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# Design and synthesis of fluorescent and biotin tagged probes for the study of molecular actions of FAF1 inhibitor

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ABSTRACT

To study the molecular action of ischemic Fas-mediated cell death inhibitor, we prepared fluorescent-tagged and biotin-tagged probes of the potent inhibitor, KR-33494, of ischemic cell death. We used the molecular modeling technique to find the proper position for attaching those probes with minimum interference in the binding process of probes with Fas-mediated cell death target, FAF1.



Ischemia is caused by contraction or occlusion of the blood vessels.[1] Although the mechanisms of cell death after ischemic attack are not fully understood yet, Fas and FasL are known to be important factors in the pathology of ischemia together with **FAF1** protein.[2–4] Through the extensive designing and derivatizing process we have identified **KR-33493** (Fig. 1) as a potent inhibitor for Fas-mediated cell death, **FAF1**.[5,6]

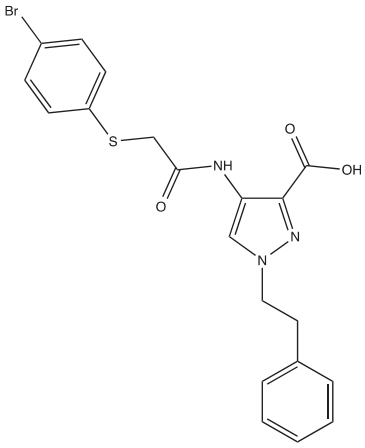


**Figure 1.** The structure of KR-33493.

In order to study the molecular action of **FAF1** inhibitor, KR-33493, in the ischemic condition, we need to prepare fluorescent-tagged and biotin-tagged probes. First, we needed to find


**Figure 2.** Molecular model for the complex of FAF1 (green) and KR-33493 compound (orange), which is obtained through the hierarchical protein structure modeling approach, based on secondary-structure enhanced Profile–Profile threading Alignment (PPA) and the iterative implementation of the Threading ASSEmbly Refinement (TASSER) program. The Phenyl rings of $Tyr^{225}$ and $Trp^{240}$ play an important role in interaction with CIF forming π–π stacking interaction.

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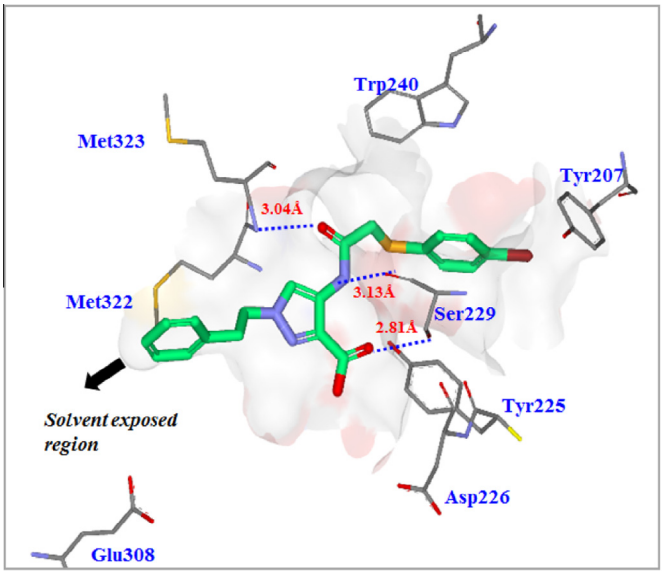
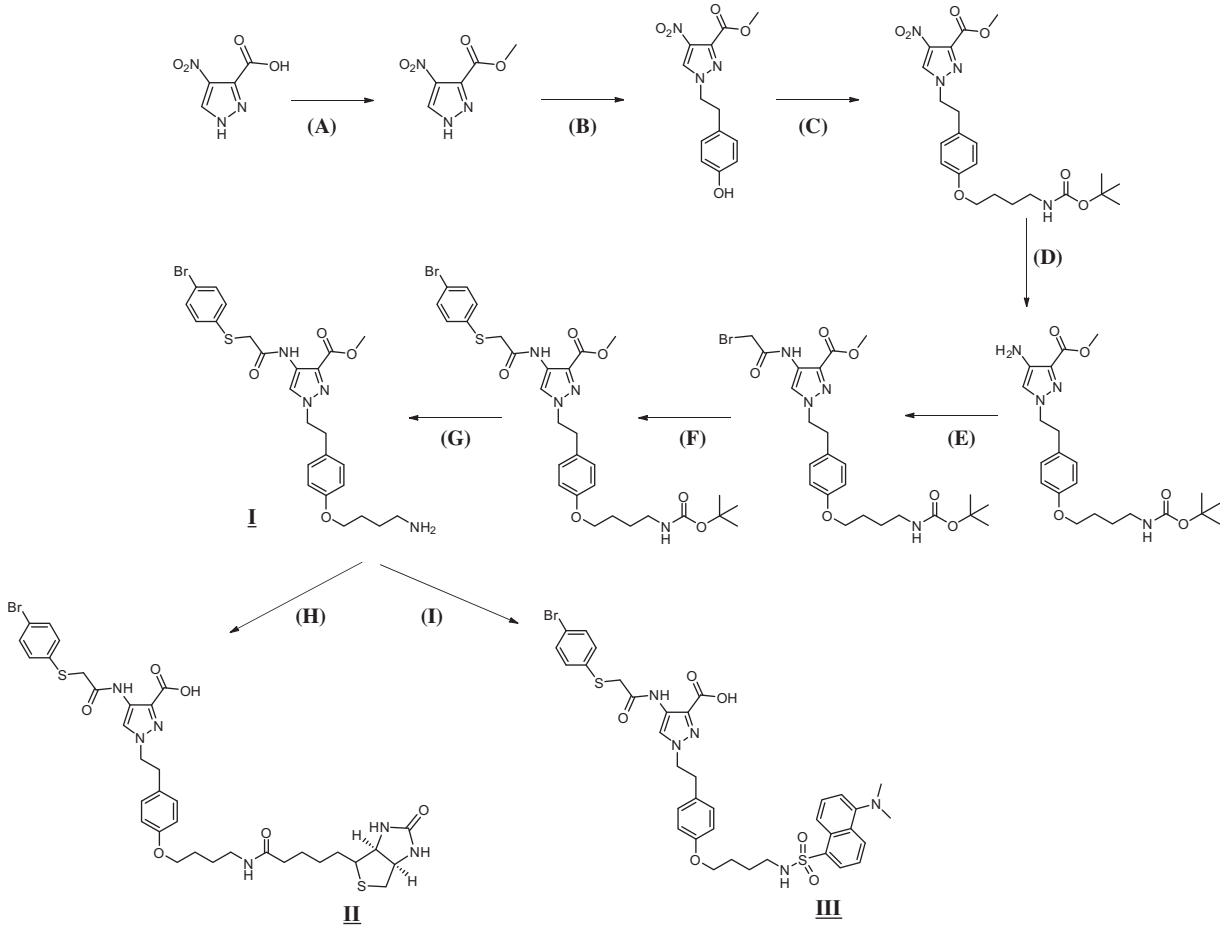



**Figure 3.** One of possible models for binding interaction of **KR-33493** with **FAF1**.

the position for the attachment of fluorescent and biotin components. The most preferred position to attach these components would be the one that has a least interference in the binding interaction of **KR-33493** with **FAF1** protein. To find the binding mode of **KR-33493** with FAF1, we initially constructed the 3D structure of FAF1 and then carried out the docking experiments as shown in Figures 2 and 3. As the sequence similarity of FAF1 with other proteins is less than 20%, we used hierarchical protein structure modeling approach based on the secondary-structure enhanced Profile–Profile threading Alignment (PPA) and the iterative implementation of the Threading ASSEmbly Refinement (TASSER) program.[7,8] Through this program, we obtained five candidate models for 3D structure of FAF1 and thus carried out molecular dynamics simulation using CHARMM force field (version 27.0) and default parameters interfaced with Accelrys Discovery Studio3.5. To decide binding sites, we used the binding site defining protocol in Accelrys Discovery Studio3.5 and defined the binding site from receptor cavity. Then, we docked our compounds including KR-33493 and an inactive compounds from our previous study,[9] and thus examined whether or not match SAR study for the compounds, and finally selected one binding model for FAF1 and an active compound as shown in Figure 1. In this binding mode the carbonyl group of Ser229 acts as a hydrogen bonding acceptor to form a hydrogen-bonding with the amide group of **KR-33493** and the hydroxyl group on the side chain of Ser229 forms another hydrogen bonding with the carboxyl group of **KR-33493**. The amine group of Met323 acts as a hydrogen-bonding donor to form hydrogen bonding with the amide carbonyl group of **KR-33493**. Especially, Met323 together with Met322 seems to provide a rather narrow hydrophobic environment.




**Scheme 1.** Reagents and condition: (A) concd sulfuric acid, MeOH, rt, 92%; (B) 4-(2-bromo-ethyl)-phenol, $Cs_2CO_3$, DMF, rt, 56%; (C) t-boc aminobutyl bromide, $Cs_2CO_3$, DMF, rt, 97%; (D) 10% Pd/C, $H_2$, MeOH, rt, 97%; (E), (F) bromoacetyl bromide, *p*-bromobenzenethiol, $NEt_3$, THF, rt, 76%; (G) trifluoroacetic acid, $CH_2Cl_2$, 0 °C, 89%; (H) (i) D-biotin, N-succinimide, EDC·HCl, DMAP, $NEt_3$, DMF, rt (ii) 6 N NaOH, MeOH, rt, 73%; (I) (i) dansyl chloride, $NEt_3$, $CH_2Cl_2$, 0 °C, (ii) 6 N NaOH, MeOH, rt, 84%.

This analysis suggests that the para position of the phenethyl part of **KR-33493** would be a good position for the attachment of biotin and fluorescent components. For the convenience reason, we decided to prepare a common linker with a proper length having an amine functional group as in the intermediate **I** in Scheme 1. We then perform the computer modeling study on the biotin attached compound **II** and dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl, group attached compound **III** to examine whether these compounds fit well in our model without interference in the binding interaction of **KR-33493** and **FAF1**. The modeling study indicates that these probes sit well in the narrow groove without significant interactions with neighboring amino acids as shown in Figure 4. The synthesis and the reaction condition for the intermediate **I** and the compounds **II** and **III** are shown in Scheme 1.

First of all, to validate whether the biotin- or dansyl-tagged KR-33493 retain the activity of the parent compound KR-33493, we examined its ability to block the cell death. The SH-SY5Y cells were seeded into 96-well plates and incubated for 24 h. The SH-SY5Y cells then were pretreated with dimethyl sulfoxide (DMSO; vehicle), KR-33493 (20 μM), biotin-tagged KR-33493 (20 μM) or dansyl tagged KR-33493 (20 μM) for 30 min. After pretreatment, cells were treated with 500 μM $H_2O_2$ for 6 h in presence of vehicle, KR-33493, biotin-tagged KR-33493 or dansyl-tagged KR-33493. Cell death was assessed by the release of lactate dehydrogenase (LDH) into the extracellular medium. Cell viability was determined by MTS assay. As shown in Figure 5, these results demonstrate that biotin- or dansyl-tagged KR-33493 retain the protective potential of parental compound KR-33493.

Also, to examine whether FAF1 is the cellular target of KR-33493 using compound **II**, the SH-SY5Y cells were transfected with the Flag-FAF1. After 36 h, cells were treated with $H_2O_2$ 500 μM for 30 min and then lysed in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 and 0.4 mM phenylmethylsulfonylfluoride. Biotinylated KR-33493

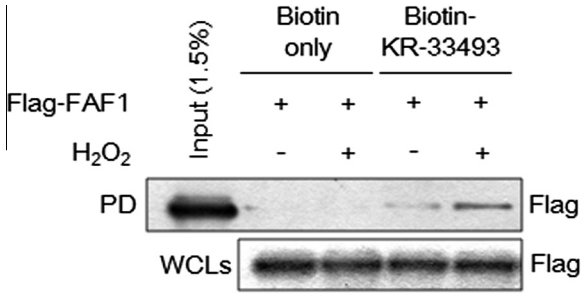



**Figure 6.** SH-SY5Y cells were transfected with Flag-FAF1. After 36 h, cells were treated with $H_2O_2$ 500 μM for 30 min and then lysed in lysis buffer. The cell lysates prepared from SH-SY5Y cells exposed to $H_2O_2$ were incubated with either biotin-bound agarose or biotinylated KR-33493 (100 μM)-bound agarose for 12 h at 4 °C. The agarose beads were then washed five times with lysis buffer and were subjected to immunoblot analysis with anti-Flag.

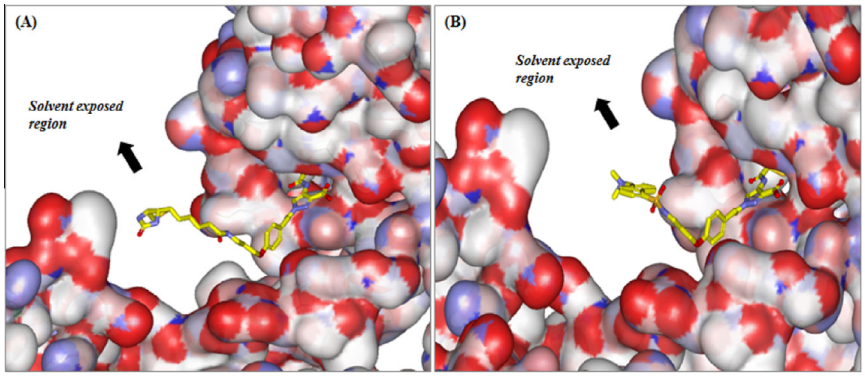



**Figure 4.** (A) The induced-fit docking model of Biotin-**KR-33493** (compound **II**) (B) Dansyl tagged compound (compound **III**). Images were made using Accelrys Discovery studio4.0.

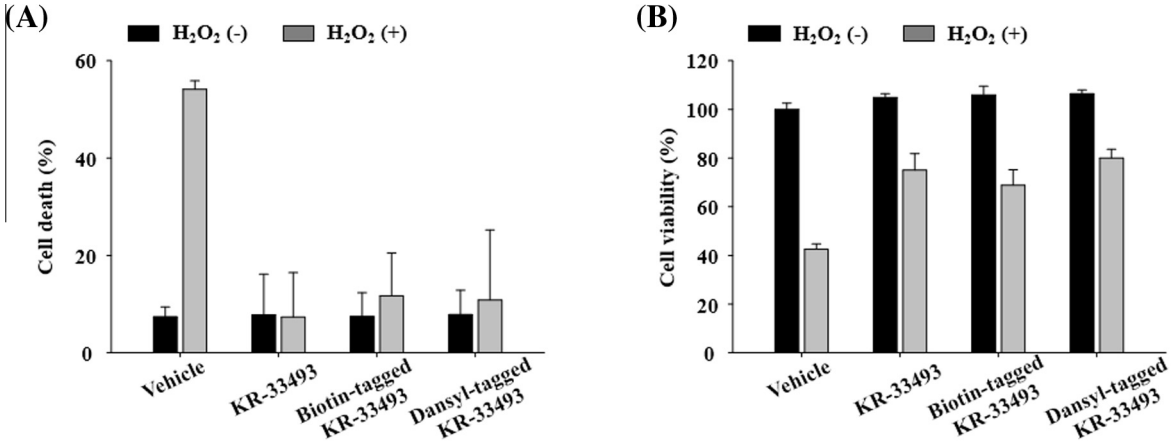



**Figure 5.** SH-SY5Y cells were pretreated with vehicle (DMSO), KR-33493 (20 μM), biotin-tagged KR-33493 (20 μM) or dansyl-tagged KR-33493 (20 μM) for 30 min. Cells then were treated with 500 μM $H_2O_2$ for 6 h in presence of vehicle or individual compounds. (A) Cell death was determined by measuring LDH release. (B) Cell viability was determined by MTS assay.

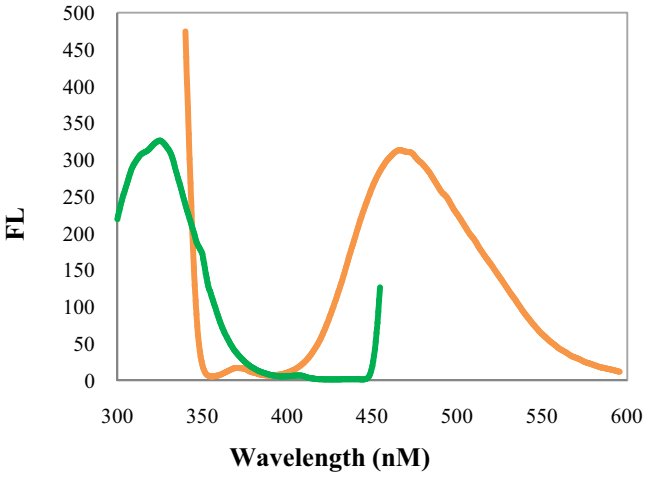



**Figure 7.** The Fluorescence Excitation Spectrum in green color (max. intensity at 325 nM) and the Dispersed Fluorescence Spectrum in brown color (max. intensity at 468 nM) taken by *Perkin Elmer LS55*.

was preincubated with 7 μl streptavidin agarose (Sigma) for 4 h at 4 °C. The beads were then isolated by centrifugation. The KR-33493-bound agrose beads were then incubated with cell lysates for 12 h at 4 °C. The beads were washed five times with lysis buffer, and compound-bound proteins were subjected to immunoblot analysis with anti-Flag. When the biotinylated KR-33493 (**II**) was incubated with cell lysates prepared from the Flag-FAF1 expressing SY-SY5Y cells exposed to $H_2O_2$ and pulled down with streptavidin-conjugated agarose beads, strong interaction between compound **II** and FAF1 was detected in lysates of $H_2O_2$-treated cells than those of non-treated cells as shown in Figure 6. This result indicated that KR-33493 targets FAF1 in a stimulus-dependent manner. Also, the dansyl group attached probe, **III**, shows high intensity spectrum with the excitation max. at 325 nM and emission max. at 468 nM as shown in Figure 7.

In this study, to study the molecular action of ischemic Fas-mediated cell death inhibitor, we prepared fluorescent-tagged and biotin-tagged probes of the potent inhibitor, KR-33494, of ischemic cell death and we confirmed FAF1is the cellular target of KR-33493 using the SH-SY5Y cells transfected with the Flag-FAF1. Afterward, we will try to use these kinds of probes to identify the biological mode of action for some molecules.

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