomerase IIα cleavage sites (Fig. 2, lane α, sites G295, T313, A343, G346, A357, G361, and others). That fact that VM-26 tends to enhance normal topoisomerase IIα cleavage sites has been noted by others (Capranico et al., 1993). Strong CQS and VM-26 stimulated cleavages can be seen at sites G295, G298, A343, G346, A357, and G361 as well as others. Moderate CQS and VM-26 cleavage sites can be seen at C396 and A399, whereas weak CQS and VM-26 cleavage sites are evident at T384 and A387. Topoisomerase IIα has been reported to have an affinity for cleavage in alternating purine-pyrimidine (RY) repeats, resulting in multiple strong cleavages (Spitzner and Mulder, 1988). For instance, there are strong VM-26 cleavages at T355, G431, and A434, which are not matched by CQS stimulated cleavage. Likewise, there are CQS cleavages at G238, A248, A266, and C242 that are not matched by comparable VM-26 stimulated cleavages. Overall, however, the two drugs show very similar patterns of topoisomerase IIα mediated DNA cleavage.

XK469-stimulated topoisomerase IIα-DNA cleavages have a pattern that is very distinct from those of CQS and VM-26. Consistent with the finding that XK469 is a strong topoisomerase IIβ poison but a poor topoisomerase IIα poison (Gao et al., 1999), the XK469 stimulated topoisomerase IIα DNA cleavages are very weak. The strongest XK469 stimulated topoisomerase IIα-DNA cleavages tend to be much weaker than those of CQS and VM-26, and, compared with the strong CQS and VM-26 cleavages, can be considered only moderate at best. Although faint XK469 cleavages often occur at sites of strong CQS and VM-26 cleavage (71% of the XK469 cleavages match CQS cleavages), the relative strength of the cleavages are very different. No XK469 cleavages were detected in the region below the DNA strand break at C413, whereas CQS and VM-26 both have uniquely strong cleavage sites in this region. One of the strongest XK469 cleavage sites occurs at G402, where CQS and VM-26 cleavages are not detectable. Other significant XK469 cleavage sites occur at T358 (just below the strong CQS, VM-26 cleavage sites at A357) and at C362 (just below the strong CQS, VM-26 cleavage site at G361). Both of the latter two sites are unique to XK469. Another strong XK469 site, at T310, corresponds to a CQS cleavage site of comparable strength.

Studies with eukaryotic topoisomerase II have indicated that DNA sequence is the primary determinant of topoisomerase II cleavage specificity and strength (Spitzner and Mulder, 1988). For DNA cleavages stimulated by topoisomerase II poisons, the strongest base preferences tend to be the −1 and +1 positions relative to the topoisomerase cleavage sites (Palumbo et al., 2002). Studies of drug-stimulated topoisomerase II mediated DNA cleavage have shown that VM-26 stimulated cleavages are favored by a C at the −1 position.

Fig. 2. Mapping of topoisomerase IIα- and IIβ-mediated DNA cleavage sites. A, topoisomerase IIα-mediated DNA cleavage was stimulated by CQS, XK469, and VM-26. The gel was run for 2 h to obtain optimum resolution of the low molecular weight DNA fragments. A 6-h electrophoresis (not shown) was used to obtain optimum resolution of high molecular weight DNA fragments. Sequencing ladders: G, guanine; A, adenine; T, thymine; C, cytosine; D, substrate DNA only; α, topoisomerase IIα with substrate DNA; Q, CQS (2 mM); X, XK469 (2 mM); V, VM-26 (100 μM). The sequencing ladders were done on the opposite strand from that used for mapping topoisomerase IIα-mediated cleavage sites (see Fig. 3), so it is necessary to translate the sequence to the opposite strand to identify the topoisomerase IIα mediated cleavage sites. Cleavage sites are identified by the base at the +1 position (5’-side of the topoisomerase IIα cleavage) and the number of the base in the cloned fragment. B, topoisomerase IIβ-mediated DNA cleavages stimulated by CQS and XK469. The same substrate DNA was used for mapping both topoisomerase IIβ and topoisomerase IIα cleavages. Again, both short (2 h, shown) and long (6 h, not shown) electrophoresis runs were made to optimize resolution in different parts of the sequence. β, topoisomerase IIβ with substrate DNA; other abbreviations are the same as in Fig. 2A. The DNA strand break at C413 occurs normally in a fraction of the DNA substrate molecules.
Fig. 3. Distribution of topoisomerase II- and drug-stimulated topoisomerase II cleavages on the 516-base pair pBR322 substrate DNA. The primer used for dideoxy DNA sequence ladders is indicated by underlining at the EcoRI end, and the 32P-labeled adenine residues incorporated into the strand for mapping topoisomerase II cleavages are indicated by bold letters and shading. The relative strengths of individual cleavages are indicated by the weight of the symbol for each drug (bold, strong cleavage; normal weight, average or moderate cleavage; gray, weak or very weak cleavage). X, XK469; Q, CQS; V, VM-26; T, topoisomerase II alone. The DNA strand-break at C413 is present in a fraction of the substrate DNA molecules before addition of enzymes or drugs (Huang et al., 2001). All +1 bases (3' relative to the topoisomerase-mediated cleavage) for the drugs are indicated by bold, beneath the symbols, indicating specific drug-stimulated DNA cleavages.
relative to the site of DNA cleavage (Pommier et al., 1991; Capranico et al., 1993; Capranico et al., 1997). Our data are consistent with this. Of the 25 strong or moderate VM-26 stimulated topoisomerase IIα cleavages, 12 have a C at the −1 position, whereas only 5.2 would be expected for random occurrence based on the frequency of C in the substrate DNA. This difference is very significant \( P = 0.002 \). Similarly, of 26 strong or moderate CQS-stimulated topoisomerase IIα cleavage sites, 12 have C at the −1 position where 5.5 would be expected \( P = 0.006 \). Of 22 XK469 stimulated topoisomerase IIα cleavage sites (most weak), 11 have C at the −1 position \( P = 0.002 \). These results indicate that XK469, like CQS and VM-26, tends to stimulate topoisomerase IIα cleavage at sites with a C at the −1 position. However, the cleavage intensity pattern for XK469-stimulated topoisomerase IIα cleavages is distinctively different from those of CQS and VM-26. In two instances, relatively strong XK469-stimulated topoisomerase IIα cleavages occur just one base to the 3’ side of strong VM-26 and CQS cleavages (at T358 and C362, Figs. 2 and 3). Although this is a striking visual feature of the sequencing ladders in Fig. 2A, the significance is not clear. XK469-stimulated topoisomerase IIβ cleavages correspond to the XK469-stimulated topoisomerase IIα cleavages in both cases, and XK469-stimulated topoisomerase IIβ cleavages also occur one base pair to the 3’ side of strong VM-26 and CQS topoisomerase IIα cleavages at T296 and C314. However, XK469-stimulated topoisomerase IIα and IIβ cleavages match exactly strong VM-26 and CQS cleavages at A248 and A343 and are offset by three base pairs at G402. Because XK469-stimulated topoisomerase IIα and IIβ cleavages often occur independently of one another and nowhere near strong VM-26 or CQS sites (see Fig. 3, row 241), a much larger data set would have to be analyzed to determine whether these one-base pair offsets relative to strong VM-26- and CQS-stimulated topoisomerase IIα cleavages are significant.

**Topoisomerase IIβ.** XK469 was compared with CQS for stimulation of topoisomerase IIβ-mediated DNA cleavage. As shown in Fig. 2, the XK469 pattern was again distinctive. CQS stimulated relatively few topoisomerase IIβ-mediated DNA cleavages, but a number of these were strong, such as the cleavages at G295, T329, A343, G346, and C396. XK469 caused a single very strong topoisomerase IIβ cleavage at T358 and numerous moderate and weak cleavages. Many of the XK469-stimulated topoisomerase IIβ DNA cleavages were also distinctly different from those stimulated by CQS. Whereas 16 of 22 (73%) of the XK469-stimulated topoisomerase IIα cleavages corresponded to CQS-stimulated topoisomerase IIα cleavages, only 3 of 20 (15%) of the XK469-stimulated topoisomerase IIβ cleavages matched the CQS-stimulated topoisomerase IIβ cleavages. In addition to the overall differences in topoisomerase IIβ cleavage sites, the cleavage intensities were also very different for CQS and XK469. XK469 stimulated one very strong topoisomerase IIβ cleavage and a number of moderate cleavages on the substrate DNA used in this study but caused only weak to moderate cleavages with topoisomerase IIα. This is consistent with the previously reported specificity of XK469 for topoisomerase IIβ (Gao et al., 1999). Although CQS stimulates DNA cleavage at many of the same sites with topoisomerase IIα and topoisomerase IIβ, the relative intensity patterns are very different. There are strong CQS stimulated cleavages at G295, T329, A343, and C396 for both isozymes, but the strong CQS-stimulated topoisomerase IIα cleavages at G235, G238, A248, A266, T310, and A357 are either missing or greatly reduced with the β isozyme. The strongest XK469-mediated topoisomerase IIβ cleavage, at T358, is also one of the stronger XK469 cleavages for topoisomerase IIα (the somewhat stronger CQS and VM-26 cleavages at A357 are indicated for topoisomerase IIα in Fig. 2A, and the XK469-topoisomerase IIα cleavage at T358 can be seen between them, one base lower on the gel). Likewise, the XK469 cleavage at G402 is apparent, with both isozymes. However, most of the numerous XK469 cleavages seen with topoisomerase IIβ do not match cleavages of similar intensity in the topoisomerase IIα experiment. Of 22 XK469 topoisomerase IIα cleavages and 20 XK469 topoisomerase IIβ cleavages, only five are common to both isozymes.

**Discussion**

Most topoisomerase poisoning assays use the detergent SDS to inactivate topoisomerases trapped in drug-stabilized topoisomerase-DNA cleavage complexes and convert them to irreversible protein-DNA crosslinks. However, CQS-stabilized topoisomerase IIα and IIβ-DNA cleavage complexes are not efficiently detected when SDS is used, and it is necessary to use chaotropic protein denaturants instead (Gao et al., 2000). Aside from this unusual feature, shared only with ICRF-193 at this time (Huang et al., 2001), CQS seems to be a typical topoisomerase II poison, resembling VM-26 in its DNA sequence specificity and lack of pronounced topoisomerase II isozyme preference. In addition to sharing many sites, the relative strengths of CQS and VM-26 stimulated topoisomerase IIα cleavages tend to be comparable, resulting in overall patterns that are quite similar, although each drug does stimulate a few significant cleavages at unique sites not shared by the other. Many of the VM-26 and CQS stimulated cleavages occur at sites normally cleaved by topoisomerase IIα in the absence of drugs.

The relation of CQS-stimulated topoisomerase IIα cleavage to VM-26 stimulated cleavage is very similar to that reported for VM-26 and mitoxantrone (Capranico et al., 1993). In general, drugs with similar shapes, and with shared pharmacophores and electronic structure, tend to have similar topoisomerase II-mediated DNA cleavage patterns, whereas topoisomerase II poisons of different chemical classes cause very different DNA cleavage patterns and/or cleavage intensity patterns (Capranico et al., 1993, 1994a, 1998; Guano et al., 1999). However, there are exceptions to this rule. Some drugs with very similar structures cause different patterns of topoisomerase-mediated DNA cleavage, and some drugs of very different structure may have similar patterns (Capranico et al., 1997). VM-26 and mitoxantrone represent an example of structurally dissimilar drugs with similar patterns of topoisomerase-mediated DNA cleavage. Both drugs tend to stimulate topoisomerase II cleavages at the same sites, often sites cleaved by topoisomerase II in the absence of drugs, yet they have no common pharmacophore, and mitoxantrone is a DNA intercalator, whereas VM-26 is not (Capranico et al., 1993). CQS represents another case of a drug with
no apparent pharmacophore in common with VM-26, yet mimics its topoisomerase IIα cleavage intensity pattern.

Many of the sites of CQS and VM-26 topoisomerase IIα cleavage also correspond to sites of ICRF-193–stimulated topoisomerase IIβ DNA cleavage (Huang et al., 2001). Among these are T203, G207, G235, G295, G298, T304, T313, A343, G346, A357, G361, C396, and A399. In addition, the patterns of ICRF-193- and CQS-stimulated topoisomerase IIβ cleavages are very similar, including not only the sites of cleavage, but the relative strengths of the cleavages. As noted under Results, the CQS cleavage intensity pattern on topoisomerase IIβ is very different from the CQS cleavage intensity pattern on topoisomerase IIα. Several topoisomerase II poisons such as VM-26 (Drake et al., 1989; Cornarotti et al., 1996), 4′-(9-acridinylamino)methanesulfon-m-anisidine (amascrine) (Marsh et al., 1996), 4-demethoxy-3′-deaminol-3′-hydroxy-4′-epi-doxorubicin I (Cornarotti et al., 1996), and other anthracycline analogs (Guano et al., 1999) show topoisomerase IIβ cleavage intensity patterns that strongly resemble their topoisomerase IIα cleavage intensity patterns. This has been interpreted as evidence that the binding of these drugs is very similar in the enzyme-DNA-drug ternary complex of both isozymes, and it has been suggested that the interactions of these drugs with the topoisomerase II isozymes may involve mainly the highly conserved active site residues of the two isozymes (Cornarotti et al., 1996; Palumbo et al., 2002). Based on this model, drugs that show topoisomerase IIβ cleavage intensity patterns that differ markedly from their topoisomerase IIα cleavage intensity patterns would interact significantly with nonconserved active site features in topoisomerase IIβ, resulting in an altered cleavage intensity pattern. The resemblance of the CQS topoisomerase IIβ cleavage intensity pattern to the ICRF-193 cleavage intensity pattern suggests that the two drugs share some similarity in their interaction with topoisomerase IIβ despite their very different structures. Flavonoid topoisomerase II poisons have also been found to have very different cleavage patterns on topoisomerase IIα and IIβ (Austin et al., 1995).

The pattern of XK469-stimulated topoisomerase IIα cleavages differs mainly in the relative strength of the cleavages. The XK469 topoisomerase IIα-mediated cleavages are generally very weak compared with those of CQS and VM-26. The strongest XK469 cleavages (which are moderate to weak compared with the strong CQS and VM-26 cleavages) do not correspond with the positions of the strongest CQS and VM-26 cleavages but often occur at positions of very weak CQS and/or VM-26 cleavage. The generally weak XK469-stimulated topoisomerase IIα cleavages are consistent with our previous finding of strong β-isozyme selectivity for XK469 (Gao et al., 1999). ICRF-193 is structurally unrelated to the quinolines but resembles XK469 in its preference for the β-isozyme of human topoisomerase II (Gao et al., 1999), although the preference is not as pronounced as that of XK469. XK469, in contrast to CQS, shows differences not only in average strength of cleavage between the two isozymes, but also many cases of differences in cleavage site specificity. The XK469 topoisomerase IIα and topoisomerase IIβ cleavage intensity patterns are quite distinct, which underscores the idea that XK469’s interaction with the topoisomerase IIβ active site is very different from its interaction with the topoisomerase IIα active site. The model discussed above would predict that XK469 interacts strongly with the topoisomerase IIβ active site features that are not found in the topoisomerase IIα active site. The fact that the XK469 topoisomerase IIα cleavage pattern is distinctively different from those of VM-26 and CQS and that its topoisomerase IIβ cleavage pattern is very different from those of CQS and ICRF-193 suggests that XK469 may have unique interactions in the active site of each topoisomerase II isozyme that are not shared by the two isozymes. The fact that both XK469 and CQS are topoisomerase II poisons suggests that the overall quinoline structure is favorable for stabilization of topoisomerase II-DNA cleavage complexes. For the quinolines, the topoisomerase II isozymes seem to play a major role in DNA cleavage site selectivity. However, the two drugs differ significantly in structural detail and chemical and electronic properties. The potential hydrogen bonding properties of XK469 and CQS are also clearly very different, not only in the modifying group on the smaller aromatic ring (-NH₂ for CQS versus -O-CH(CH₃)₂-CO-H for XK469) but also on the linking group between the two aromatic ring systems (-NH-SO₂- for CQS versus -O- for XK469). The differences in structure and electronic properties may account not only for the marked differences in topoisomerase II-mediated DNA cleavage site selectivity for these drugs but also for the differences in isozyme preference and trapping of cleavage complexes by different protein denaturants. Quinolines seem to hold unusual promise as topoisomerase II poisons with IIIs properties.

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**Address correspondence to:** Dr. Robert M. Snapka, The Ohio State University, Department of Radiology, 103 Wiseman Hall, 400 West 12th Avenue, Columbus, OH 43210. E-mail: snapka.1@osu.edu