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Discovery of a Small Molecule-Dependent Photolytic Peptide

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Supporting Information Placeholder

ABSTRACT: We accidentally found that YM-53601, a known small-molecule inhibitor of squalene synthase (SQS), selectively depletes SQS from mammalian cells upon ultraviolet (UV) irradiation. Further analyses indicate that the photo-depletion of SQS requires its short peptide segment located at the COOH terminus. Remarkably, when the 27-amino acid peptide was fused to GFP or unrelated proteins at either NH_2 or COOH termini, such fusion proteins were selectively depleted when the cells are treated both with YM-53601 and UV exposure. Product analysis and ESR experiments suggest that the UV irradiation promotes a homolytic C-O bond cleavage of the aryl ether group in YM-53601. It is likely that the radical species generated from UV-activated YM-53601 abstract hydrogen atoms from the SQS peptide, leading to the photolysis of the entire protein. The pair of the SQS peptide and YM-53601 discovered in the present study paves the way for designing a new small-molecule-controlled optogenetic tool.

Exogenous control of protein degradation by small molecules permits conditional depletion of particular proteins in living cells or organisms. Such powerful techniques, exemplified by PROTAC[1-3], auxin-inducible degron (AID)[4], and ligand-induced degradation (LID)[5], have been used for investigating biological regulatory mechanisms and even for developing pharmaceuticals. Herein we report the discovery of a small molecule-dependent photolytic peptide that might lead to development of a new optogenetic strategy that allows temporal and spatial elimination of protein products.

We fortuitously found such a peptide during the course of an unrelated research project on squalene synthase (SQS), an enzyme that catalyzes the reductive dimerization of two molecules of farnesyl pyrophosphate (FPP) to form squalene[6]. Squalene synthesis is the first committed step in cholesterol biosynthesis, making SQS an attractive pharmacological target for hypocholesterolemic therapies[7]. One of the prominent small-molecule inhibitors of SQS is YM-53601 (Figure 1A), which was developed by Yamanouchi Pharmaceutical decades ago[8-9].

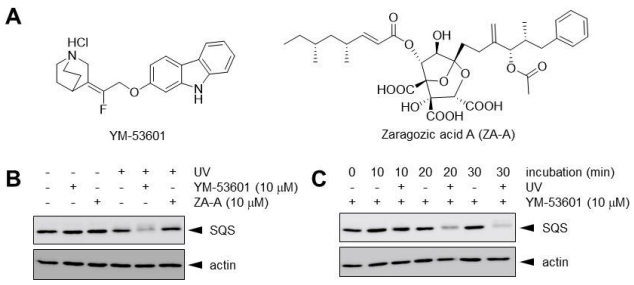

Figure 1. Photo-depletion of SQS with YM-53601. (A) Chemical structures of YM-53601 and zaragozic acid A (ZA-A). (B) Effects of YM-53601 and UV irradiation on the protein levels of SQS. HEK293 cells were pre-treated with YM-53601 or ZA-A for 1 h, and the samples were then irradiated with UV (365 nm, 0.093 J/cm²/s) on ice for 30 min. The lysates were immunoblotted with the indicated antibodies. (C) Time-course experiments. HEK293 cells were pre-treated with 10 μM of YM-53601 for 1 h and then further incubated in the presence or absence of UV exposure (365 nm, 0.093 J/cm²/s) for 0, 10, 20 or 30 min. The lysates were immunoblotted with the indicated antibodies.

As shown in Figure 1B, the level of SQS was reduced when human HEK293 cells were treated with both YM-53601 and UV irradiation (365 nm, 0.093 J/cm²/s), while YM-53601 treatment or UV irradiation alone had no detectable effects on the SQS levels. Zaragozic acid A (ZA-A) (Figure 1A), another inhibitor of SQS[10-11], failed to affect the SQS levels with or without UV irradiation, indicating that the photo-depletion of SQS is unique to YM-53601. Time-course experiments showed that 20-min UV exposure is required for the elimination of SQS (Figure 1C), whereas the actin protein levels were unchanged

even after 30-min UV irradiation. We also similarly observed SQS photo-depletion in a number of other cultured mammalian cells including HeLa cells and MEF cells (Figure S1A and S1B).

To identify segments of SQS that are required for its YM-53601-induced photo-depletion, a series of NH_2-terminal and/or COOH-terminal deletion mutants of SQS (amino acids 1-370, 31-417, and 31-370) (Figure 2A) were expressed in HEK293 cells and subjected to YM-53601 and UV irradiation. As shown in Figure 2B-2E, the combination of YM-53601 and UV irradiation depleted the FLAG-tagged NH_2-terminal deletion mutant of SQS as much as the FLAG-tagged full length SQS. On the other hand, the identical treatment exerted no significant effects on the COOH-terminal deletion mutants. These results indicate that the COOH-terminal segment of SQS (amino acids 371-417) is required for the YM-53601-dependent photo-depletion of SQS.

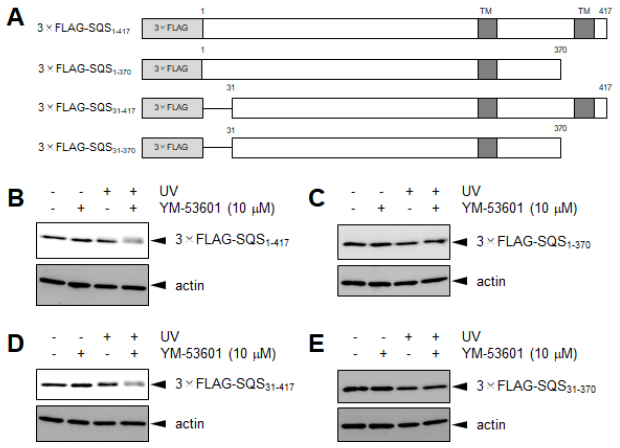

Figure 2. Importance of the COOH-terminal region of SQS. (A) Schematic representation of SQS deletion mutants. (B-E) Effects of YM-53601 and UV irradiation on the protein levels of the SQS deletion mutants. HEK293 cells were transfected with 3×FLAG-SQS_{1-417} (B), 3×FLAG-SQS_{1-370} (C), 3×FLAG-SQS_{31-417} (D), or 3×FLAG-SQS_{31-370} (E). After 24 h, the cells were treated with DMSO or 10 μM of YM-53601 for 1 h, and then irradiated with UV (365 nm, 0.093 J/cm²/s) on ice for 30 min. The lysates were immunoblotted with the indicated antibodies.

We next examined whether the COOH-terminal segment of SQS is sufficient for the photo-depletion of other proteins. Remarkably, a simple attachment of the COOH-terminal segment of SQS (371-417) to the COOH-terminus of EGFP (Figure 3A) rendered the EGFP fusion protein as responsive as SQS to YM-53601 and UV irradiation (Figure 3C), whereas EGFP itself had no detectable effects (Figure 3B). Linking the identical SQS segment to the NH_2 terminus of EGFP similarly rendered the EGFP fusion responsive to YM-53601 and UV irradiation (Figure S2). When the COOH-terminal segment of SQS was fused with GSK3β, an unrelated protein kinase important in cell signaling, similar effects were observed (Figure S3), raising the idea that the 47 amino-acid peptide could be used as a tag for the photo-depletion.

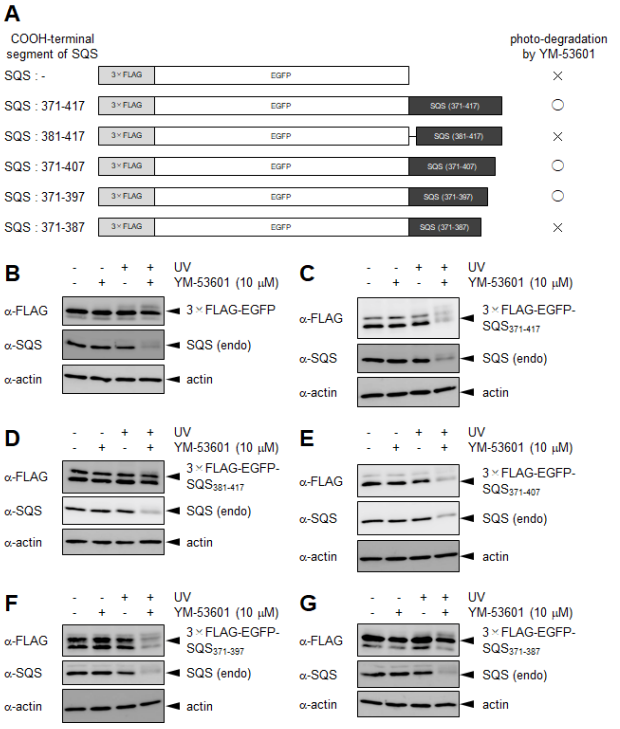

Figure 3. The YM-53601-dependent depletion of the EGFP proteins fused with SQS peptides. (A) Schematic representation of the EGFP-SQS fusion proteins. (B-G) Effects of YM-53601 and UV irradiation on the protein levels of the EGFP-SQS fusions. HEK293 cells were transfected with 3×FLAG-EGFP (B), 3×FLAG-EGFP-$SQS_{371-417}$ (C), 3×FLAG-EGFP-$SQS_{381-417}$ (D), 3×FLAG-EGFP-$SQS_{371-407}$ (E), 3×FLAG-EGFP-$SQS_{371-397}$ (F), 3×FLAG-EGFP-$SQS_{371-387}$ (G). After 24 h, the cells were treated with DMSO or 10 μM of YM-53601 for 1 h, and then irradiated with UV (365 nm, 0.093 J/cm²/s) on ice for 30 min. The lysates were immunoblotted with the indicated antibodies.

To find the minimal SQS peptide sequence required for the photo-depletion, we fused a series of short SQS peptides to EGFP and analyzed their responses to YM-53601 and UV irradiation. While removal of the NH_2-terminal 10 amino acids from the 47 amino-acid SQS peptide abolished the response to YM-53601 and UV irradiation (Figure 3D; EGFP-$SQS_{381-417}$), its COOH-terminal truncation of up to 20 amino acids was tolerated (Figure 3F; EGFP-$SQS_{371-397}$). Further COOH-terminal truncation impaired the effect of YM-53601 and UV irradiation (Figure 3G; EGFP-$SQS_{371-387}$). These results suggest that fusion of the 27 amino-acid segment of SQS (amino acids 371-397) is sufficient for the YM-53601-dependent photo-depletion of the fused proteins.

We next investigated the mode of action. The observed protein elimination is most likely mediated by protein degradation since the depletion was relatively rapid (~20 min) and promoter-independent. Cells possess three major protein degradation machineries: ubiquitin-proteasome, autophagy, and protease pathways. To examine whether these pathways are involved, we analyzed the effects of known inhibitors for each pathway on the YM-53601-dependent photo-depletion of SQS. All of MG-132 (proteasome inhibitor), chloroquine (lysosome inhibitor), E-64d (lysosome inhibitor), bafilomycin A (lysosome inhibitor), NH_4Cl (lysosome inhibitor), and a protease inhibitor cocktail failed to inhibit the YM-53601-dependent photolysis of SQS (Figure S4), suggesting that the photolysis of SQS is independent from these cellular protein degradation machineries.

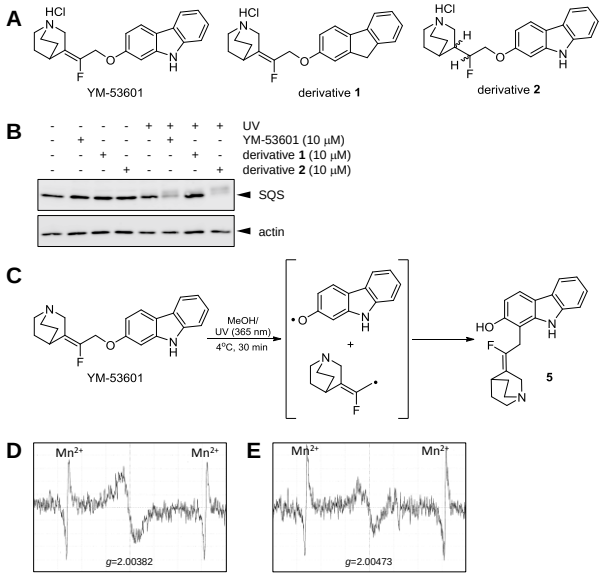

Figure 4. Photoreaction of YM-53601. (A) Chemical structures of YM-53601 and its derivatives (**1** and **2**). (B) Effects of YM-53601 derivatives on the photolysis of SQS. HEK293 cells were pre-treated with DMSO, YM-53601 (10 μM), derivative **1** (10 μM), or derivative **2** (10 μM) for 1 h and then irradiated with UV (365 nm, 0.093 J/cm²/s) on ice for 30 min. The lysates were immunoblotted with the indicated antibodies. (C) Photoproduct analysis of YM-53601. YM-53601 was irradiated with UV (365 nm, 0.093 J/cm²/s) for 30 min in MeOH at 4 °C. The main product was purified by HPLC, and its structure was determined by NMR and MS analysis. (D and E) ESR spectra of YM-53601 (D) and derivative **1** (E). Mn^{2+} was used as an internal standard.

Requirement of UV irradiation implies involvement of photoreaction of YM-53601. Two derivatives of YM-53601 were chemically synthesized: in derivative **1**, the nitrogen atom of carbazole was replaced by a carbon atom, and the fluorinated C-C double bond was saturated in derivative **2** (Figure 4A). Cellular thermal shift assays confirmed that both derivatives **1** and **2** bind and stabilize endogenous SQS protein in cells as much as YM-53601 does, indicating their target engagement in cells comparable to that of YM-53601 (Figure S5A-S5C). However, derivative **1** failed to induce the photolysis of SQS, highlighting the importance of the electron-rich carbazole structure for its photolytic ability. On the other hand, derivative **2** maintained its ability to induce the photolysis of SQS at a level comparable to YM-53601, indicating no contribution of the fluorinated double bond in the photolytic activity.

Carbazole derivatives have been reported to generate radicals through UV irradiation[12-13]. We hypothesized that the YM-53601-dependent photolysis of SQS may be initiated by the reactive radicals generated from UV-activated YM-53601. To analyze the photoreaction of YM-53601, we photo-irradiated a MeOH solution of YM-53601 at 365 nm at 4 °C and separated its reaction products by reverse-phased HPLC (Figure S6A, S6B). MS analysis of the major product revealed a compound of *m/z* = 337.1703, which is identical to that of YM-53601, suggesting the formation of a photo-rearrangement product. NMR profiles of the product are indeed consistent with the structure of a product of the Photo-Claisen rearrangement (**5**) (Figure S6C), in which the homolytic C-O bond cleavage products of allyl phenyl ether are recombined to form *ortho* or *para*-substituted phenols[14] (Figure 4C). ESR analysis further supported the radical formation process. YM-53601 upon UV irradiation displayed an ESR signal consistent with the formation allyl and phenoxy radicals (g=2.00382)[15] (Figure 4D). The ESR signals from derivative **1** were weaker than that from YM-53601 (Figure 4E), which is in a good agreement with its inability to photolyse SQS. These results suggest that the UV irradiation of YM-53601 generates a pair of phenoxy and allyl radicals to initiate the photolysis of SQS.

The photoreaction of a mixture of YM-53601 and $SQS_{371\text{-}397}$ was next investigated *in vitro* (Figure 5A). Since $SQS_{371\text{-}397}$ is quickly oxidized in PBS solution, the cysteine residue (C374) was replaced with a serine residue (C374S) (Figure 5A). The resulting mutant peptide maintained its ability to induce the YM-53601-dependent photolysis of its EGFP fusion protein (Figure S7A and S7B). The mutant peptide, $SQS_{371\text{-}397(C374S)}$, was suspended in PBS in the presence or absence of YM-53601, irradiated at 365 nm, and then subjected to HPLC analysis. In the absence of YM-53601, $SQS_{371\text{-}397(C374S)}$ remained stable upon UV irradiation even for 60 min (Figure 5C). In contrast, addition of YM-53601 induced decomposition of $SQS_{371\text{-}397(C374S)}$ (Figure 5D). Co-decomposition of YM-53601 and $SQS_{371\text{-}397(C374S)}$ was observed within 10-min UV irradiation. The co-decomposition appears to be selective to $SQS_{371\text{-}397(C374S)}$ since YM-53601 has less effects on the stability of a control peptide, $SQS_{1\text{-}30}$ (Figure S8).

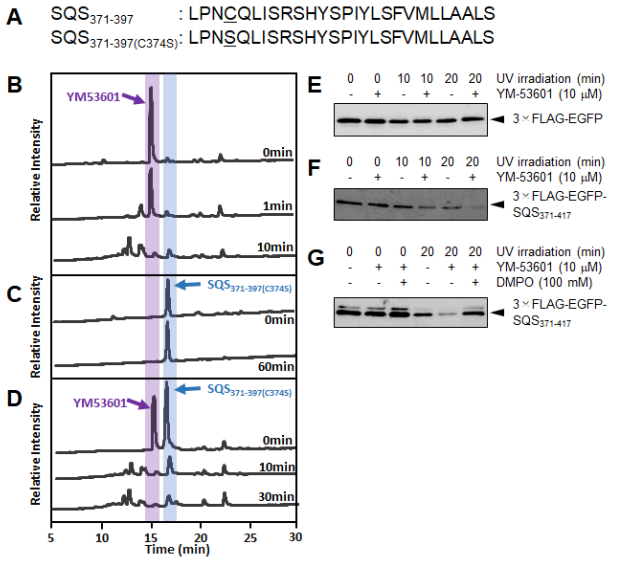

Figure 5. YM-53601-dependent photolysis of $SQS_{371-397}$ and EGFP-$SQS_{371-417}$ *in vitro*. (A) The amino acid sequences of $SQS_{371-397}$ and $SQS_{371-397(C374S)}$. (B-D) YM-53601-dependent photolysis of $SQS_{371-397}$ *in vitro*. The peptide and YM-53601 were dissolved in PBS and irradiated with UV (365 nm, 0.093 J/cm²/s) for the indicated time. The samples were directly analyzed by HPLC at 280 nm. YM-53601 (5 μM) alone (B); $SQS_{371-397(C374S)}$ alone (20 μM) (C); a mixture of YM-53601 (5 μM) and $SQS_{371-397(C374S)}$ (20 μM) (D). The positions of YM-53601 and $SQS_{371-397(C374S)}$ are highlighted. (E-G) YM-53601-dependent photolysis of EGFP-$SQS_{371-417}$ *in vitro*. 3×FLAG-EGFP (E) and 3×FLAG-EGFP-$SQS_{371-417}$ (F and G) were immunopurified from HEK293 cells. Each purified protein was mixed with DMSO or 10 μM of YM-53601 (E-G) in the presence or absence of 100 mM of DMPO (G). The samples were irradiated with UV (365 nm, 0.093 J/cm²/s) for the indicated time. The proteins were detected with an α-FLAG antibody.

We performed similar experiments with the EGFP fusion of $SQS_{371-417}$ *in vitro*. We purified 3×FLAG-EGFP and its fusion of $SQS_{371-417}$ from HEK293 cell lysates through immunoprecipitation, and used the purified proteins for *in vitro* experiments. 3×FLAG-EGFP itself exhibited no detectable photolysis either in the presence or absence of YM-53601 (Figure 5E and S9A). In contrast, UV irradiation induced decomposition of purified 3×FLAG-EGFP-$SQS_{371-417}$ in a YM-53601-dependent manner (Figure 5F and S9B). DMPO (5,5-dimethyl-1-pyrroline N-oxide), a radical scavenger[16], rescued 3×FLAG-EGFP-$SQS_{371-417}$ from the decomposition, demonstrating that the photolysis is mediated by radical species.

To confirm that no additional cellular components are necessary for this degradation event, we next bacterially expressed and purified 6×His-EGFP and its fusion of $SQS_{371-417}$ for *in vitro* experiments (Figure S10). Western blot analysis of the products demonstrated that UV irradiation induced decomposition of 6×His-EGFP-$SQS_{371-417}$ in a YM-53601- and time-dependent manner whereas 6×His-EGFP exhibited no detectable effects. Surprisingly, silver-stained gels of the samples displayed no particular partial fragments of 6×His-EGFP-$SQS_{371-417}$. Such a pattern of radical-mediated protein decomposition has been documented in the literature[17-19], in which reactive radical species are capable of inducing complete protein degradation through hydrogen abstraction from the protein backbone. It is likely that the radical species generated from UV-activated YM-53601 abstract hydrogen atoms from the SQS peptide, leading to the photolysis of the entire protein. It remains unclear why the radical reaction of the SQS peptide could deplete the entire proteins without the cellular protein degradation machineries.

Our study reported herein uncovered a small molecule-dependent photolytic peptide. Cellular thermal shift experiments with the SQS deletion mutants indicated that this COOH-terminal peptide of SQS is required for the interaction with YM-53601 in cells (Figure S11). Circular dichroism (CD) spectra of $SQS_{371-397(C374S)}$ revealed its α-helix-forming property, which was promoted by addition of trifluoroethanol (TFE) (Figure S12). However, we were unable to detect direct physical interaction between YM-53601 and $SQS_{371-397(C374S)}$ as measured by CD or isothermal titration calorimeter (ITC) experiments, indicating that the isolated peptide is not sufficient to exhibit detectable affinity to YM-53601 (Figure S12).

This observation prompted us to extend our structure-activity relationship study with two additional analogs of YM-53601: compound **5** and 2-hydroxycarbazole. Cellular thermal shift assays indicate that compound **5** displayed weak affinity to SQS while 2-hydroxycarbazole exhibited no detectable thermal shifts (Figure S13A and S13B). Intriguingly, both of the molecules depleted SQS upon UV exposure although 2-hydroxycarbazole was less potent than compound **5** (Figure S13C), suggesting that the photo-sensitivity of carbazole in addition to the SQS affinity plays a significant role in the SQS depletion. It is likely that the photolysis of SQS is occurring through a photo-sensitization reactivity of carbazole and the SQS peptide.

Why the SQS peptide is highly responsive to the photoreaction remains unknown. It has been demonstrated that the radical-mediated oxidation of glutamate, aspartate, and proline residues leads to peptide bond cleavage[20-21]. The amino acid sequence of $SQS_{371-397}$ includes only two proline residues and no glutamate or aspartate residues. Metal ion coordination may also catalyze radical-mediated oxidation reactions[21-25]; however $SQS_{371-397}$ lacks in metal chelating amino acids except one histidine residue. Further studies are needed for understanding how

the SQS peptide induces protein degradation in response to radicals.

The pair of the SQS peptide and YM-53601 provides a fresh perspective for designing a new small-molecule-controlled optogenetic tool. In such tool development, three major technical hurdles remain to be surmounted. First, the specificity of the protein photo-depletion needs to be examined and optimized at a proteome level. Second, YM-53601 induces the photolysis of endogenous SQS in addition to SQS-tagged proteins. Third, prolonged UV irradiation generally damages cells (Figure S14). Design of visible light-sensitive YM-53601 analogs and optimization of the SQS peptide may provide potential solutions in future development.

ASSOCIATED CONTENT

Supporting Information.
The Supporting Information is available free of charge on the ACS Publications website. Supplemental figures and experimental details (PDF)

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Notes
The authors declare no competing financial interest.

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