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# A rationally designed peptide enhances homologous recombination *in vitro* and resistance to DNA damaging agents *in vivo*

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## ABSTRACT

**The RecA family of proteins is essential in homologous recombination, a critical step in DNA repair. Here, we report that a rationally-designed small peptide based on the crystal structure of *Escherichia coli* RecA–DNA complex can promote homologous recombination through the enhancement of both RecA-mediated strand assimilation and three-strand exchange activity. Among 17 peptides tested, peptide #3 with the amino acid sequence of IRFLTARRR has the most potent activity in promoting the RecA-mediated D-loop formation by ~7.2-fold at 37°C. Other peptides such as IRFLTAKKK and IRLLTARRR also have similar, albeit lower, activities. Therefore, hydrophobicity and poly-positive charges, and the space between them in those small peptides are crucial features for such activities. The enhancement of recombination by these peptides appears to be a general phenomenon as similar results were seen by using different plasmids. Remarkably, peptide #3 alone without RecA can also promote the D-loop formation at elevated temperature. Cell viability assays showed that the peptide elevates mammalian cell resistance to two cytotoxic DNA drugs, cisplatin and doxorubicin. The rescue of viability may result from increased DNA repair efficiency. Such peptides may find future biological applications.**

## INTRODUCTION

Homologous recombination is a major, error-free repair process for DNA double strand breaks and collapsed DNA replication forks. It also serves to generate genetic diversity in meiosis. The RecA family proteins (also called recombinases), including prokaryotic RecA (1), archaeal RadA (2), eukaryotic Rad51 (3) and meiosis-specific DMC1 (4), play a major role in homologous recombination. The process of homologous recombination is widely conserved across all three kingdoms of life (5–8). The proposed mechanism of homologous recombination starts with a 3′ single-stranded tail generated by a nuclease at double strand break sites, and single-stranded DNA (ssDNA) binding protein (SSB) in the prokaryote, or replication protein A (RPA) in the eukaryote, coats to the ssDNA tail, thereby circumventing the formation of a single-stranded secondary DNA structure. Subsequent to the displacement of SSB or RPA by the recombinase, a continuous nucleoprotein filament forms and performs a homology search on chromosomal DNA. The nucleoprotein filament further invades the homologous double-stranded DNA (dsDNA) substrate, a process often called strand assimilation or D-loop formation. Finally, the three-strand exchange reaction occurs between the single-stranded and homologous double-stranded DNA substrates (9).

The mechanism of homologous recombination is also controlled and influenced by a number of mediator proteins in different species, including BRCA2 and BRCA1, which are recombinase regulators in higher eukaryotes (10,11); the Rad51 paralog, Rad55–Rad57 heterodimer complex, which helps the nucleoprotein filament to bind the homologous dsDNA substrate (8,12); Dmc1, which acts together with Hop2 to promote meiotic DNA cross-over (13), and other Rad51 paralogs, which play different roles in the maintenance of genome stability (14–16). However, several reports have demonstrated that overexpression of Rad51 proteins can partially rescue the defects resulting from the BRCA1$^{-/-}$ mutant in a DT40 cell, and bypass the deficiencies of rad55 and rad57 mutant yeast strains (17,18). Similarly, the defects of dmc1 and rad51 paralogs mutants can be counteracted by overexpression of Rad51 (13,15,16).

Recently, Jayathilaka *et al.* (19) identified a small molecule that can enhance homologous recombination

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efficiency by specifically stimulating the formation of human Rad51–ssDNA nucleoprotein filaments based on the high-throughput screening of small molecules (20). The compound not only increases the strand assimilation activity of human Rad51 proteins *in vitro*, but also promotes the resistance of human cells to DNA damaging agents *in vivo*.

*Escherichia coli* RecA protein was the first recombinase to be discovered and although it has been studied thoroughly over the past 30 years, a number of unanswered questions remain. In 2008, Chen *et al*. (21) solved the important crystal structures of *E. coli* RecA, RecA–ssDNA and RecA–dsDNA that provide a platform for us to rationally design a small peptide (IRFLTARRR) based on the interactions of RecA filaments and DNA. Here, we report on the studies of this peptide, which not only enhances the homologous recombination process *in vitro* via stabilizing the DNA strand assimilation (D-loop) structure, but also promotes two human cell lines (A375 and MCF-7) to resist the DNA damaging agents cisplatin and doxorubicin *in vivo*.

## MATERIALS AND METHODS

### DNA substrates and chemical reagents

The ϕX174 viral (+) and replicative form I were purchased from New England Biolabs (USA). The PA1655 and PA1656 ssDNAs (50 nt) and plasmids DNA GW1 and pUC18 used in the strand assimilation assay have been described previously (22–24). Spermine, streptomycin, AM-PNP, phosphocreatine, Arg-Arg 4-methoxy-B-naphthylamide HCl, 1-naphthyl-acetyl spermine HCl, *N′*-glutathionyl-spermidine disulfide and Thiazolyl blue tetra-zolium bromide (MTT powder) were purchased from Sigma-Aldrich (USA). SYBR-Green II was purchased from Invitrogen (USA).

### Proteins and peptides

The *E. coli* RecA protein and proteinase K were purchased from New England Biolabs and *E. coli* SSB was from Promega. All peptides used in this work were synthesized by the Peptide Synthesis Facility of Institute of Biological Chemistry, Academia Sinica and purified by HPLC. The final products attained >95% purity and were confirmed by ESI-MS.

### Strand assimilation, ATPase assay and three-strand exchange assay

The RecA-mediated strand assimilation or D-loop formation assay and ATPase assay were performed according to the previously described methods (25). Unless otherwise stated, strand assimilation assay of the peptide #3 alone was performed without changing any reaction conditions apart from using peptide #3 instead of RecA proteins. The three-strand exchange assay was performed as follows. The reaction solutions contained 25 mM HEPES–KOH (pH 7.0), 1 mM DTT, 5 % glycerol, 10 mM $Mg(OAc)_2$, 12 mM phosphocreatine, 10 U/ml creatine phosphokinase, 6 μM RecA, 9 μM (in nucleotides) ϕX174 viral (+) and different amounts of peptide#3, as indicated. These mixtures were pre-incubated for 5 min. The reactions were initiated by adding 4.5 μM (in base pairs) dsDNA (ϕX174RF I, XhoI-treated) after simultaneously mixing 0.9 μM SSB and 3 mM ATP for 5 min. All reactions were incubated at 37°C. Aliquots (8 μl) were removed at the indicated times and quenched with 1.1 μl of SDS (10%) and 0.9 μl of proteinase K (20 mg/ml). The samples were analyzed by electrophoresis for 1.5 h at 4 V/cm on a 0.8 % agarose gel in Tris–acetate–EDTA buffer (40 mM Tris, 1 mM $Na_2$–EDTA, and 20 mM acetic acid, pH 8.0). The substrates and products were visualized by SYBR-Green II staining. Those bands were quantified by Quantify One (BIO-RAD, USA).

### Electrophoretic mobility shift assay

Each reaction solution contained 13.6 μM (in nucleotides) ϕX174 virion ssDNA, 20 mM $Mg(OAc)_2$, 4.5 % glyceol, 20 mM HEPES–KOH (pH 7.0), 1 mM DTT and 2 mM AMP-PNP. The reaction was started by adding 4.6 μM RecA and increasing amounts of peptide #3 (0.5, 5, 10 μM). The reactions were incubated at 37°C for 30 min and resolved by electrophoresis for 50 min at 50 V on a 0.8% agarose gel. The gel was stained with SYBR-Green II and subsequent visualization was conducted by UV illumination. As depicted in Figure 4C, the different forms of DNA, including bubble (2 + 3), single-stranded (2), D-loop mimic (1 + 2 + 3), double-stranded (2 + 4) and double-stranded with a homologous single-stranded (1 + 2 + 4) DNA, were composed of (1) gaagcccgatataggt gacagacgatatgagcccggg, (2) gagttttatcgcttccatgacgcagctcat atcgtctgtcacctatatcgatgagtcgaaaaattatcttgataa, (3) ttatcaa gataatttttcgactcatcagaaatatccgaaagtgttaacttctgcgtcatggaa gcgataaaactc or (4) ttatcaagataatttttcgactcatcgatataggtgac agacgatatgagctgcgtcatggaagcgataaaactc. Those DNA substrates were heated at 95°C for 3 min and annealed by cooling slowly to room temperature. The reactions with or without peptide #3 were incubated at 37°C and the electrophoretic mobility shift assay (EMSA) was performed in 6% native poly-acrylamide gel. After running in 0.5× TBE buffer in 100 V for 60 min, the gel was stained with SYBR-Green II for 20 min.

### Cell survival assay

The human breast adenocarcinoma cell line MCF-7 was cultured in α-MEM and supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate and 10% FBS at 37°C, in 5% $CO_2$. Human melanoma cell line A375 was maintained in DMEM supplemented with 4.5 g/l glucose, 2 mM glutamine and 10% FBS at 37°C, in 5% $CO_2$. Using a protocol modified from that described previously (26), cell survival assay was carried out for Prolactin resistant to cisplatin. 6000 cells per well were plated in 96-well plates. In the following day, cells were incubated with different amounts of peptide #3, #8, #10 or #12 for 24 h in a treatment medium. After peptide pre-treating, DNA damaging reagents, cisplatin (2.4 μM and 4.8 μM) or doxorubicin (10 nM and 100 nM), as specified in various experiments, were added

for an additional 4 days. Cytotoxicity was determined by MTT (Thiazolyl blue tetra-zolium bromide) assay.

## RESULTS AND DISCUSSION

### Rationally designed peptides based on RecA–ssDNA structure

Although the first *E. coli* RecA protein structure was solved in 1992 (27), a detailed understanding of the interaction sites between RecA and its DNA substrate remained elusive until recently (21). The crystal structures of RecA–ssDNA and RecA–dsDNA complexes showed that each nucleotide triplet is bound by the continuous protomers of the RecA filament [Figure 2 and Supplementary Figure 7 in ref. (21)]. The backbone of the nucleotide triplet is almost fully buried by the L1 and L2 loops plus portion of N-terminal domain of the RecA filament.

As shown in Figure 1A, all three phosphate groups of the nucleotide triplet were bound by the consecutive positive charges of the RecA filament through strong ionic bonds [Figure 1A and ref. (21)]. Figure 1B represents the multiple sequence alignment of RecA homologs from *Sulfolobus solfataricus* (*Sso*RadA), *Methanococcus voltae* (*Mv*RadA), *Pyrococcus furiosus* (*Pf*Rad51), *Homo sapiens* (*Hs*Rad51 and *Hs*Dmc1), *Saccharomyces cerevisiae* (*Sc*Dmc1), *Gluconacetobacter polyoxogenes* (*Gp*RecA), *Gluconobacter oxydans* (*Go*RecA) and *E. coli* (*Ec*RecA). The areas of the L1 and L2 loops are indicated and the numerous positively charged amino acids between them are also involved in ssDNA binding directly or indirectly (21). Many hydrophobic amino acids in the L2 loop (in yellow, Figure 1A) forming the hydrophobic environment, do not interact with phosphate groups of ssDNA, but are embedded between the bases of two nucleotide triplets to fill and stabilize the inter-triplet gap. The above interactions between RecA filament and ssDNA cause the Watson–Crick edges of the bases to expose to the solvent, a process necessary for the subsequent base pairing of homologous recombination. However, the structures of the L2 loop in all solved eukaryotic recombinases are disordered, perhaps due to the lack of DNA substrates.

The characteristics of these interactions were instrumental in formulating the design of small peptides that mimic the continuous positive charges and hydrophobic mass of the RecA filament as mentioned above. Accordingly, a series of small peptides were designed (Figure 2C) and examined as to whether they have the ability to stimulate homologous recombination through strand assimilation assay, a central reaction of the RecA-mediated homologous recombination.

### Peptide #3 stimulates the RecA-mediated strand assimilation

Among the peptides tested, peptide #3 with the amino sequence of IRFLTARRR consisting of hydrophobic parts followed by positively charged amino acids, has the highest activity. As shown in Figure 2A, when a RecA-mediated strand assimilation assay was performed with increasing concentrations of peptide #3, the result displays a significant enhancement of the D-loop signal in comparison to that of RecA alone as a positive control. Peptide #3 stimulates the RecA-mediated D-loop activity, detected not only in the plasmid GW1 plus the PA1656 primer system, but also in the pUC18 plasmid (22) plus the PA1655 primer system (Figure 2A and B, respectively). The results show that the enhancement induced by peptide #3 expresses no DNA sequence specificity. The quantitation of the D-loop signal indicated that even when a low concentration (0.5 μM) of peptide #3 was added, the signal increased by >4-fold to that of the control's (RecA alone). This enhancement effect reached a plateau of ~10-fold between 25 and 50 μM of peptide #3 concentration and there is some reduced stimulation at higher concentrations (Figure 2A).

Next, we changed the sequence of peptide #3 to identify the functional motifs which had contributed to enhancement. All synthesized peptides were measured for their D-loop enhancement as shown in Figure 2C. When the sequence of peptide #3 was changed from IRFLTARRR to IRFLTAKKK (peptide #3 and peptide #4, respectively), the stimulation of the strand assimilation activity was reduced somewhat, though it was still 2.5-fold higher than for RecA alone. This is a reasonable result because both Arg and Lys are positively charged amino acids. The different degrees of RecA-mediated D-loop activity enhancement could have resulted from their slightly dissimilar physical and chemical properties. Reducing or increasing the number of Arg in the C-terminus (peptide #13 or #5 and peptide #6, respectively), showed no apparent enhancement (peptide #13) or inhibition (peptide #5 and peptide #6) of RecA-mediated D-loop activities.

Replacement of different hydrophobic amino acids in peptide #3 also affected enhancement. Both peptide #11 and #12 totally lose enhancement capabilities due to the Phe being deleted or replaced with Ala from peptide #3. We also changed the Phe of peptide #3 to Leu (peptide #14), Tyr (peptide #16) and Trp (peptide #17). The results indicate they still exert a slight enhancement effect at the 50 μM concentration.

We also tested the peptide #12, peptide #13 and peptide #17 over a range of concentrations, similar to the concentrations of peptide #3 in the Figure 2A. The results showed that peptide #12 and peptide #13 exhibited no enhancement at any tested concentrations, whereas the peptide #17 showed a maximal stimulation (~3-fold) at 25 μM concentration (data not shown).

The distance between the Phe and the three Arg of peptide #3 was evaluated by inserting Ala following Phe (peptide #15), which also produced a reduction in enhancement. Based on the above observations, we concluded that the hydrophobic core, the poly-positively charged part, and the distance between them are all important factors in enhancement. This is consistent with our design based on the structure of RecA–ssDNA complex (Figure 1A).

It could be argued that the high positive charge of peptide #3 may induce DNA binding in a non-specific manner and might result in an unexpected enhancement of RecA-mediated strand assimilation. As controls,

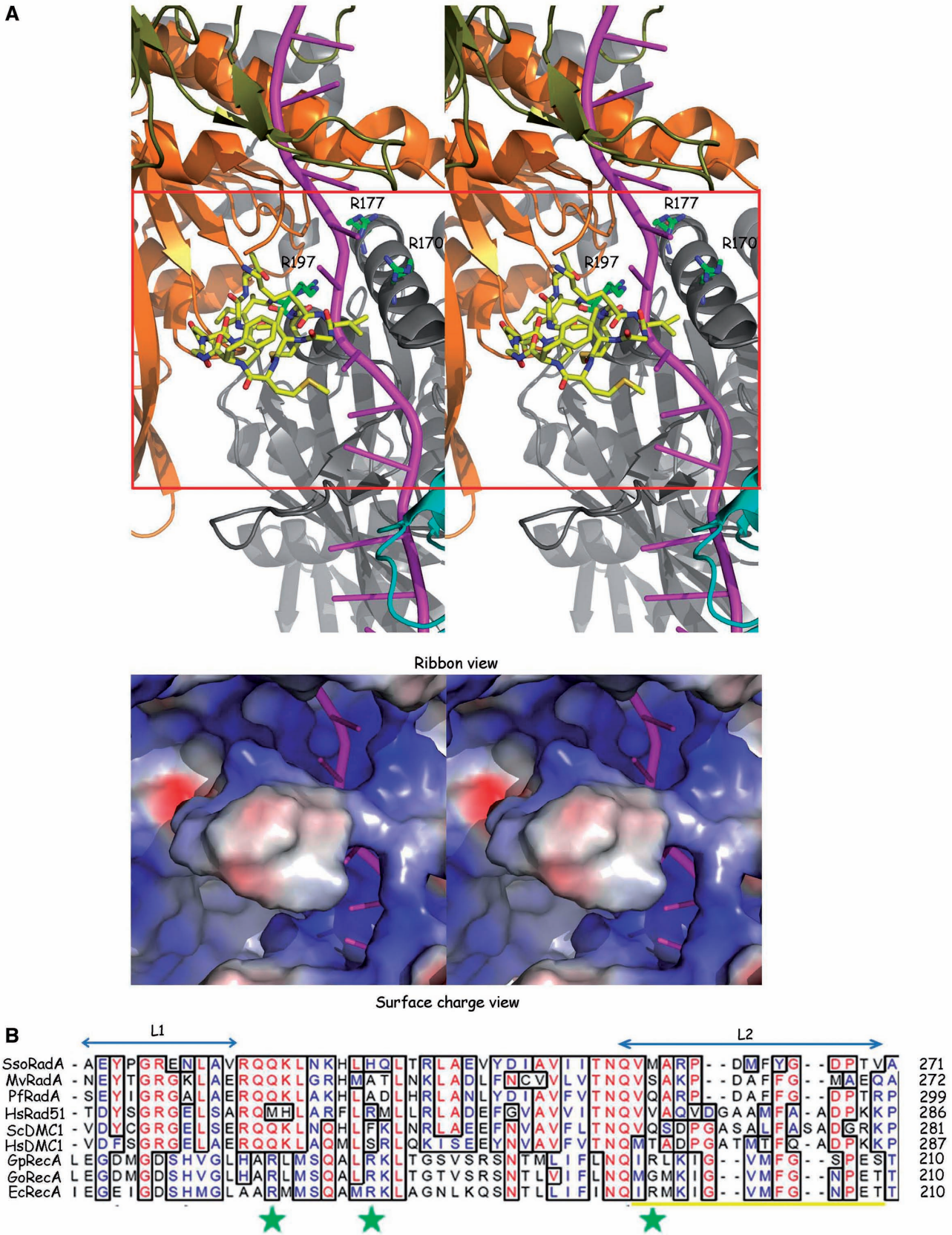


**Figure 1.** The complex structure of *E. coli* RecA bound with a single-stranded DNA. (**A**) The ribbon and surface charge potential diagrams of RecA–ssDNA (PDB: 3CMW). The red frame on top of Figure 1A is highlighted as ribbon, while surface charge views and all images are drawn as stereo views by software PyMol. A group of stick amino acids (in yellow) form the hydrophobic mass to embed in the base stacking of ssDNA (in magentas). The negatively charged sugar-phosphate backbone of ssDNA is also stabilized by many positively charged amino acids of RecA, including R170, R177 and R197 (in green). (**B**) Multiple sequence alignment of RecA family proteins from *S. solfataricus* (*Sso*RadA), *M. voltae* (*Mv*RadA), *P. furiosus* (*Pf*Rad51), *H. sapiens* (*Hs*Rad51 and *Hs*Dmc1), *S. cerevisiae* (*Sc*Dmc1), *G. polyoxogenes* (*Gp*RecA), *G. oxydans* (*Go*RecA) and *E. coli* (*Ec*RecA). The yellow line and stars show a group of hydrophobic amino acids and three Arg residues, respectively. The ssDNA binding loops L1 and L2 are indicated.

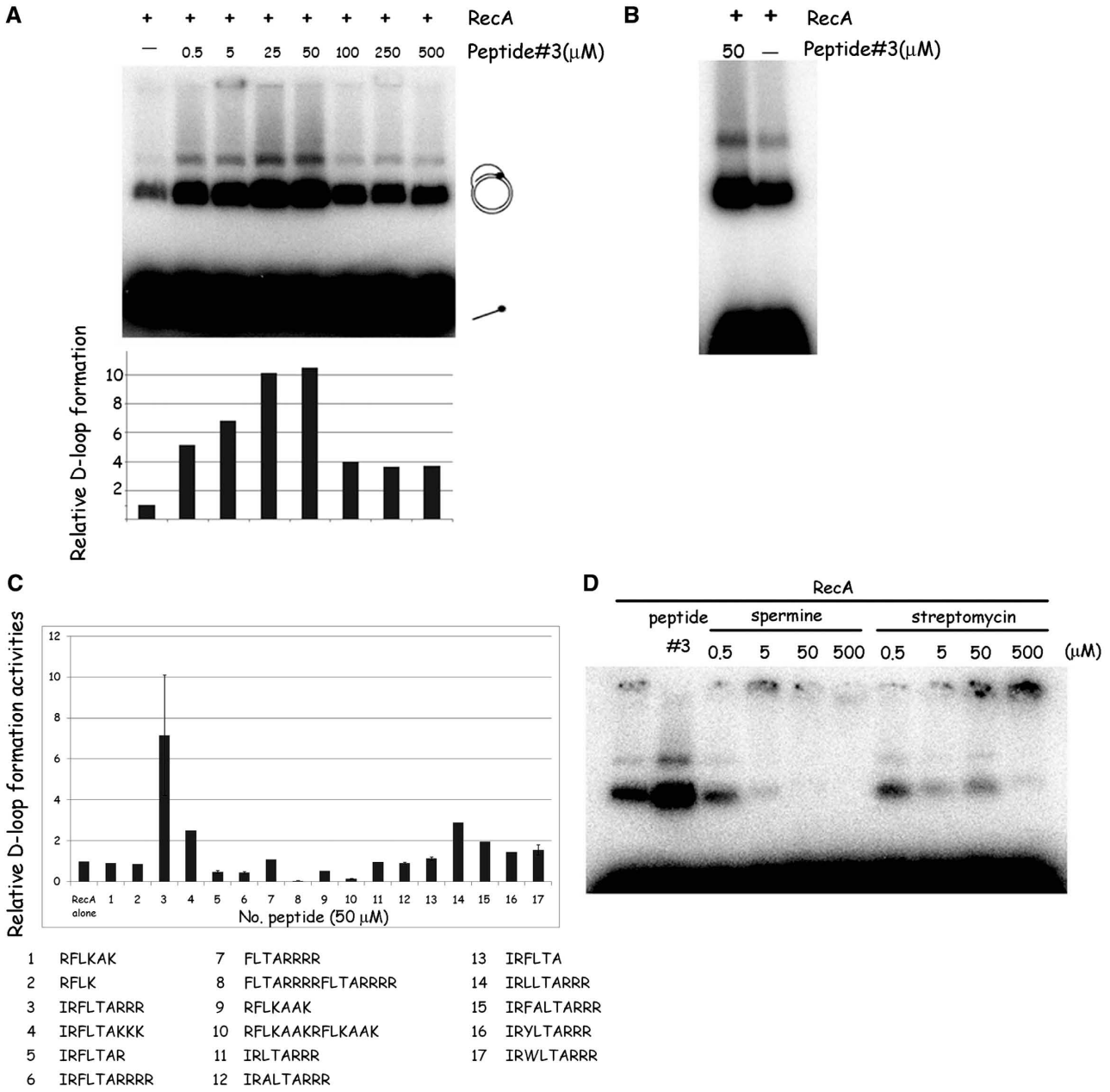


**Figure 2.** Peptide #3 enhances the strand assimilation activity due to its composition of positively charged amino acids following the hydrophobic amino acids. (**A** and **B**) Peptide #3 increases the activity of RecA-mediated strand assimilation. The reactions were carried out using GW1 (A) or pUC18 plasmids (B) and the concentration of peptide #3 was indicated. The strand assimilation activities were normalized when no peptide #3 was added as the control. More detailed information about strand assimilation assay is described in 'Materials and Methods' section (**C**). Positively charged amino acids following a hydrophobic environment and the space between them are critical motifs for the enhancement of peptide #3. Seventeen different peptides, containing some amino acid modifications, were synthesized for the RecA-mediated strand assimilation assay. The amino acid sequences of all the peptides are shown. The amount of strand assimilation is normalized (RecA alone as the control) to measure the relative enhancement and error bars were shown. (**D**) The enhancement effect of peptide #3 is not solely due to its poly-positive charge. Spermine and streptomycin have many amino groups and they display poly-positive charges in neutral pH. Instead, they exerted considerable high inhibition of RecA-mediated strand assimilation even in low concentration conditions. The concentration of spermine and streptomycin are indicated.

two positively charged compounds, spermine and streptomycin, and peptide #8 with eight Arg were then examined in order to determine whether they enhance strand assimilation like peptide #3 (Figure 2C and D). Interestingly, all of them intensely inhibited the RecA-mediated strand assimilation, even at the low concentration of 0.5 μM (spermine and streptomycin). The inhibition generated by the spermine, streptomycin as well as the highly positively charged peptide #8 may result from the aggregation of RecA proteins or DNA (28).

In order to test whether the characteristic properties of peptide #3 can be found in small compounds, we tested Arg-Arg 4-methoxy-B-naphthylamide HCl, 1-naphthylacetyl spermine HCl and *N*′-glutathionyl-spermidine disulfide. They have a hydrophobic core followed by positive charges and were tested for any promotion in the

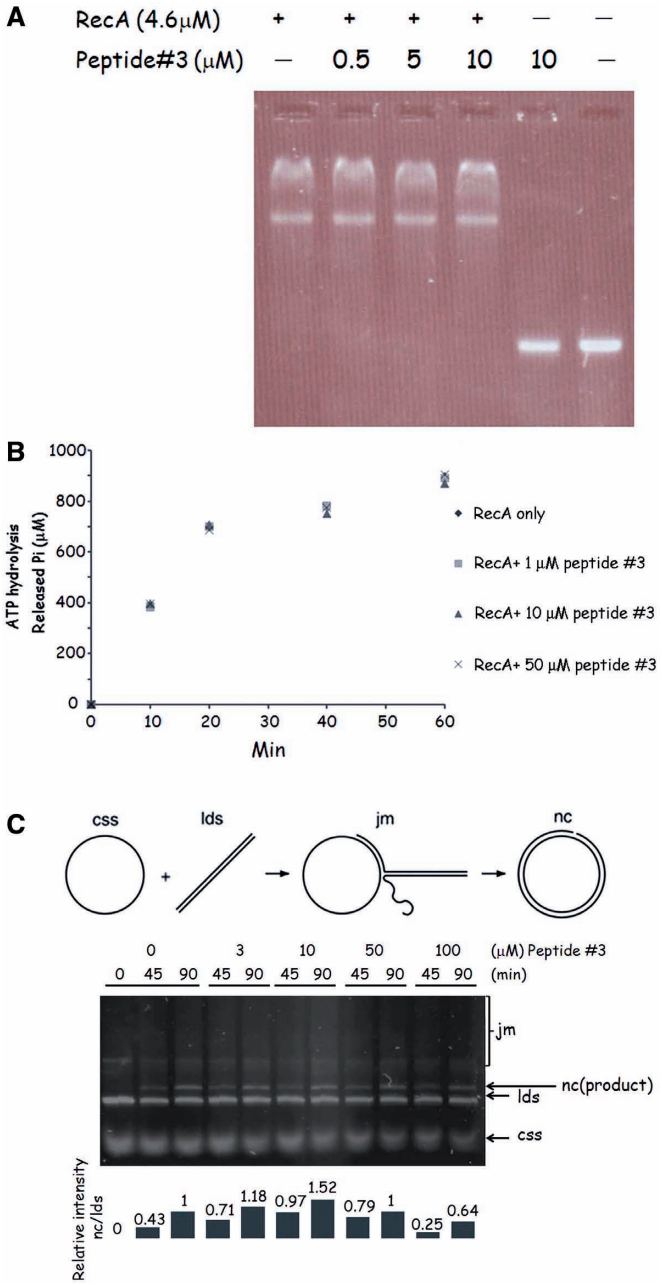


**Figure 3.** Peptide #3 can enhance both RecA-mediated strand assimilation and three-strand exchange. (**A**) EMSA analysis: formation of RecA–ssDNA filament with or without peptide #3. ΦX 174 was incubated with RecA proteins, peptide #3, or both. The resulting products were separated on an agarose gel and visualized by staining with SYBR-Green II (Invitrogen, USA). (**B**) Time-course experiments of RecA-mediated ATPase assay with or without peptide #3. ATP hydrolysis was initiated by adding 7.5 mM ATP (with 1 nM [$\gamma$-$^{32}$P]ATP) at 37°C. At different time points, 0.5 μl aliquots were withdrawn and spotted on a thin layer of chromatography paper to separate [$\gamma$-$^{32}$P]ATP from free $^{32}$P-labeled inorganic phosphate. (**C**) The representative gel shows the RecA-mediated three-strand exchange activity with increasing amounts of peptide #3 (0, 3, 10, 50 and 100 μM). The reaction solutions contained an ATP-regeneration system (ATP, phosphocreatine and creatine phosphokinase), $Mg(OAc)_2$, RecA proteins, ssDNA (ϕX174), dsDNA (ϕX174RF I, XhoI-treated) and SSB. All reactions were incubated at 37°C. At a determined time, the reactions were stopped by treating with SDS and proteinase K. The samples were analyzed by electrophoresis on an agarose gel using TAE buffer. The substrates and products were visualized by SYBR-Green II (Invitrogen) staining. The relative band intensity was shown above each bar when the intensity of RecA without peptides in 90 min was normalized. See the 'Materials and Methods' section for more detailed information. For abbreviations: css, circular single-stranded DNA; lds, linear duplex DNA; jm, joint molecule DNA (D-loop); nc, nicked circular duplex DNA (products).

RecA-mediated strand assimilation. Our data showed that none of these three compounds can enhance the D-loop formation (data not shown). We will use high-throughput compound screening method in order to find compounds with properties similar to those of peptide #3 in the future. Interestingly, such a strategy had been developed to screen compounds that inhibit DNA recombination (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1385).

Taken together, we suggest that peptide #3 adopts a unique conformation in the recombination system which serves to stimulate RecA-mediated strand assimilation.

### Peptide #3 stimulates RecA activities by a mechanism that does not influence the ATP hydrolysis

The ssDNA binding of RecA requires the presence of ATP or ATP analogs and $Mg^{2+}$. The activity of RecA-mediated D-loop formation can be stimulated by reducing or preventing the hydrolysis of ATP (29,30). Hence, two experiments were performed to determine whether the enhancement promoted by peptide #3 is related to ATP hydrolysis. EMSA, carried out using ϕX174 virion ssDNA (Figure 3A), showed that peptide #3 does not interfere with binding between RecA filaments and ssDNA. Furthermore, there is little interaction between peptide #3 and ssDNA under EMSA conditions.

A second experiment was conducted to determine possible association of RecA-mediated ATP hydrolysis with increasing concentrations of peptide #3 (Figure 3B). The data, collected at different time points, indicate that peptide #3 has no effect upon the rate of ATP hydrolysis. In sum, the two experiments suggest that the enhancement capabilities of peptide #3 are not related to the inhibition of ATP hydrolysis. We then compared the behavior of peptide #3 with that of a peptide capable of stabilizing the Rad51 filament derived from a fragment of BRCA2 (31,32). The BRCA2 peptide was observed to inhibit filament depolymerization via a reduction in the Rad51 ATPase activity and produce a further increase in ionizing-radiation (IR)-induced Rad51 foci *in vivo*. Other previous reports also showed that $Ca^{2+}$ can enhance recombinase activity by way of inhibiting ATP hydrolysis (30,33). This clearly suggests that the enhancement produced by peptide #3 differs in its mechanism from the BRCA2-derived peptide or the effect of $Ca^{2+}$.

Having demonstrated its capacity to stimulate RecA-mediated strand assimilation (Figure 2A), we continued to examine Peptide #3 to ascertain if it could enhance the three-strand exchange activity as well. As shown in Figure 3C, RecA protein was able to produce joint molecule DNA (jm; intermediate) and nicked circular duplex DNA (nc, products). When different amounts of peptide #3 were added to the three-strand exchange reactions, the ratio of nc (product) to lds (substrate) was notably raised up to 50 μM. Evidently, the

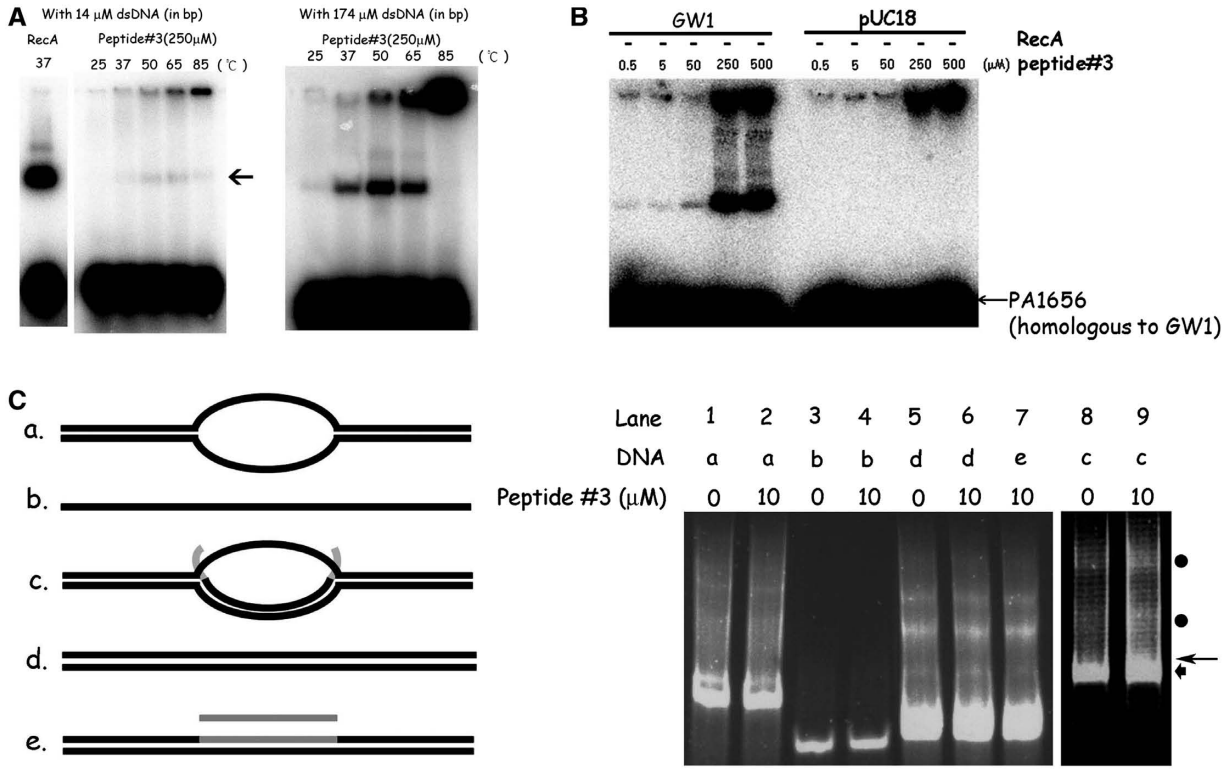


**Figure 4.** Peptide #3 alone expresses strong strand assimilation activity due to its preference for the D-loop or D-loop mimic structure. (**A**) Strand assimilation assay was performed with peptide #3 alone at increasing temperatures. Peptide #3 showed low and high strand assimilation activity with 14 μM and 174 μM dsDNA (GW1) subtracts, respectively. The RecA protein expressed strand assimilation activity here as a positive control. (**B**) Peptide #3 showed DNA sequence-dependent strand assimilation activity that eliminated the possibility of non-specific interactions between peptide #3 and DNA substrates. We used $^{32}$P-labeled P1656 which is homologous to GW1 as the ssDNA substrate to perform strand assimilation assay. The results showed no D-loop signal when using the non-homologous pUC18 plasmids as the dsDNA substrate. (**C**) Peptide #3 expresses a preference for the D-loop or D-loop mimic DNA structure. EMSAs were performed with, bubble (a), single-stranded (b), D-loop mimic (c), double-stranded (d) or double-stranded with a homologous single-stranded (e) DNA. The concentration of each peptide #3 used is indicated. The long and short arrows represented the peptide bound and unbound DNA, respectively. Two spots are the speculated DNA concatemers.

peptide #3 we designed stimulated not only RecA-mediated D-loop formation, but also three-strand exchange activity.

Many RecA structures in different conformations have been solved (21,27,34,35), but the exact structure of RecA in the stage of strand assimilation remains unclear. Although the rationally designed peptide #3 was based on the structure of the RecA–ssDNA complex (Figure 1A), it produces a remarkable effect on strand assimilation enhancement and three-strand exchange (Figures 2 and 3). Structural characterization of the interactions of the RecA–ssDNA–dsDNA ternary complex should provide better insights into the mechanism of peptide #3′s enhancement activity.

### Peptide #3 alone can induce strand assimilation activity

Strand assimilation assay of peptide #3 alone, devoid of RecA proteins, was performed at different temperatures (Figure 4A). Peptide #3 promoted a small extent of D-loop formation (as the arrow in Figure 4A indicates) under standard conditions of 14 μM (in base pairs) dsDNA substrate GW1, while a much greater D-loop signal appeared with 174 μM (in base pairs) GW1. The D-loop signal was increased from 25°C to 85°C, although for reasons which remain unclear, the DNA seemed to be aggregated at the top of the gel at high temperature reactions. Because peptide #3 alone shows low or no D-loop signal (below 37°C) under standard conditions, we can exclude the possibility that the enhancement of RecA-mediated D-loop formation (Figure 2A) only results from the effect of peptide #3 alone.

The high concentration of peptide #3 (250 μM) and non-specific binding of the ssDNA probe and the dsDNA substrate at high temperature demands consideration. Experiments were conducted on concentration-dependent peptide #3 at 65°C using two different dsDNA substrates (GW1 and pUC18) and the ssDNA probe PA1656 (50 nt), which is homologous to GW1 (Figure 4B). Only with high concentrations of peptide #3 and GW1, was the D-loop signal becoming evident. However, no D-loop signal was detected using pUC18 substrate, even when treated with high concentrations of peptide #3. This result eliminated the skepticism due to the non-specific binding between the ssDNA probe and dsDNA substrate at high temperature. Furthermore, peptide #3 alone does not show any three-strand exchange activity (data not shown).

Why peptide #3 is able to stimulate RecA-mediated homologous recombination (Figures 2A and 3C) and also promotes strand assimilation only by itself

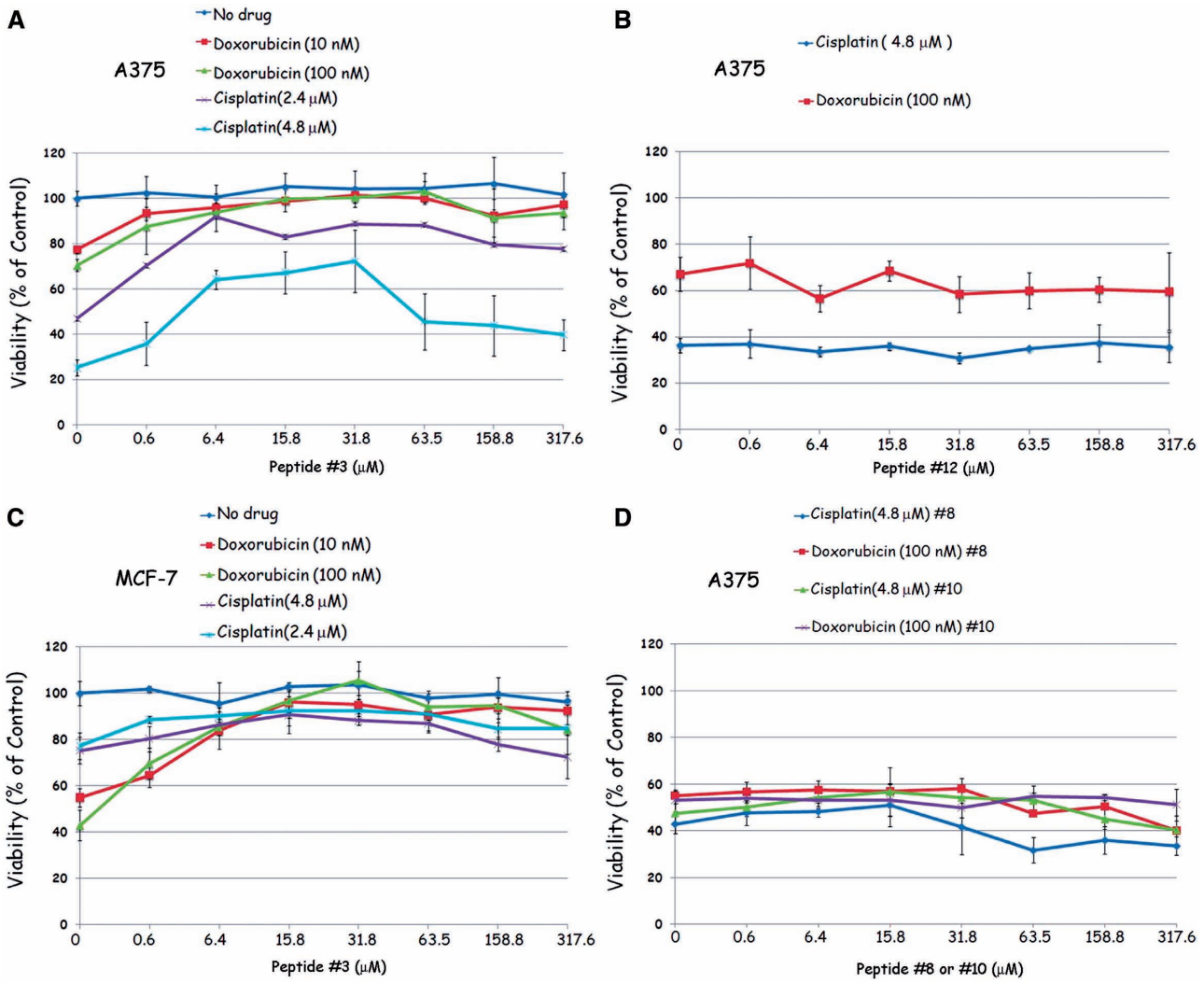


**Figure 5.** Peptide #3 rescues the viability of A375 and MCF-7 cells after treatment with DNA damaging agents. In these four illustrations, cells were treated with the drugs alone for 4 days or pre-treated with increasing amount of peptide #3, #8, #10 or #12 for 24 h prior to exposure to the drugs for 4 days. Cell viability was determined by the MTT assay. Two cell lines, A375 (**A, B** and **D**) and MCF-7 (**C**) and two DNA damaging agents (cisplatin and doxorubicin) were used for these experiments. The concentrations of cisplatin and doxorubicin used in the treatment are indicated. Averages of three independent experiments are plotted. Error bars represent standard deviations. The viability of the cells treated with DMSO was represented as the control.

(Figure 4A)? The work of Chang *et al.* (36) has proven that there is no D-loop signal in the absence of any recombinase, even at 65°C. In Figure 4A, the D-loop signal was increased by peptide #3 alone in accordance with the raising of the temperature. We assume that increased temperature could transiently open the two strands of dsDNA substrate (e.g. GW1), thereby presenting a window of opportunity for the homology ssDNA probe to temporarily invade the dsDNA substrate. Concurrently, peptide #3 may stabilize the three-strand DNA conformation. However, at high temperature, peptide #3 seems to induce the non-specific interactions between ssDNA probes and dsDNA substrates, which generate high molecular weight aggregates that accumulated at the top of the wells (Figure 4A and B). Conversely, in the absence of peptide #3, this conformation will cease immediately due to the annealing of the two strands of dsDNA substrate. The three-strand DNA structure is also created by the RecA nucleoprotein filament after invading the homologous sequence of the dsDNA substrate. This may be why peptide #3 is able to enhance RecA-mediated strand assimilation and three-strand exchange at 37°C. In the more stable three-strand DNA structure, peptide #3 promotes a stronger D-loop signal and increases the product obtained from three-strand exchange.

To test our hypothesis, we designed a series of DNA structures according to the previous report (37), including bubble (a), single-stranded (b), D-loop mimic (c), double-stranded (d) or double-stranded with a homologous single-stranded (e) as shown in Figure 4C. The results of EMSA analysis indicated that peptide #3 alone prefers to bind with the D-loop mimic DNA structure, though the binding was weak (lane 8 and 9, Figure 4C). We assumed each peptide #3 may bind three

nucleotides according to our concept of the rational design. There are 25 nucleotides in the region of D-loop mimic DNA structure (the scheme c). It means that each DNA molecule could bind a maximum of eight peptides and the molecular weight of this complex would only increase ~6 kDa comparing to that of unbound DNA molecule (~61.7 kDa). Therefore, there is only a little band shifting when running the gel (lane 8 and 9, Figure 4C). However, no band shifting was detected in the other DNA structures (lane 1–7, Figure 4C). In addition to the band of D-loop mimic DNA structure, we observed that there are two other bands which are higher molecular weight than D-loop mimic DNA structure in the lane 9. We speculate these two bands may be some kinds of DNA concatemers induced by the peptides, because the molecular weight of these two bands is much too high for a single peptide–DNA complex.

Based on the RecA–DNA crystal structures, ssDNA and dsDNA are arranged in a B-DNA-like conformation, though it contains variations from that of a typical B-DNA (21). These variations influence the binding of peptide #3 and the D-loop mimic DNA structure (Figure 4C). Furthermore, the D-loop mimicking DNA structure we designed might not totally represent the actual D-loop structure that RecA filaments created. We suggest this is the reason for the weak affinity of peptide #3 to the D-loop mimic DNA structure (Figure 4C). So, the RecA-mediated D-loop enhancement of peptide #3 may have resulted from its interaction with the D-loop DNA structure initiated by the recombinases.

The design of peptide #3 is based on the characteristic interactions between RecA (L1, L2 loops and the area between them) and ssDNA (Figure 1A). The enhancement activity of peptide #3 is due to its stabilizing effect on the D-loop DNA structure rather than influencing the binding of RecA and ssDNA (Figures 3 and 4). It seems to imply that the L1, L2 loops and the area between them play important yet unknown roles in the process of recombinase-mediated D-loop formation. In the future, thorough investigation of the interaction of D-loop DNA structure and peptide #3 will supply us with a better understanding of the complexities of the D-loop DNA structure and the recombinase.

### Peptide #3 promotes resistance toward DNA damaging agents

The mechanism of homologous recombination is presumably similar in eukaryotes and prokaryotes. Hence, we next examined whether peptide #3 promotes homologous recombination in eukaryotic cells. Two mammalian cell lines, A375 (melanoma) and MCF-7 (breast cancer), were treated by two cytotoxic DNA damaging drugs, cisplatin and doxorubicin for 4 days after pre-incubating with peptide #3 for 24 h. Cisplatin- and doxorubicin-induced cytotoxicity directly resulted from a double-stranded break that seemed to be rescued by peptide #3 (Figure 5A and C).

As a control, the viability of the cells treated with neither DNA damaging drugs nor peptide #3 was 100%. In Figure 5A, pre-treatment of A375 cells with a low peptide #3 dose (6.4 μM) protected 90% of the cells from 2.4 μM cisplatin, a drug dose that reduced the viability by 50%. When the cells were treated with 4.8 μM cisplatin, the viability of the cells was rescued by peptide #3 (from 0 to 31.8 μM) from 25% to 70%.

When treated with the doxorubicin (10 nM and 100 nM), 15.8 μM peptide #3 enabled the A375 cells to completely recover viability. Unlike the intense suppression of the effect of cisplatin on the A375 cells, peptide #3 was only moderately effective in MCF-7 cells (Figure 5C). However, the reduction of viability in MCF-7 cells resulting from doxorubicin treatment, was fully rescued by 31.8 μM peptide #3. The results also showed that the treatment of peptide #3 without DNA damaging drugs does not have any impact on the viability of both A375 and MCF-7 cells (Figure 5A and C).

Peptide #8 and peptide #10 strongly inhibited the RecA-mediated D-loop formation (Figure 2C). We tested peptide #8 or #10 to see whether they have any biological effect on mammalian cells treated with DNA damage agents. However, those peptides essentially had no effect (Figure 5D). It is likely that the strong inhibitory effect of the RecA-mediated D-loop formation of those two peptides *in vitro* was due to their very high positively charged nature, causing non-specific DNA aggregation. Therefore, these two peptides likely do not inhibit homologous recombination *in vivo*, thus do not lead to increased effect for DNA damage agents.

Peptide #12, which has no effect on the enhancement of strand assimilation (Figure 2C) was then examined, and the data showed a failure to rescue viability in A375 cells (even with high dosages) when treated with cisplatin or doxorubicin (Figure 5B). These results taken together imply that peptide #3 specifically is able to promote mammalian cells to repair DNA damage induced by DNA damaging agents.

The linkage between the recovery of cell viability promoted by peptide #3 and the promotion of homologous recombination would appear to be considerable. Peptide #3 must penetrate the cell membrane and enter into the nucleus to execute its function. It has been well documented that positively charged small peptides exhibit excellent membrane and nuclear translocation properties (38,39). Some of them have become reliable delivery tools for therapeutic macromolecules such as peptides, proteins, and nucleic acids (39). It is reasonable to speculate that peptide #3, with its poly-positive charges, may penetrate the cell membrane, get into the nucleus, and further promote DNA repair via the mechanism of homologous recombination. Taken together, peptide #3 seems to protect cells from double strand breaks caused by DNA damaging agents *in vivo* using a mechanism similar to that *in vitro* seen in our biochemical findings.

One small compound, RS-1, identified by Jayathilaka *et al.* (19) can enhance the activity of human Rad51 proteins under the condition that stimulates the formation of Rad51–ssDNA nucleoprotein. Although RS-1 can promote D-loop formation, it seems to result from increased formation or stabilization of the nucleoprotein. Our data clearly suggest the enhancement stimulated by the peptide #3 directly comes from increasing D-loop

formation efficiency and, moreover, stimulating three-strand exchange activity.

Human Rad51AP1 and Hop2-Mnd1 also promote the D-loop formation of the Rad51 proteins (22,37,40,41). It is possible that the enhancement promoted by peptide #3 results from its imitation of Rad51AP1 or Hop2-Mnd1. In the future, structural investigations of the interaction between Rad51AP1 (or Hop2-Mnd1) and Rad51 may supply more clues to test this possibility.

Interactions of several small peptides derived from the DNA binding loop L2 of RecA proteins and single-stranded or double-stranded DNA have been shown with different experimental designs in previous reports (42–44). Although those peptides also contain hydrophobic amino acids that are indispensable for their functions, they seem to have different mechanisms from those of our peptides. For example, peptide #3 has a high affinity with D-loop mimic DNA but has little interactions with single-stranded or double-stranded DNA (Figures 3A and 4C). However, previously reported peptides (42–44) can interact with both single-strand and double-strand DNA. It is possible that the peptide #3 may stabilize the D-loop structure via sequestering the displaced ssDNA or arresting the annealing of original dsDNA.

The single-stranded or double-stranded DNA bound to RecA proteins is extended about 1.5 times relative to a canonical DNA resulted from intercalating several hydrophobic amino acids of L2 loop into base-base stacking (21,45,46). Recently, it has been proven that this kind of DNA structure can be induced by some homologous pairing proteins, including bacterial RecO, viral RecT and human Rad51 (46). The non-canonical DNA structure may also be generated by peptide #3 by stacking the DNA base with the hydrophobic amino acids in the peptide. Future structural work of the DNA in complex with peptide #3 will answer this question.

In summary, our data indicate that our rationally designed specific peptides (particularly peptide #3) from the RecA–DNA structure can enhance strand assimilation activity and three-strand exchange of the recombinase-mediated recombination relying on the ability of its preference for the D-loop DNA structure. The peptide also enhances resistance toward DNA damaging drugs in mammalian cells. Peptide #3 may prove to be a very useful tool in homologous recombination related issues, including gene targeting, complementing the deficiencies of homologous recombination accessory proteins, elevating cellular resistance to DNA-damaging agents and so forth. Based on the properties we have discovered in this study (a hydrophobic patch and a poly-positive change region separated by certain distance), important avenues of research have been opened for further improvement of the peptide or extensive screening of small molecules possessing similar structural characteristics.

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