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DIDS, a chemical compound that inhibits RAD51-mediated homologous pairing and strand exchange

Takako Ishida[1], Yoshimasa Takizawa[1], Takashi Kainuma[1], Jin Inoue[2], Tsutomu Mikawa[2,3], Takehiko Shibata[2], Hidekazu Suzuki[4], Satoshi Tashiro[4] and Hitoshi Kurumizaka[1,*]

[1]Laboratory of Structural Biology, Graduate School of Advanced Science and Engineering, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, [2]RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, [3]RIKEN SPring-8 center, 1-1-1 Kouto, Sayo, Sayo, Hyogo 679-5148 and [4]Department of Cellular Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

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ABSTRACT

RAD51, an essential eukaryotic DNA recombinase, promotes homologous pairing and strand exchange during homologous recombination and the recombinational repair of double strand breaks. Mutations that up- or down-regulate *RAD51* gene expression have been identified in several tumors, suggesting that inappropriate expression of the RAD51 activity may cause tumorigenesis. To identify chemical compounds that affect the RAD51 activity, in the present study, we performed the RAD51-mediated strand exchange assay in the presence of 185 chemical compounds. We found that 4, 4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) efficiently inhibited the RAD51-mediated strand exchange. DIDS also inhibited the RAD51-mediated homologous pairing in the absence of RPA. A surface plasmon resonance analysis revealed that DIDS directly binds to RAD51. A gel mobility shift assay showed that DIDS significantly inhibited the DNA-binding activity of RAD51. Therefore, DIDS may bind near the DNA binding site(s) of RAD51 and compete with DNA for RAD51 binding.

INTRODUCTION

Genomic DNA is continuously under attack from exogenous and endogenous mutagens, such as ionizing radiation, oxygen-free radicals, DNA cross-linking reagents and DNA replication failure. Such mutagens cause double-strand breaks (DSBs), which induce chromosome aberrations and tumorigenesis if they are not repaired appropriately (1,2). Homologous recombinational repair (HRR) is an accurate pathway for DSB repair without base substitutions, deletions and insertions (3–5).

RAD51 is an essential protein for the HRR pathway (6). The *RAD51*-gene knockout in mice results in early embryonic lethality (7,8). In chicken DT40 cells, the *RAD51*-knockout causes cell death, with the accumulation of unrepaired DSBs (9). These facts indicated that RAD51 is actually required for DSB repair in higher eukaryotes. Consistent with the importance of RAD51 in the HRR pathway, mutations in the *RAD51* gene have been identified in several tumors (10–14). Most of the *RAD51* mutations in tumor cells were found in its non-coding region, suggesting that improper up- and down-regulation of the RAD51 activity may be a source of tumorigenesis. A missense RAD51 mutation, in which Arg150 is replaced by Gln (R150Q), was also found in patients with bilateral breast cancer (10,15). In addition, the Tyr315 residue of RAD51 was found to be constitutively phosphorylated by the BCR/ABL fusion protein, which is derived from the translocation of the *c-ABL* gene from chromosome 9 to the *BCR* gene locus on chromosome 22 (Philadelphia chromosome) in leukemia patients (16). These findings strongly suggest the involvement of the RAD51 activity in tumorigenesis or tumor progression.

During HRR, RAD51 assembles onto single-stranded DNA (ssDNA) tails, which are produced at the DSB sites, and forms a helical filamentous polymer. This RAD51-ssDNA filament then binds to intact double-stranded DNA (dsDNA), and a nascent heteroduplex is formed between the ssDNA and the complementary strand of dsDNA within the filament (homologous pairing). The heteroduplex region is then extended by RAD51

*To whom correspondence should be addressed. Tel: +81 3 5369 7315; Fax: +81 3 5367 2820; Email: kurumizaka@waseda.jp

with ATP hydrolysis (strand exchange). These RAD51-mediated recombination reactions, such as homologous pairing and strand exchange, are the key steps in DSB repair through the HRR pathway (17–21). Therefore, alterations of the RAD51-mediated recombination reactions by chemical compounds may result in the suppression of tumorigenesis and/or tumor progression.

To identify chemical compounds that regulate the RAD51 recombinase activity, in the present study, we screened 185 chemical compounds for their effects on RAD51-mediated strand exchange *in vitro*. We found that 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) efficiently inhibited the RAD51-mediated homologous pairing and strand-exchange reactions.

MATERIALS AND METHODS

Proteins

Hexahistidine-tagged human RAD51 was overexpressed in the *Escherichia coli* strain JM109 (DE3), which also carried an expression vector for the minor tRNAs (Codon(+)RIL, Stratagene, La Jolla, CA, USA). The RAD51 expressed in the *E. coli* strain was purified by a four-step method, as described previously (22). In this method, the purified RAD51 lacked the hexahistidine tag. Human RPA was produced in *E. coli* cells, and was prepared according to the published protocol (23). Protein concentrations were determined using the Bradford method (24), with bovine serum albumin as the standard protein.

DNAs

The ϕX174 phage ssDNA and dsDNA used in the DNA-binding and strand-exchange assays were purchased from New England Biolabs (Ipswich, MA, USA). All of the DNA concentrations are expressed in moles of nucleotides.

Assay for strand exchange

The ϕX174 circular ssDNA (20 μM) was incubated with RAD51 (6 μM) in the presence of a chemical compound at 37°C for 10 min, in 10 μl of 26 mM HEPES buffer (pH 7.5), containing 45 mM NaCl, 0.03 mM EDTA, 0.6 mM 2-mercaptoethanol, 3% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 0.1 mg/ml bovine serum albumin, 2 mM $CaCl_2$, 20 mM creatine phosphate and 75 μg/ml creatine kinase. After this incubation, 2 μM RPA was added to the reaction mixture, which was incubated at 37°C for 10 min. The reactions were then initiated by the addition of 20 μM ϕX174 linear dsDNA, and were continued for 60 min. The reactions were stopped by the addition of 0.1% SDS and 1.97 mg/ml proteinase K (Roche Applied Science, Basel, Switzerland), and were further incubated at 37°C for 20 min. After adding 6-fold loading dye, the deproteinized reaction products were separated by 1% agarose gel electrophoresis in 1× TAE buffer at 3.3 V/cm for 4 h. The products were visualized by SYBR Gold (Invitrogen, Carlsbad, CA, USA) staining. When the reactions were performed with the ^{32}P-labeled dsDNA, the gels were dried, exposed to an imaging plate and visualized using an FLA-7000 imaging analyzer (Fujifilm, Tokyo, Japan).

The D-loop formation assay

To prevent the dsDNA substrates from undergoing irreversible denaturation, superhelical dsDNA (pB5Sarray DNA), which contained 11 repeats of a sea urchin 5S rRNA gene (207-bp fragment) within the pBlueScript II SK(+) vector, was prepared by a method avoiding alkaline treatment of the cells harboring the plasmid DNA (25,26). For the ssDNA substrate, the following high-performance liquid chromatography (HPLC)-purified oligonucleotide was used: 50-mer, 5′-GGA ATT CGG TAT TCC CAG GCG GTC TCC CAT CCA AGT ACT AAC CGA GCC CT-3′. The 5′-end of the oligonucleotide was labeled with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA) in the presence of [γ-^{32}P]ATP. RAD51 (0.1 μM) was incubated with the indicated amount of DIDS at 37°C for 5 min, in 7 μl of reaction buffer, containing 22 mM HEPES–NaOH (pH 7.5), 15 mM NaCl, 0.01 mM EDTA, 0.2 mM 2-mercaptoethanol, 1% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 2 mM AMPPNP and 0.1 mg/ml BSA. The ^{32}P-labeled 50-mer oligonucleotide (1 μM) was then added, and the samples were further incubated at 37°C for 5 min. The pB5Sarray superhelical dsDNA (10 μM) was added along with 9 mM $MgCl_2$ to initiate the homologous-pairing reaction. The reactions were continued at 37°C for 30 min, and were stopped by the addition of 0.1% SDS and 1.97 mg/ml proteinase K (Roche Applied Science). After deproteinization at 37°C for 15 min, the reaction products were separated by 1% agarose gel electrophoresis in 1× TAE buffer at 3.3 V/cm for 2.5 h. The gels were dried, exposed to an imaging plate, and visualized using an FLA-7000 imaging analyzer (Fujifilm).

ATPase activity

RAD51 (6 μM) was incubated with 1 mM ATP (Roche Applied Science) in 26 mM HEPES buffer (pH 7.5), containing 45 mM NaCl, 1 mM $MgCl_2$, 3% glycerol, 0.03 mM EDTA, 0.6 mM 2-mercaptoethanol, 1 mM dithiothreitol and 0.1 mg/ml bovine serum albumin, in the presence or absence of ssDNA. In the ssDNA-dependent reaction, the ϕX174 circular ssDNA (40 μM) was used as the substrate. The reaction was performed at 37°C. After a 10 min preincubation in the absence of ATP, the reaction was initiated by adding 1 mM ATP. At each indicated time, a 20 μl aliquot of the reaction mixture was removed and mixed with 30 μl of 100 mM EDTA, to quench the reaction. The amount of inorganic phosphate released was determined by a colorimetric assay. Briefly, a 500 μl aliquot of a malachite green solution [0.034%(w/v) malachite green oxalate, 1.05%(w/v) hexaammonium heptamolybdate tetrahydrate, and 0.1%(w/v) polyvinyl alcohol in 1 M HCl] was mixed with 50 μl of sample solution (i.e. the reaction mixture quenched with EDTA). After 1 min, 50 μl of 34%(w/v) sodium citrate dihydrate was added to stop further color development. The absorbance

at 655 nm was measured with a 96-well micro plate reader (Bio-Rad Laboratories, Hercules, CA, USA). A 1 mg/ml phosphate ion standard solution (Wako Pure Chemical Industries, Osaka, Japan) was used to prepare the phosphate standards.

Assays for DNA binding

The ϕX174 circular ssDNA (40 μM) or ϕX174 linear dsDNA (10 μM) was mixed with RAD51 in 10 μl of 24 mM HEPES buffer (pH 7.5), containing 30 mM NaCl, 1 mM $MgCl_2$, 2% glycerol, 0.02 mM EDTA, 0.4 mM 2-mercaptoethanol, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 1 mM ATP, 20 mM creatine phosphate, 75 μg/ml creatine kinase and 2 mM $CaCl_2$. The reaction mixtures were incubated at 37°C for 15 min, and were then analyzed by 0.8% agarose gel electrophoresis in 1× TAE buffer (40 mM Tris–acetate and 1 mM EDTA) at 3.3V/cm for 2.5 h. The bands were visualized by ethidium bromide staining. For the RPA-ssDNA binding assay, the ϕX174 circular ssDNA (40 μM) was mixed with RPA in 10 μl of 20 mM HEPES buffer (pH 7.5), containing 10 mM KCl, 1 mM $MgCl_2$, 2% glycerol, 1.2 mM dithiothreitol and 0.1 mg/ml bovine serum albumin.

Gel filtration

RAD51 (45 μg) was incubated with 100 μM of DIDS, and the samples were analyzed by Superdex 200 HR 10/30 (GE Healthcare Biosciences, Uppsala, Sweden) gel filtration chromatography. The elution buffer contained 20 mM HEPES–NaOH (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol, and the flow rate was 0.5 ml/min.

Surface plasmon resonance analysis

The interaction between RAD51 and DIDS was detected with a Biacore T100 surface plasmon resonance biosensor (GE Healthcare Biosciences). RAD51 was immobilized to the sensor surface of a CM-5 sensor chip by the random amine coupling method. Surface activation with *N*-hydroxysuccinimide and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (GE Healthcare Biosciences) was followed by an injection of RAD51 (30 μg/ml), which was diluted in 10 mM NaOAc (pH 4.7) prior to injection. Unliganded sites on the chip were then blocked with ethanolamine. A 100 μl (10 min) injection of RAD51 resulted in the immobilization of about 20 000 response units (RU) of RAD51. Subsequently, the interaction experiments were carried out in 20 mM HEPES buffer (pH 7.5) containing 1 mM dithiothreitol, at a flow rate of 30 μl/min. A 30 μl (1 min) aliquot of each of the different concentrations of DIDS (1.25, 2.5, 5, 10, 20 μM) was injected onto the chip sequentially, without regeneration (kinetic titration). The baseline response of the non-treated surface of the chip was subtracted from the response obtained from the RAD51 surface. Kinetic parameters, such as k_a and k_d, were calculated with the Biacore T100 Evaluation Software (GE Healthcare Biosciences).

RESULTS

DIDS is a potential inhibitor of RAD51-mediated strand exchange

To identify chemical compounds that affect the recombinase activity of RAD51, we employed the strand-exchange assay. In this assay, ϕX174 phage circular ssDNA (5386 bases) and linearized ϕX174 dsDNA (5386 base pairs) were used as DNA substrates, and the reactions were conducted in the presence of RPA. In this combination of DNA substrates, both intermediate (joint molecule; JM) and complete strand-exchange products (nicked circular; NC) were potentially detectable (Figure 1A). However, under the reaction conditions used in this study, only the JM product was detected, because 5% methanol (as a solvent for chemical compounds) and low concentrations of salt, which are not optimal for the reaction, were present in the reaction mixture (Figure 1B).

We then performed the RAD51-mediated strand exchange in the presence of each of 185 chemical compounds, which were selected as potential antitumor chemical compounds by the program of Scientific Research on Priority Areas, Cancer, Japan (Supplementary Table 1). As shown in Figure 2A, most of the chemical compounds did not affect the RAD51-mediated strand exchange; however, we found that DIDS (Figure 2A, #137 and B) was a potential inhibitor for the RAD51-mediated strand exchange. Careful titration experiments confirmed that DIDS actually inhibited the RAD51-mediated strand exchange (Figure 3A and B). DIDS also inhibited the

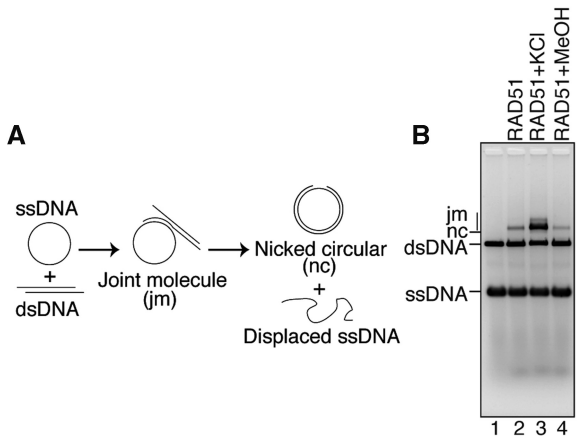

Figure 1. The strand-exchange assay with human RAD51. (**A**) A schematic diagram of the strand-exchange assay. (**B**) The strand exchange activity of RAD51 under the three different conditions. The ϕX174 circular ssDNA (20 μM) was incubated with RAD51 (6 μM) at 37°C for 10 min. After this incubation, 2 μM RPA was added to the reaction mixture, which was incubated at 37°C for 10 min. The reactions were then initiated by the addition of 20 μM ϕX174 linear dsDNA. The DNA products were then deproteinized, and were separated by 1% agarose gel electrophoresis in 1× TAE buffer at 3.3 V/cm for 4 h. The products were visualized by SYBR Gold (Invitrogen) staining. Joint molecules and nicked circular DNA are indicated by jm and nc, respectively. Lane 1 indicates a negative control experiment without RAD51. Lanes 2 and 3 indicate experiments with RAD51 in the absence and presence of 0.2 M KCl, respectively. Lane 4 indicates an experiment with 5% methanol in the absence of 0.2 M KCl.

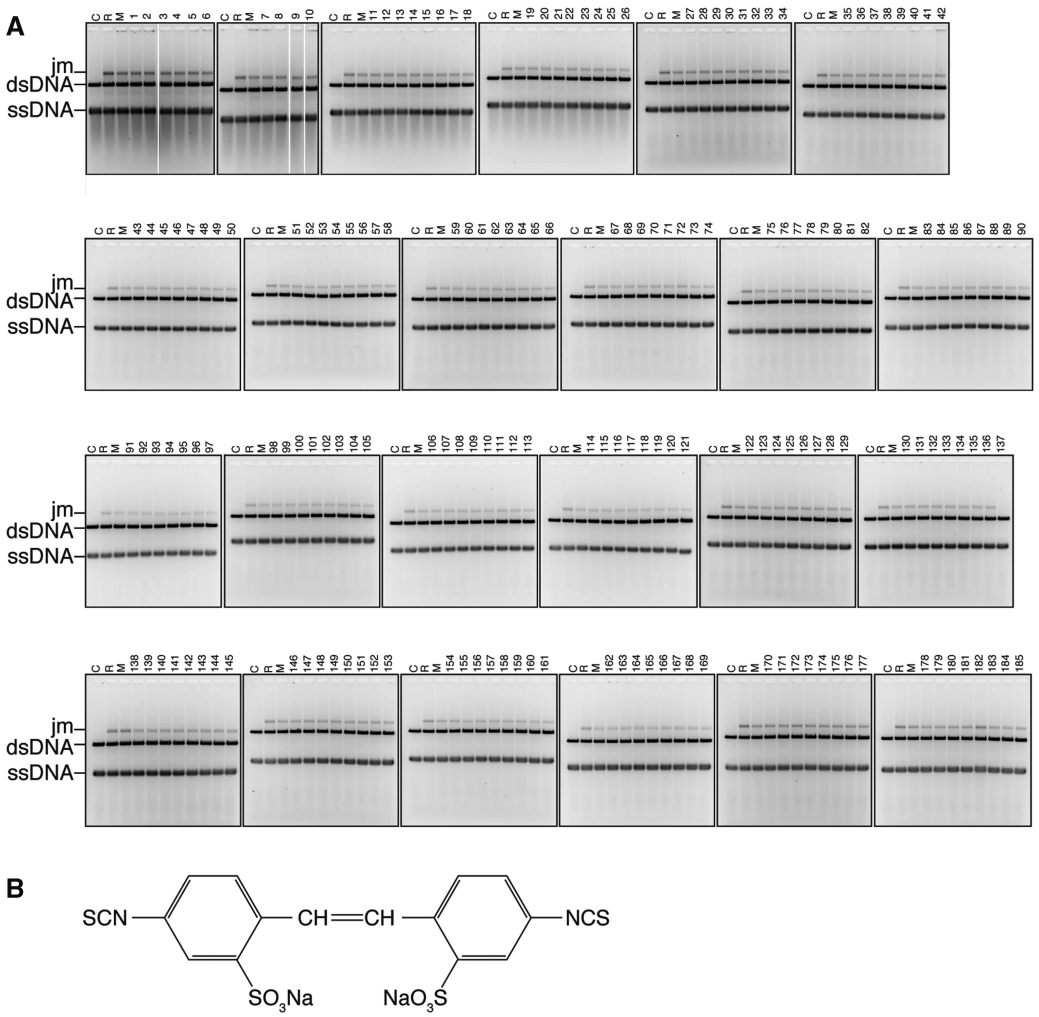

Figure 2. The strand-exchange assay in the presence of chemical compounds. (**A**) Each chemical compound (10 μM, lanes 1–185) was incubated with RAD51 (6 μM), and the strand-exchange reaction was performed under the conditions containing 5% methanol at 37°C for 1 h with 20 μM ssDNA, 20 μM dsDNA and 2 μM RPA. Lanes C, R and M indicate negative control reactions without RAD51, complete reactions and complete reactions with 5% methanol, respectively. The chemical compounds are listed in Supplementary Table 1. (**B**) Structure of 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS).

RAD51-mediated strand exchange under the conditions with 0.2 M KCl, which significantly stimulates the strand exchange (Figure 3C and D).

We next tested whether the reaction order of the DIDS addition affected the strand-exchange inhibition. Time-course experiments were performed. Since we used ^{32}P-labeled dsDNA in this assay, the JM products and dsDNA were detected using an FLA-7000 imaging analyzer (Fujifilm). As shown in Figure 4A and B, DIDS significantly inhibited the RAD51-mediated strand exchange, when it was added before the RAD51 was incubated with the ssDNA. DIDS moderately inhibited the strand exchange, when it was added after the RAD51was incubated with the ssDNA (just before RPA addition) (Figure 4C and D). Modest, but clear, inhibition was observed when DIDS was added before dsDNA (after RPA addition) (Figure 4E and F). Therefore, DIDS actually inhibits the RAD51-mediated strand exchange in various reaction orders, although the inhibition efficiencies depend on the reaction order.

DIDS inhibits homologous pairing by RAD51

We then tested whether DIDS inhibits homologous pairing by RAD51, because the homologous-pairing step needs to occur just before strand exchange. To do so, we performed the D-loop formation assay. In this assay, a ^{32}P-labeled ssDNA 50-mer and superhelical dsDNA were used as substrates (Figure 5A), and the reactions were performed in the absence of RPA. As shown in Figure 5B and C, DIDS inhibited the RAD51-mediated D-loop formation in a concentration-dependent manner. These results indicated that DIDS inhibits homologous pairing, as well as strand exchange, by RAD51 *in vitro*.

DIDS directly binds to RAD51

To determine whether DIDS directly binds to RAD51, we performed a surface plasmon resonance (SPR) analysis. As shown in Figure 6A, DIDS directly interacted with the RAD51-conjugated sensor chip. The association rate constant (k_a) and the dissociation rate constant

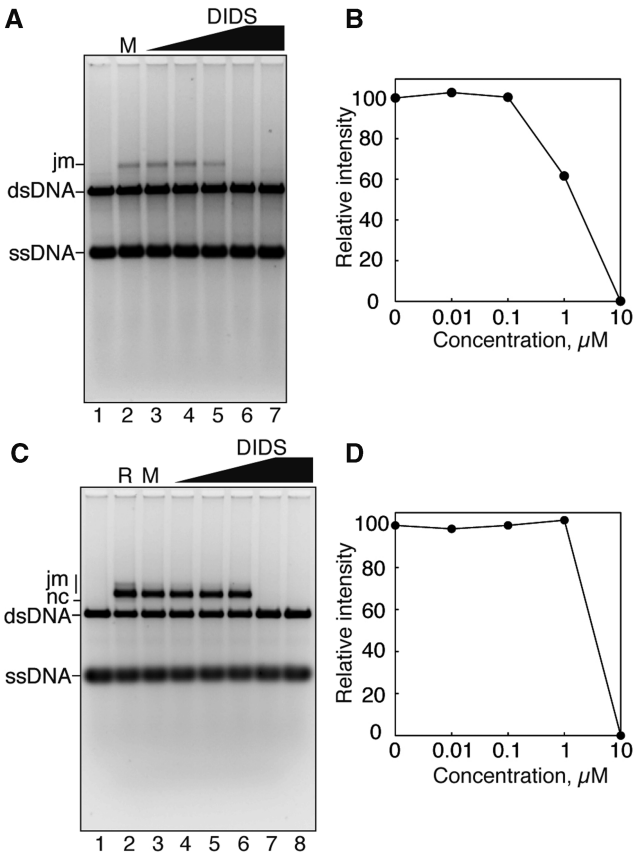

Figure 3. DIDS efficiently inhibits RAD51-mediated strand exchange. (**A**) DIDS titration experiments in the absence of 0.2 M KCl. The ϕX174 circular ssDNA (20 μM) was incubated with RAD51 (6 μM) in the presence of DIDS at 37°C for 10 min. After this incubation, 2 μM RPA was added to the reaction mixture, which was incubated at 37°C for 10 min. The reactions were then initiated by the addition of 20 μM ϕX174 linear dsDNA. The DNA products were then deproteinized, and were separated by 1% agarose gel electrophoresis in 1× TAE buffer at 3.3 V/cm for 4 h. The products were visualized by SYBR Gold (Invitrogen) staining. Joint molecule is indicated by jm. Lane 1 indicates a negative control experiment without RAD51. Lane 2 indicates an experiment with RAD51 and 5% methanol in the absence of DIDS. DIDS concentrations were 0.01 μM (lane 3), 0.1 μM (lane 4), 1 μM (lane 5) and 10 μM (lane 6). Lane 7 indicates an experiment with 10 μM DIDS in the absence of RAD51. (**B**) Graphic representation of the experiments shown in (A). The band intensities of the jm product were quantified as the peak volumes of densitometric scans. The jm peak volumes relative to that in the reaction without DIDS (A, lane 2) were plotted against the DIDS concentration. (**C**) The strand-exchange assay in the presence of 0.2 M KCl. Lane 1 indicates a negative control experiment without RAD51. Lanes 2 and 3 indicate control experiments without DIDS with RAD51 in the absence and presence of 5% methanol, respectively. DIDS concentrations were 0.01 μM (lane 4), 0.1 μM (lane 5), 1 μM (lane 6) and 10 μM (lane 7). Lane 8 indicates an experiment with 10 μM DIDS in the absence of RAD51. (**D**) Graphic representation of the experiments shown in (C). The band intensities of the jm products were quantified as the peak volumes of densitometric scans. The jm peak volumes relative to that in the reaction without DIDS (C, lane 3) were plotted against the DIDS concentration.

(k_d) obtained from the sensorgram (Figure 6A) are $1 \times 10^3\,M^{-1}s^{-1}$ and $2 \times 10^{-3}\,s^{-1}$, respectively. Therefore, the K_D value of the RAD51–DIDS interaction is 2 μM. The DIDS binding did not disrupt the RAD51 multimer, which eluted near the void volume from the Superdex 200 gel filtration column (Figure 6B). This RAD51 multimer is known to be disrupted by the BRC4 peptide (27), which binds to the RAD51–RAD51 interface (28–30). Therefore, DIDS may bind a different region than the BRC4-binding surface of RAD51.

DIDS inhibits the RAD51-DNA binding

We then examined whether DIDS affected the RAD51-DNA binding. Consistent with the results from the homologous-pairing and strand-exchange assays, DIDS significantly inhibited the ssDNA binding and dsDNA binding by RAD51 (Figure 7A and B). A 1 μM concentration of DIDS, which moderately inhibited strand exchange (Figure 3A and B), also moderately inhibited the RAD51-DNA binding, especially for the dsDNA (Figure 7B, lane 6). In addition, 10 μM DIDS, which significantly inhibited strand exchange (Figure 3A and B), also significantly inhibited both RAD51-ssDNA binding and RAD51-dsDNA binding (Figure 7A, lane 6 and B, lane 7). These inhibitory effects of DIDS on the RAD51-DNA binding did not depend on the presence of ATP or its analogs, AMPPNP and ATPγS (Figure 7C and D). In contrast, DIDS did not inhibit the RPA-ssDNA binding in the presence of 0.1–20 μM DIDS (Figure 7E). These results strongly suggested that DIDS inhibits the RAD51-mediated strand exchange by preventing RAD51-DNA binding, and not by inhibiting RPA-ssDNA binding, although RPA is required for the efficient promotion of the RAD51-mediated strand exchange.

DIDS stimulates the ATP hydrolyzing activity of RAD51 in the absence of DNA

RAD51 hydrolyzes ATP in the presence of ssDNA or higher concentrations of salt (1.5 M NaCl) (31). In contrast, RAD51 does not efficiently hydrolyze ATP without DNA under low salt conditions. Interestingly, under the conditions without DNA and high salt, 10 μM DIDS stimulated ATP hydrolysis by RAD51 (Figure 8A). This may imply that DIDS binds near the DNA binding site of RAD51 and stimulates the ATPase activity. If so, then the DIDS may compete with ssDNA for RAD51 binding, and may inhibit the ssDNA-dependent ATP hydrolysis by RAD51, once the ATP hydrolyzing reaction has commenced. As shown in Figure 8B, the progress of the ssDNA-dependent ATP hydrolysis reaction by RAD51 was clearly inhibited just after the addition of DIDS (30 min). Therefore, DIDS may bind to a region close to the DNA binding site(s) of RAD51, and directly compete with DNA for RAD51 binding.

DISCUSSION

Increased expression of RAD51 has been reported in immortalized and tumor cells (32), and chromosome rearrangements, probably resulting from the enhanced homologous recombination, are found in these cells. Consistently, the overexpression of RAD51 enhances cell growth and gene targeting in mouse hybridoma cells (33), and promotes aneuploidy and multiple chromosomal rearrangements in mouse embryonic stem cells (34).

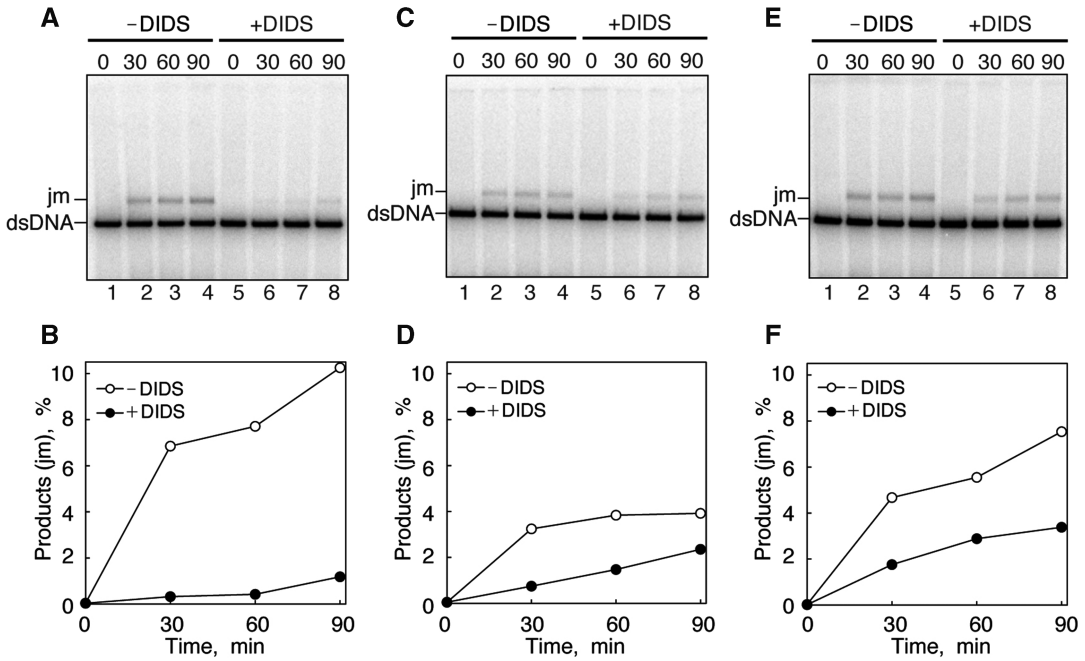

Figure 4. Effects of the DIDS reaction order on RAD51-mediated strand exchange. The ϕX174 circular ssDNA (20 μM) was incubated with RAD51 (6 μM) at 37°C for 10 min. After this incubation, 2 μM RPA was added to the reaction mixture, which was incubated at 37°C for 10 min. The reactions were then initiated by the addition of 20 μM ϕX174 linear dsDNA containing 2 μM ^{32}P-labeled ϕX174 linear dsDNA, in the absence of 0.2 M KCl. The reactions were stopped and deproteinized at the indicated times, and the products were separated by 1% agarose gel electrophoresis in 1× TAE buffer at 3.3 V/cm for 4 h. The jm products and dsDNA labeled by ^{32}P were visualized and quantified using an FLA-7000 imaging analyzer (Fujifilm). (**A**) RAD51 was incubated with DIDS (10 μM) at 37°C for 10 min. After the addition of the ϕX174 circular ssDNA, RPA was added to the reaction mixture. The ϕX174 linear dsDNA was then added to initiate the reaction. The reactions were continued for the indicated times. Lanes 1–4 indicate positive control experiments with RAD51 and 5% methanol. Lanes 5–8 indicate experiments with RAD51 and DIDS (and 5% methanol). Reaction times were 0 min (lanes 1 and 5), 30 min (lanes 2 and 6), 60 min (lanes 3 and 7) and 90 min (lanes 4 and 8). (**B**) Graphic representation of the experiments shown in (A). Closed and open circles indicate experiments with and without DIDS, respectively. (**C**) RAD51 was incubated with the ϕX174 circular ssDNA at 37°C for 10 min. After this incubation, DIDS (10 μM) was added, followed by the addition of RPA. The ϕX174 linear dsDNA was then added to initiate the reaction. (**D**) Graphic representation of the experiments shown in (C). Closed and open circles indicate experiments with and without DIDS, respectively. (**E**) RAD51 was incubated with the ϕX174 circular ssDNA at 37°C for 10 min, and RPA was added. DIDS was added, and then ϕX174 linear dsDNA was added to initiate the reaction. (**F**) Graphic representation of the experiments shown in (E). Closed and open circles indicate experiments with and without DIDS, respectively.

RAD51 overexpression also contributes to chemoresistance in human soft tissue sarcoma cells (35). BRCA1 is involved in the HRR pathway. Intriguingly, RAD51 overexpression bypasses BRCA1 function in BRCA1-deficient cells, and up-regulation of RAD51 was found in BRCA1-deficient tumors (36). In addition, constitutive phosphorylation of the RAD51-Tyr315 residue in leukemia cells enhanced the recombination activity, which induces chromosomal translocation (16). These data strongly suggest that an elevated RAD51 level and/or activity causes genome instability and tumor progression.

To identify chemical compounds that suppress the recombinase activity of RAD51, we performed the RAD51-mediated strand-exchange assay in the presence of each of 185 chemical compounds, which were selected as potential antitumor chemical compounds by the program of Scientific Research on Priority Areas, Cancer, Japan. We found that DIDS significantly inhibits both homologous pairing and strand exchange by RAD51 *in vitro*, probably by inhibiting the DNA-binding activity of RAD51. The BRC4 polypeptide derived from BRCA2 is known to inhibit DNA binding by RAD51 (28). This polypeptide directly binds to RAD51 at the monomer–monomer interface of the RAD51 filament, and disassembles the filament (27–30). Our SPR analysis revealed that DIDS also directly binds to RAD51. In contrast to the BRC4 polypeptide, a gel filtration analysis revealed that DIDS did not disrupt the RAD51 multimer, which is known to be disrupted by the BRC4 polypeptide (27). Therefore, DIDS may bind to a different RAD51 surface from the BRC4-binding region.

Our results presented here suggest that DIDS may bind near the DNA binding sites of RAD51. In addition, we found that DIDS inhibits the RAD51-dsDNA binding more efficiently than the RAD51-ssDNA binding. This fact implies that DIDS may bind to the secondary DNA binding site specific for dsDNA within the RAD51 filament (37–40). Another possibility is that DIDS may bind to the dsDNA-binding path, which may function to guide DNA into the RAD51 filament. Previously, we and others proposed that the N-terminal domain of RAD51 (as well as that of a meiosis-specific homologue, DMC1) constitutes such a dsDNA-binding path (37–42). We found that 50% inhibition of the RAD51-mediated

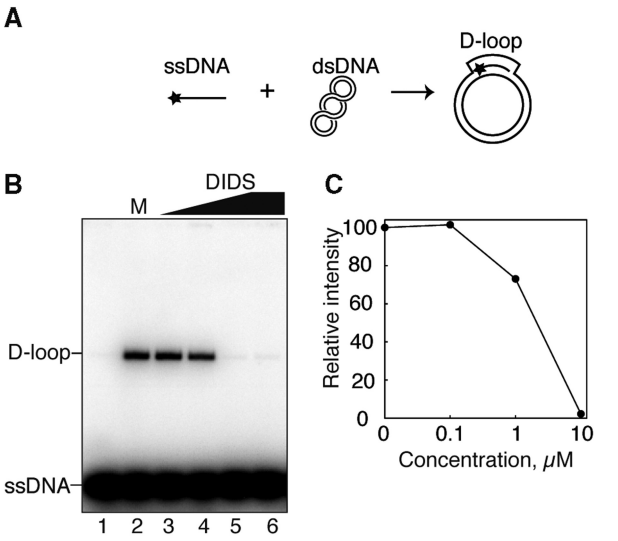

Figure 5. DIDS inhibits homologous pairing by RAD51. (**A**) The D-loop formation assay. Asterisks indicate the ^{32}P-labeled end of the ssDNA 50-mer. (**B**) The reactions were conducted with 0.1 μM RAD51 in the presence of increasing amounts of DIDS. Lanes 1 and 6 indicate negative control experiments without and with 10 μM DIDS in the absence of RAD51, respectively. Lane 2 indicates a positive control experiment with RAD51 in the presence of 5% methanol. Lanes 3–5 indicate experiments with RAD51 and DIDS. The DIDS concentrations were 0 μM (lanes 1 and 2), 0.1 μM (lane 3), 1 μM (lane 4) and 10 μM (lanes 5 and 6). (**C**) Graphic representation of the experiments shown in (B).

strand exchange was observed at a ratio of about 1–2 μM DIDS to 6 μM RAD51, suggesting the binding of approximately 1 DIDS per turn of the RAD51 filament. Therefore, DIDS may inhibit the dsDNA binding to the RAD51 filament on the dsDNA-binding path that runs between two consecutive N-terminal domains in the filament structure. Further structural studies will be required to understand the molecular mechanism of the DIDS-mediated alteration of the RAD51 activities.

DIDS reportedly binds to the outer cell membrane surface, and inhibits Cl^- channels (43). However, no DIDS function in the nucleus has been reported, thus far. We confirmed that DIDS was efficiently imported into the nuclei of human cultured cells (H.S. and S.T. unpublished results). Actually, DIDS was toxic for the cultured cells (H.S. and S.T. unpublished results), and thus it was difficult to evaluate the contribution of the DIDS-mediated RAD51 suppression, because of its cytotoxicity. Therefore, DIDS may be a potential lead compound for developing RAD51-specific anticancer drugs. Jayathilaka *et al.* (44) reported that 3-[(benzylamino)sulfonyl]-4-bromo-N-(4-bromophenyl)benzamide (RS-1) stimulated RAD51 activity. RS-1 also directly binds to RAD51, but, in contrast to DIDS, it stabilizes the RAD51-DNA binding. These two chemical compounds, DIDS and RS-1, which have opposite effects on the RAD51 activity, may have broad potential applications in both research and medical settings.

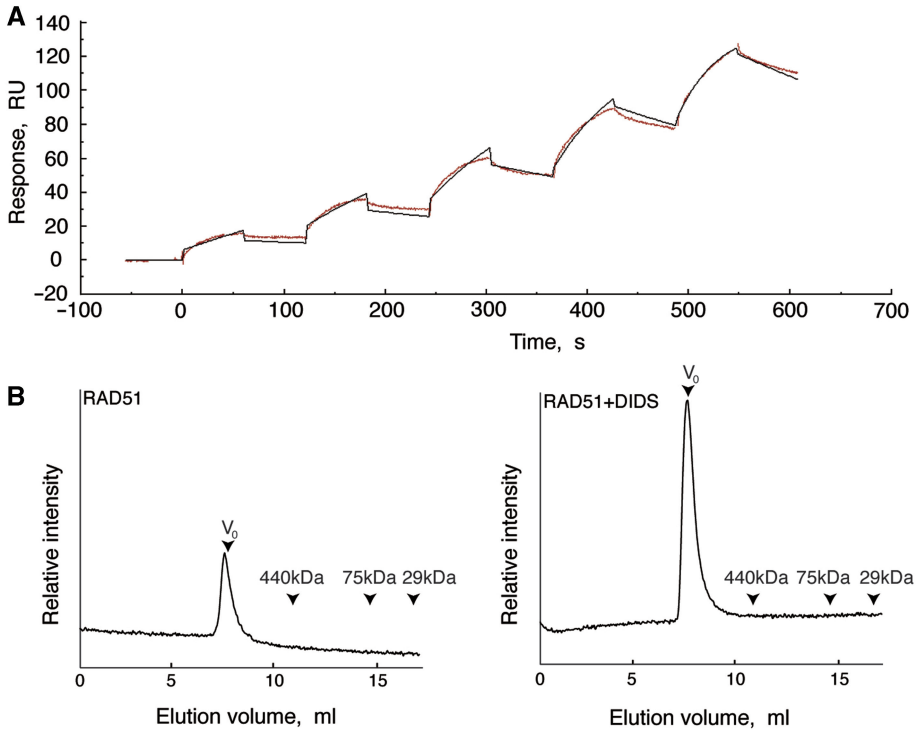

Figure 6. DIDS binds to RAD51 without interfering the RAD51 oligomerization. (**A**) SPR analysis. The RAD51-conjugated sensorchip was used. Sensorgram of a kinetic titration analysis between RAD51 and DIDS presented with a solid line. The DIDS concentrations were 1.25, 2.5, 5, 10 and 20 μM. DIDS (30 μl) was applied to the chip sequentially without regeneration. Kinetic parameters were obtained by curve fitting with a red line. (**B**) Gel filtration analysis. The left panel indicates the elution profile of RAD51 from the Superdex 200 column. The right panel indicates the elution profile of RAD51 previously incubated with 10 μM DIDS. The peak intensity of the RAD51–DIDS complex (right panel) was higher than that of RAD51 alone (left panel), because DIDS contains two aromatic rings.

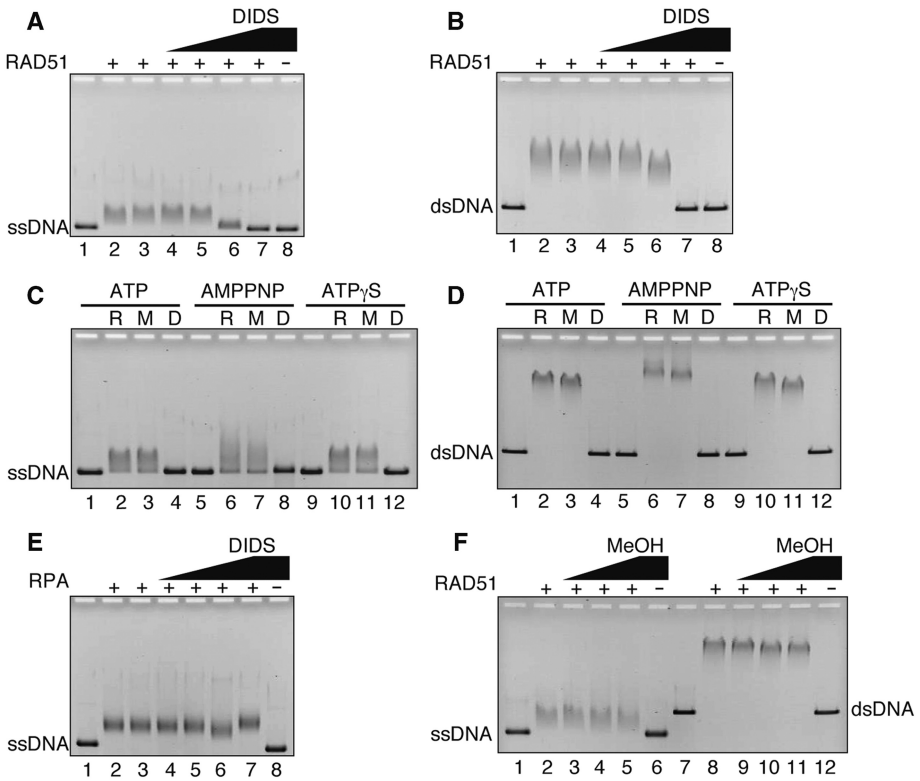

Figure 7. DIDS inhibits DNA binding by RAD51. (**A**) The ssDNA-binding experiments in the presence of ATP. The ϕX174 circular ssDNA (40 μM) was incubated with RAD51 (2 μM) in the presence of DIDS at 37°C for 15 min. The samples were analyzed by 0.8% agarose gel electrophoresis in 1× TAE buffer. The bands were visualized by ethidium bromide staining. Lane 1 indicates a negative control experiment without RAD51. Lane 2 indicates an experiment with RAD51 alone. Lane 3 indicates an experiment with RAD51 and 5% methanol. DIDS concentrations were 0.1 μM (lane 4), 1 μM (lane 5), 10 μM (lane 6) and 20 μM (lane 7). Lane 8 indicates an experiment with 20 μM DIDS in the absence of RAD51. (**B**) The dsDNA-binding experiments in the presence of ATP. The linear ϕX174 dsDNA (10 μM) was incubated with RAD51 (1 μM) in the presence of DIDS at 37°C for 15 min. Lane 1 indicates a negative control experiment without RAD51. Lane 2 indicates an experiment with RAD51 alone. Lane 3 indicates an experiment with RAD51 and 5% methanol. DIDS concentrations were 0.01 μM (lane 4), 0.1 μM (lane 5), 1 μM (lane 6) and 10 μM (lane 7). Lane 8 indicates an experiment with 10 μM DIDS in the absence of RAD51. (**C**) The ssDNA-binding experiments in the presence of ATP, AMPPNP and ATPγS. The ϕX174 circular ssDNA (40 μM) was incubated with RAD51 (2 μM) at 37°C for 15 min. Lanes 1–4, lanes 5–8 and lanes 9–12 represent experiments with ATP, AMPPNP and ATPγS, respectively. Lanes 1, 5 and 9 indicate negative control experiments without RAD51. Lanes 2, 6 and 10 indicate experiments with RAD51 alone. Lanes 3, 7 and 11 indicate experiments with RAD51 and 5% methanol. Lanes 4, 8 and 12 indicate experiments with RAD51 and DIDS (20 μM). (**D**) The dsDNA-binding experiments in the presence of ATP, AMPPNP and ATPγS. The linear ϕX174 dsDNA (10 μM) was incubated with RAD51 (1 μM) in the presence of DIDS at 37°C for 15 min. Lanes correspond to those in (C). The DIDS concentration was 10 μM. (**E**) Effect of DIDS on the ssDNA binding of RPA. The ϕX174 circular ssDNA (40 μM) was incubated with RPA (0.5 μM) in the presence of DIDS at 37°C for 15 min. Lanes 1 and 8 indicate negative control experiments without RPA. Lanes 2 and 3 indicate experiments with RPA in the absence and presence of 5% methanol, respectively. The DIDS concentrations were 0.1 μM (lane 4), 1 μM (lane 5), 10 μM (lane 6) and 20 μM (lanes 7 and 8). (**F**) The ssDNA-binding and dsDNA-binding experiments with RAD51 were performed in the presence of methanol. Lanes 1–6 and lanes 7–12 indicate experiments with ssDNA and dsDNA, respectively. Lanes 1, 6, 7 and 12 are negative controls without RAD51, and lanes 2 and 8 are positive controls with RAD51 in the absence of methanol. Methanol concentrations were 2.5% (lanes 3 and 9), 5% (lanes 4 and 10) and 10% (lanes 5, 6, 11 and 12).

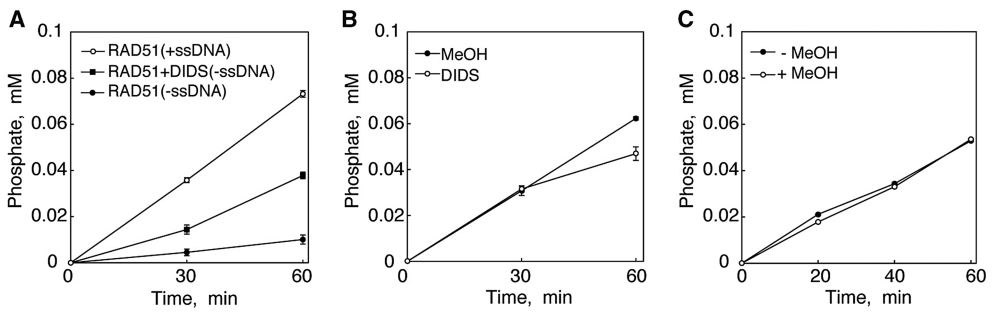

Figure 8. RAD51-mediated ATP hydrolysis with DIDS. (**A**) Open and closed circles indicate positive and negative control experiments without DIDS, in the presence and absence of ssDNA (40 μM), respectively. Closed squares indicate the experiments with DIDS (10 μM) in the absence of ssDNA. The averages of three independent experiments are represented with standard deviation values. (**B**) DIDS (20 μM) was added at 30 min after the ATP hydrolyzing reaction was initiated. Reactions were conducted at 37°C in the presence of ssDNA (40 μM). Closed and open circles indicate experiments with 5% methanol and DIDS, respectively. (**C**) Five percent methanol did not affect the ssDNA-dependent ATP hydrolysis by RAD51. Closed and open circles indicate experiments without and with 5% methanol, respectively.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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