Light-induced stabilization of microtubules by photo-crosslinking of a Tau-derived peptide†

Soei Watari, Hiroshi Inaba, Tomonori Tamura, Arif Md. Rashedul Kabir, Akira Kakugo, Kazuki Sada, Itaru Hamachi and Kazunori Matsuura

For light-induced stabilization of microtubules (MTs) to manipulate cells, a photo-reactive diazirine group was conjugated to a Tau-derived peptide, a motif binding on the inside of MTs. Ultraviolet (UV) light irradiation induced significant stabilization of MTs via the formation of a covalent bond of the peptide and showed toxicity.

Microtubules (MTs) are tubular cytoskeletal elements with a 15 nm inner diameter that are formed by the polymerization of tubulin dimers. MTs play important roles in various cell functions such as cell division and shape and intracellular transport, being associated with motor proteins (kinesin and dynein). Malfunction of MTs is linked to various pathological conditions, including neurodegenerative diseases, while controlling MT stabilization/destabilization is effective for the treatment of MT-related diseases and cancers. Taxol (paclitaxel) is a well-known anticancer drug, which stabilizes MTs by binding to a hydrophobic pocket of β-tubulin inside of the MTs and inhibiting their dynamic instability. However, Taxol has several limitations including a complex synthesis due to its complex structure, low water solubility and side effects resulting from its adverse outcomes on normal cells.

In this technique, light irradiation converts photoaffinity labelling agent could increase the binding affinity of TP to tubulin and stabilize MTs by light-induced covalent bond formation. In this study, we have conjugated a photo-reactive diazirine (DA), which forms a carbene upon UV light irradiation, at the N-terminus of TP (DA-TP). This strategy allowed stabilization of MTs via covalent bond formation upon UV light irradiation in vitro and in living cells. DA-TP has high water solubility and allows large structural changes of MTs by photoaffinity labelling of DA.

This DA-TP was synthesised by Fmoc solid-phase chemistry followed by the introduction of a DA moiety at its N-terminus on the resin.
DA-TP changed upon UV light irradiation (Fig. S3, ESI†), confirming the generation of carbene as reported previously.31 The dissociation constant ($K_d$) of DA-TP to tubulin was estimated as 6.1 \( \mu \)M (Fig. S4, ESI†), by measuring the fluorescence change of the intrinsic tryptophan of tubulin as reported previously.32 The affinity is similar to that of TMR-labelled TP ($K_d \approx 6.0 \mu$M). To evaluate the binding of DA-TP to MTs using confocal laser scanning microscopy (CLSM), the cysteine residue of DA-TP was modified with TMR (DA-TP-TMR) (Scheme S1 and Fig. S1d, S2d, ESI†). The MTs were prepared by preincubation with tubulin, Alexa Fluor 488-labelled tubulin (AF-tubulin) and DA-TP-TMR, and subsequent polymerization by guanosine-5’-[(3,β)-methylene]triphosphate (GMPCPP), a GTP analogue used to form stable MTs (Fig. 2a). The binding of DA-TP-TMR to MTs was confirmed by measuring the co-localisation of TMR and AF (Fig. 2b). When Taxol was added to the DA-TP-TMR-encapsulated MTs, the TMR fluorescence on the MTs was significantly reduced, indicating binding of DA-TP-TMR to the interior pocket of the MTs like Taxol. When UV light was applied to the DA-TP-TMR-encapsulated MTs for 10 min, the TMR fluorescence was not reduced by the addition of Taxol. The amount of DA-TP-TMR bound to the MTs was estimated by analysing the fluorescence intensity ratio ($I_{TMR}/I_{AF}$) from the CLSM images (Fig. 2c). The $I_{TMR}/I_{AF}$ ratio was significantly reduced upon Taxol treatment in the absence of UV light irradiation, while this ratio remained unchanged when UV light irradiation was applied for 10 min. These results indicate a covalent bond formation of DA-TP-TMR to the Taxol-binding pocket of the MTs upon UV light irradiation, remaining unaffected by Taxol administration. To further confirm a covalent bond formation between DA-TP-TMR and tubulin, SDS-PAGE with fluorescence scanning was performed (Fig. 2d and Fig. S18, ESI†). Following UV light irradiation of the DA-TP-TMR and tubulin mixture, TMR fluorescence was observed at the band corresponding to tubulin, contrary to non-UV-irradiated controls. These results confirm a covalent bond formation between DA-TP-TMR and tubulin upon UV light irradiation. Molecular mechanics calculations by a MacroModel module predicted the potential binding sites of DA of DA-TP-TMR to tubulin (Supporting text and Fig. S5, ESI†).

The effect of DA-TP on the formation efficiency of MTs was estimated using MTs prepared with GTP, which are generally unstable. DA-TP-encapsulated MTs were prepared with GTP and the supernatant (tubulin) and pellet (MTs) were separated by ultracentrifugation and analysed by SDS-PAGE to estimate the formation efficiency of MTs (Fig. S6, ESI†). Treatment of DA-TP increased the MT formation efficiency (62%) compared to controls without any additive (39%). Following UV light irradiation to the DA-TP-encapsulated MTs, the MT formation efficiency further increased (74%) reaching levels similar to Taxol treatment. These results indicate that DA-TP stabilizes MTs, while UV light irradiation further enhances its stabilization effect.

Stabilization of GTP MTs by DA-TP was further evaluated using CLSM. Although GTP MTs were unstable, with aggregates only observed without additive, DA-TP induced the formation of MTs similar to Taxol (Fig. 3a). Conversely, the formation of MTs was not observed when UV light was applied to the mixture.
of tubulin and DA-TP after the addition of GTP (Fig. S7, ESI†).
Interestingly, UV light irradiation to DA-TP-encapsulated MTs induced the formation of longer and more rigid MTs. Analysis of MTs using the CLSM images revealed that applying UV light irradiation to the DA-TP-encapsulated MTs increased both their contour length (1.6-fold) and persistence length (1.4-fold), compared to controls without UV irradiation (Fig. S8, ESI†). Under depolymerization conditions of MTs (4 °C), DA-TP-encapsulated MTs without UV light irradiation were also completely depolymerized and formed dot-like aggregates (Fig. 3b). By contrast, DA-TP-encapsulated MTs exposed to UV light irradiation resulted in long, rigid and stable MTs. The increased amount of DA-TP triggered by UV light also generated longer and more rigid MTs (Fig. S10, ESI†). Two control peptides (i.e., TP modified with DA at different positions and actin-binding peptide, Lifeact, modified with DA) were not bound to the Taxol-binding pocket and showed no effects on the stabilization of MTs with and without UV light irradiation (Supporting text and Fig. S11, S12, ESI†). Thus, the covalent bond formation between DA-TP and MTs induced by UV light irradiation resulted in long, rigid and stable MTs. The increased amount of DA-TP triggered by UV light also generated longer and more rigid MTs (Fig. S10, ESI†). Two control peptides (i.e., TP modified with DA at different positions and actin-binding peptide, Lifeact, modified with DA) were not bound to the Taxol-binding pocket and showed no effects on the stabilization of MTs with and without UV light irradiation (Supporting text and Fig. S11, S12, ESI†). Thus, the TP sequence with a DA at the N-terminus is important for the light-induced stabilization of MTs by covalent bond formation.

The motile properties of DA-TP-encapsulated GMPCPP MTs driven by ATP on kinesin-coated substrates were next analysed by fluorescence microscopy (Fig. S13, ESI†). The velocity of DA-TP-encapsulated GMPCPP MTs increased 1.3 and 1.7-fold, respectively, before and after UV light irradiation compared to control MTs. The persistence length of GMPCPP MTs was also increased by the treatment of DA-TP and subsequent UV light irradiation. The increased velocity of GMPCPP MTs was presumably due to an increased rigidity of the MTs as we have reported previously.23–25

After confirming the UV light-induced stabilization of the DA-TP-encapsulated MTs in vitro, the binding of DA-TP-TMR to human hepatoma HepG2 cells and the resulting effects on the cells were evaluated. Before introducing DA-TP-TMR, demecolcine was applied to the cells to depolymerize intracellular MTs,33,34 thus allowing for the binding of DA-TP-TMR to tubulin and subsequent repolymerization. We confirmed that intracellular MTs were depolymerized by demecolcine and then 10 μM DA-TP-TMR was applied to the cells using ProteoCarry. Intracellular MTs were stained with Tubulin Tracker Deep Red and cell nuclei were stained with Hoechst 33342. Scale bars: 10 μm. (c) Toxicity of DA-TP-TMR to HepG2 cells. HepG2 cells treated with 10 μM DA-TP-TMR or Taxol as above were further incubated for 24 h and their viability was determined using the WST assay. Demecolcine-treated cells were used as standards. Error bars represent the standard deviation (N = 3). *P < 0.05 compared to DA-TP-TMR without UV light irradiation or only UV light irradiation for the same duration, two-tailed Student’s t-test.

Fig. 3 CLSM images of GTP MTs prepared with DA-TP. Taxol or without any additives (a) after polymerization and (b) further incubation at 4 °C for 15 min. UV light was applied for 5 min. Contour and persistence lengths of MTs were determined from CLSM images (Fig. S8g and S9e, ESI†). Preparation concentrations: 2 μM tubulin, 2 μM AF-tubulin, 8 μM DA-TP or Taxol. Scale bars, 10 μm.

Fig. 4 Effects of DA-TP-TMR on HepG2 cells. CLSM images showing HepG2 cells (a) upon introduction of DA-TP-TMR and (b) after UV light irradiation (5 min) and further incubation for 15 h. Intracellular MTs were depolymerized by demecolcine and then 10 μM DA-TP-TMR was applied to the cells using ProteoCarry. Intracellular MTs were stained with Tubulin Tracker Deep Red and cell nuclei were stained with Hoechst 33342. Scale bars: 10 μm. (c) Toxicity of DA-TP-TMR to HepG2 cells. HepG2 cells treated with 10 μM DA-TP-TMR or Taxol as above were further incubated for 24 h and their viability was determined using the WST assay. Demecolcine-treated cells were used as standards. Error bars represent the standard deviation (N = 3). *P < 0.05 compared to DA-TP-TMR without UV light irradiation or only UV light irradiation for the same duration, two-tailed Student’s t-test.

Effect of demecolcine treatment on MTs.

Fig. 4 Effects of DA-TP-TMR on HepG2 cells. CLSM images showing HepG2 cells (a) upon introduction of DA-TP-TMR and (b) after UV light irradiation (5 min) and further incubation for 15 h. Intracellular MTs were depolymerized by demecolcine and then 10 μM DA-TP-TMR was applied to the cells using ProteoCarry. Intracellular MTs were stained with Tubulin Tracker Deep Red and cell nuclei were stained with Hoechst 33342. Scale bars: 10 μm. (c) Toxicity of DA-TP-TMR to HepG2 cells. HepG2 cells treated with 10 μM DA-TP-TMR or Taxol as above were further incubated for 24 h and their viability was determined using the WST assay. Demecolcine-treated cells were used as standards. Error bars represent the standard deviation (N = 3). *P < 0.05 compared to DA-TP-TMR without UV light irradiation or only UV light irradiation for the same duration, two-tailed Student’s t-test.
treated with MT stabilizers.15,35 However, the cell abnormalities were not observed when DA-TP-TMR was incubated without UV light irradiation (Fig. S16, ESI†). These findings indicate that the binding of DA-TP-TMR to intracellular MTs and the stabilization of MTs by UV light irradiation induced the observed cellular abnormalities. The toxicity of DA-TP-TMR was further evaluated using the WST assay (Fig. 4c). Viability was greatly reduced in cells treated with DA-TP-TMR and UV light irradiation, whereas DA-TP-TMR without UV light irradiation had no apparent effect. Although UV light irradiation without DA-TP-TMR also showed some cytotoxicity, the combination of DA-TP-TMR and UV light irradiation had greater effects. It is suggested that DA-TP-TMR bound to MTs in cells combined with UV light irradiation stabilized MTs, thus inhibiting cell proliferation.

DA-TP and Taxol reduced cell viability in a concentration-dependent manner; however, the tendency was not completely matched (Fig. S17, ESI†). The diffusion of DA-TP-TMR into the cytoplasm was also observed in the CLSM image (Fig. 4a), it may be possible that parts of DA-TP-TMR bind intracellular molecules non-specifically and inhibit their activity by photo-affinity labelling.

In conclusion, we showed that DA-conjugated TP formed covalent bonds to MTs and stabilized their structure upon UV light irradiation. Binding of DA-TP-TMR to intracellular MTs together with a strong toxicity was observed upon UV light irradiation, indicating that DA-TP-TMR can induce MT stabilization within cells. Although Taxol has low water solubility and is difficult to synthesize, DA-TP has the advantages of good water solubility and being relatively easy to synthesize. In addition, DA-TP can stabilize MTs only when it forms a covalent bond to MTs by photoaffinity labelling. Thus, it is expected that MTs will be stabilized only at the light-exposed areas. This technique will lead to various applications, such as the development of MT-stabilizing drugs with minimal side effects and local cell manipulation. Because prolonged UV light irradiation causes damage to cellular tissues, stabilization of MTs by visible light irradiation is required in the future.

This work was supported by KAKENHI (No. 19K15699 for H. I.) from the Japan Society for the Promotion of Science (JSPS), ACT-X (JPMJAX2012 for H. I.) and FOREST Program (JPMJFR2034 for H. I.) from the Japan Science and Technology Agency (JST).

Conflicts of interest
There are no conflicts to declare.

References
1 A. Desai and T. J. Mitchison, Annu. Rev. Cell Dev. Biol., 1997, 13, 83–117.
2 E. Karsenti, Nat. Rev. Mol. Cell Biol., 2008, 9, 255–262.
3 C. Conde and A. Cáceres, Nat. Rev. Neurosci., 2009, 10, 319–332.
4 A. Akhmanova and M. O. Steinmetz, Nat. Rev. Mol. Cell Biol., 2015, 16, 711–726.
5 G. J. Brouchard and L. M. Rice, Nat. Rev. Mol. Cell Biol., 2018, 19, 451–463.
6 M. A. Jordan and L. Wilson, Nat. Rev. Cancer, 2004, 4, 253–265.
7 The Role of Microtubules in Cell Biology, Neurobiology, and Oncology, ed. T. Fojo, Humana Press, Totowa, NJ, 2008.
8 S. Manzoor, A. Bilal, S. Khan, R. Ullah, S. Iftikhar, A.-H. Emwas, M. Alazmi, X. Gao, A. Jawaid, R. S. Z. Saleem and A. Faisal, Sci. Rep., 2018, 8, 3305.
9 M. O. Steinmetz and A. E. Prota, Trends Cell Biol., 2018, 28, 776–792.
10 C. Dumontet and M. A. Jordan, Nat. Rev. Drug Discovery, 2010, 9, 790–803.
11 A. Janke and M. O. Steinmetz, EMBO J., 2015, 34, 2114–2116.
12 M. Borowiak, W. Nahaboo, M. Reynolds, K. Nekolla, P. Jalinot, J. Hasserodt, M. Rebherg, M. Delattre, S. Zahler, A. Vollmar, D. Trauner and O. Thom-Simensional, Cell, 2015, 162, 403–411.
13 J. van Stiphout, R. A. Chabbert, D. A. Estinger, H. Wang, M. K. Hahn and T. Wittmann, Nat. Cell Biol., 2018, 20, 252–261.
14 R. C. Adikes, R. A. Hallett, B. F. Saway, B. Kuhlman and K. C. Slep, J. Cell Biol., 2018, 217, 779–793.
15 A. Müller-Deku, J. C. M. Meiring, K. Loy, Y. Kraus, C. Heise, R. Bingham, K. I. Jansen, X. Qu, F. Bartolini, L. C. Kapitein, A. Akhmanova, A. Ashfield, D. Trauner and O. Thom-Simensional, Nat. Cell Biol., 2020, 11, 46–50.
16 L. Gao, J. C. M. Meiring, Y. Kraus, M. Wranik, T. Weinert, S. D. Pritzl, R. Bingham, E. Ntouliou, K. I. Jansen, N. Olieric, J. Standfuss, L. C. Kapitein, T. Lohmüller, A. Ashfield, A. Akhmanova, M. O. Steinmetz and O. Thom-Simensional, Cell Chem. Biol., 2021, 28(228–241), e6.
17 A. Sailer, J. C. M. Meiring, C. Heise, L. N. Pettersson, A. Akhmanova, J. Thom-Simensional and O. Thom-Simensional, Angew. Chem. Int. Ed., 2021, 60, e202114614.
18 L. Gao, J. C. M. Meiring, C. Heise, A. Rai, A. Müller-Deku, A. Akhmanova, J. Thom-Simensional and O. Thom-Simensional, Angew. Chem., Int. Ed., 2022, 61, e202114614.
19 L. Gao, J. C. M. Meiring, A. Varady, I. E. Ruider, C. Heise, M. Wranik, C. D. Velasco, J. A. Taylor, B. Terni, T. Weinert, J. Standfuss, C. C. Cumber, A. Lloret, M. O. Steinmetz, A. R. Bausch, M. Distel, J. Thorn-Simensional, A. Kakugo, K. Sada and O. Thom-Simensional, J. Am. Chem. Soc., 2022, 144, 5614–5628.
20 F. Kotzyna-Hibert, I. Kapfer and M. Goeldner, Angew. Chem., Int. Ed. Engl., 1995, 34, 1296–1312.
21 J. Das, Chem. Rev., 2011, 111, 4405–4417.
22 S. Rao, L. He, S. Chakravarty, J. Ojima, G. A. Orr and S. B. Horwitz, J. Biol. Chem., 1999, 274, 37990–37994.
23 C.-P. O. Steinmetz, C. Wang, I. Ojima and S. B. Horwitz, J. Nat. Prod., 2018, 81, 600–606.
24 H. Inaba, T. Yamamoto, A. Md., R. Kabir, A. Kakugo, K. Sada and K. Matsuura, Chem. – Eur. J., 2018, 24, 14958–14967.
25 H. Inaba, T. Yamamoto, T. Iwasaki, A. M. R. Kabir, A. Kakugo, K. Sada and K. Matsuura, Chem. Commun., 2019, 55, 9072–9075.
26 H. Inaba, Y. Sueki, M. Ichikawa, A. M. R. Kabir, T. Iwasaki, H. Shimematsu, A. Kakugo, K. Sada and K. Matsuura, bioRxiv, 2022, DOI: 10.1101/2022.01.27.476107.
27 H. Inaba, M. Yamada, M. Rashid, A. Md. R. Kabir, A. Kakugo, K. Sada and K. Matsuura, Chem. Lett., 2020, 52, 5251–5258.
28 H. Inaba and K. Matsuura, Bull. Chem. Soc. Jpn., 2021, 94, 2100–2112.
29 H. Inaba, T. Yamamoto, T. Iwasaki, A. Md., R. Kabir, A. Kakugo, K. Sada and K. Matsuura, ACS Omega, 2019, 4, 11245–11250.
30 H. Inaba, M. Nagata, K. Juliano-Miyake, A. M. R. Kabir, A. Kakugo, K. Sada and K. Matsuura, Polyhedron, 2020, 52, 1143–1151.
31 T. Seifert, M. Malo, J. Lengqvist, C. Sihlbom, E. M. Jarho and K. Luthman, J. Med. Chem., 2016, 59, 10794–10799.
32 P. Mondal, G. Das, J. Khan, K. Pradhan, R. Mallesh, A. Saha, B. Jana and S. Ghosh, ACS Chem. Neurosci., 2019, 10, 2609–2620.
33 S. Dutertre, M. Ababou, R. Onderlecq, J. Delic, B. Chatton, C. Jaulin and M. Amor-Guérêt, Oncogene, 2000, 19, 2731–2738.
34 G. E. Deyave, P. Murmann, M. Hoechli, T. Tanaka and C. W. Heizmann, Biochim. Biophys. Acta, 2000, 1498, 220–232.
35 T. J. Mitchison, Mol. Biol. Cell, 2012, 23, 1–6.