



Bifunctional chimera for ligand-directed photo-degradation of oncogenic microRNA†

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Targeted inhibition of oncogenic microRNAs provides a promising anti-cancer approach. Here, we report a bifunctional chimera for ligand-directed regulation of target oncogenic precursor microRNA through photo-degradation. Chimeric TGP-210-Ppa with photosensitizer pyropheophorbide a (Ppa) linked with the ligand of the oncogenic precursor miR-210 was able to bind specifically to oncogenic pre-miRNA and produce $^1\text{O}_2$ under red light irradiation to degrade the target pre-miRNA. This bifunctional chimera-based modification of precursor microRNA serves as a unique method for target gene regulation since photo-irradiation was able to provide temporal-spatial resolution. We demonstrated that TGP-210-Ppa prevented the generation of functional miR-210 in breast cancer cells in a photocontrollable manner. This also successfully reversed the downstream oncogenic signaling pathway mediated by miR-210 to promote cancer cell death.

As a class of endogenous gene regulators, microRNAs (miRNAs) have been found to extensively participate in cancer progression.¹ Suppression of oncogenic miRNAs thus represents a promising anti-cancer strategy.^{2,3} In addition to antisense oligonucleotides capable of blocking miRNA function through Watson-Crick base pairing-based hybridization,⁴ small molecules that directly bind to the secondary structures of miRNAs are emerging as a novel type of miRNA inhibitor.⁵ For example, xanthone derivatives,⁶ aminoglycosides,^{7,8} and ether-amide derivatives⁹ have been reported to selectively bind and inhibit target miRNAs in living cells. To further enhance the potency of small-molecule inhibitors, the Disney group developed bifunctional chimeras containing small-molecule ligands of precursor miRNAs for targeted miRNA degradation.^{10,11} Ribonuclease-targeting chimera (RIBOTAC) comprising a small-molecule miRNA ligand linked with an RNase recruiter is validated to

induce miRNA degradation by bringing RNase in close proximity to the target miRNA.^{12,13} This approach has been demonstrated to degrade oncogenic miRNAs such as miR-96,¹⁴ miR-210,¹⁵ miR-17-92,¹⁶ and miR-21.¹² These achievements highlight the promise of small-molecule chimeras for miRNA inhibition.

Singlet oxygen ($^1\text{O}_2$) is a reactive oxygen species (ROS) that is highly detrimental toward biomolecules, including nucleic acids, proteins and lipids.^{17,18} Exposure to $^1\text{O}_2$ is known to cause oxidative damage to adjacent biomolecules and lead to malfunction of the oxidized biomolecules.^{19,20} Photosensitizers with strong absorption at long wavelengths have been widely adopted for biomedical applications to generate $^1\text{O}_2$ in spatio-temporal- and dose-controllable manners.^{21,22} The integration of photosensitizers with photoirradiation to produce excessive $^1\text{O}_2$ has led to the discovery of photodynamic therapy (PDT) as a powerful anti-cancer modality.^{23,24} A more deliberate approach, named chromophore-assisted light inactivation (CALI), has been developed to selectively inactivate proteins in living cells by pre-installation of photosensitizers onto the target followed by meticulous light illumination to generate $^1\text{O}_2$.^{25,26} However, the possibility of using $^1\text{O}_2$ to precisely inactivate miRNAs in living cells has been little reported.

Here we report a novel small-molecule chimera, **TGP-210-Ppa**, for targeted inhibition of miR-210 in living cancer cells (Scheme 1). **TGP-210-Ppa** consists of the small-molecule ligand, **TGP-210**, against precursor miR-210 (pre-miR-210) and the organic photosensitizer, pyropheophorbide a (Ppa). Upon ligand-directed binding of **TGP-210-Ppa** to pre-miR-210, red light irradiation is implemented to excite Ppa to produce $^1\text{O}_2$ to oxidize riboguanosine (rG) into 8-oxo-rG, causing the mutation and dysfunction of pre-miR-210.

The chemical structure of **TGP-210-Ppa** is shown in Fig. 1a. The two functional units, **TGP-210** and Ppa were readily conjugated together using an amide condensation strategy with ethylenediamine as the linker (Fig. S1, ESI†). The structure of the purified **TGP-210-Ppa** was confirmed by electrospray ionization-mass spectrometry (ESI-MS) (Fig. S2, ESI†). The

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Scheme 1 Schematic illustration on the working mechanism of the ligand-directed red-light regulation of oncogenic microRNA.



Fig. 1 (a) Chemical structure of **TGP-210-Ppa**. (b) Fluorescence spectra of **TGP-210-Ppa** after irradiation with 670 nm light (70 mW cm^{-2}) for 5 min. (c) The increase of SOSG fluorescence at 530 nm after incubation with **TGP-210-Ppa** and irradiation by 670 nm light (70 mW cm^{-2}) for 0–20 min. $\lambda_{\text{ex}} = 488 \text{ nm}$. Data are shown as mean \pm SEM ($n = 3$).

ultraviolet-visible (UV-Vis) spectroscopy of **TGP-210-Ppa** shows the characteristic absorption of **TGP-210** at $\sim 350 \text{ nm}$ and two characteristic absorption peaks of **Ppa** at $\sim 400 \text{ nm}$ and $\sim 660 \text{ nm}$ (Fig. S3, ESI[†]). The fluorescence spectrum shows that **TGP-210-Ppa** has similar fluorescence emission to that of **Ppa** (Fig. S4, ESI[†]). These results suggest that the conjugation of **TGP-210** to **Ppa** does not change the photophysical properties of the photo-sensitizer significantly.

We next examined whether **TGP-210-Ppa** was able to generate $^1\text{O}_2$ upon red-light irradiation and induce photo damage to its target pre-miR-210. Using the $^1\text{O}_2$ sensor green (SOSG) as the fluorescent indicator, we confirmed the capability of

TGP-210-Ppa to generate $^1\text{O}_2$ in Phosphate Buffered Saline (PBS) buffer upon red light irradiation. As shown in Fig. 1b, irradiation of **TGP-210-Ppa** in PBS buffer containing SOSG using light of wavelength around 670 nm increased the fluorescence signal around 530 nm, which is characteristic of the fluorescence signal enhanced by $^1\text{O}_2$ on SOSG. Time dependence curve of the fluorescence enhancement *versus* irradiation time is shown in Fig. 1c. Subsequently, 9,10-anthracendi-propionic acid (ADPA) was used as a capture probe to evaluate the efficiency of the $^1\text{O}_2$ generation. As shown in Fig. S5 (ESI[†]), the absorption intensity of ADPA at 378 nm gradually decreased as the irradiation time increased in the presence of the **TGP-210-Ppa** and MB, suggesting ADPA decomposition. According to the decay curves of the ADPA absorption, the $^1\text{O}_2$ quantum yield of the **TGP-210-Ppa** was about 0.327.²⁷ We then chose red light irradiation for 5 min as the standard condition to induce $^1\text{O}_2$ -based oxidation.

Since nucleobases are known to be susceptible to $^1\text{O}_2$,²⁸ we evaluated the generation of oxidized nucleobases in pre-miR-210. Whether pre-miR-210 treated with **TGP-210-Ppa** upon red light irradiation results in the oxidation of nucleobases in pre-miR-210 was examined. We used LC-ESI-MS/MS to analyze the hydrolysis products of pre-miR-210 with or without **TGP-210-Ppa** under light irradiation (Fig. S6, ESI[†]). It is noteworthy that only 8-oxo-rG was detected as the oxidized nucleobase form in the hydrolyzed products of pre-miR-210 treated with **TGP-210-Ppa** and light irradiation. This is consistent with previous evidence that, among the four nucleobases in RNA, guanine is a major substrate of $^1\text{O}_2$.²⁹ Gel-shift assay revealed that excessive $^1\text{O}_2$ generated in the system by the presence of 200 equivalent of **TGP-210-Ppa** to pre-miR-210, together with 15 min light irradiation induced strand breakage (Fig. S7, ESI[†]), which is in agreement with previously reported results.^{30,31} Overall, these results demonstrate that **TGP-210-Ppa** is able to cause oxidative damage to pre-miR-210 under photoirradiation.

The intracellular applications of **TGP-210-Ppa** were then explored. An MTT assay showed that **TGP-210-Ppa** had no obvious cytotoxicity in the dark at the concentration up to $25 \mu\text{M}$ (Fig. 2a). Confocal fluorescence imaging and flow cytometry showed that **TGP-210-Ppa** was able to be taken up



Fig. 2 (a) Relative viabilities of MDA-MB-231 cells incubated with different concentrations of **TGP-210-Ppa** for 48 h before light exposure. (b) Confocal microscopic images of MDA-MB-231 cells treated with **TGP-210-Ppa** for 4 h followed by exposure to 670 nm light for 5 min. Scale bar: $20 \mu\text{m}$.

by cancer cell lines such as breast cancer MDA-MB-231 cells (Fig. S8, ESI†). Intracellular generation of $^1\text{O}_2$ by **TGP-210-Ppa** upon exposure to red light irradiation was monitored using the $^1\text{O}_2$ -sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). As shown in Fig. 2b, the irradiation of MDA-MB-231 cells incubated with **TGP-210-Ppa** using light of 670 nm for 5 min significantly enhanced the fluorescence signal from the $^1\text{O}_2$ indicator inside the cells. We next investigated whether the *in-situ* generation of $^1\text{O}_2$ was able to regulate the intracellular pre-miR-210 since it was the target of **TGP-210-Ppa** for proximity-based regulation. Furthermore, to study the binding consequences of adding the Ppa moiety to **TGP-210**, binding affinities were measured by microscale thermophoresis (MST) to the targets with **TGP-210** or **TGP-210-Ppa**.^{15,32} The results indicate that **TGP-210-Ppa** maintained selective binding to RNA with a K_d of 392 nM to pre-miR-210, which is modestly weaker compared with **TGP-210** with a K_d of 265 nM (Fig. S9, ESI†).

The protocol to evaluate the efficacy of the ligand-directed red-light regulation of intracellular pre-miR-210 is illustrated in Fig. 3a. Quantitative reverse transcription polymer chain reaction (qRT-PCR) results indicated the relative abundance of various RNAs in MDA-MB-231 cells treated with **TGP-210-Ppa** with or without red light irradiation. Cells treated by **TGP-210** or Ppa were used as a control to exclude the effect caused by the ligand or photo-sensitizer alone (Fig. 3b). Without exposure to light irradiation, both **TGP-210** and **TGP-210-Ppa** raised the intracellular level of pre-miR-210 dose-dependently, which

could be attributed to the ligand-mediated blockade of pre-miR-210 processing due to the occupancy of enzymatic sites by **TGP-210-Ppa**.³³ Cells treated by Ppa alone did not show obvious changes in the intracellular pre-miR-210 level even after red light irradiation.

For cells treated by **TGP-210-Ppa** with a concentration as low as 10 nM, red light irradiation induced a significant decrease of intracellular pre-miR-210 level (Fig. 3c). Higher concentrations of **TGP-210-Ppa** elevated the pre-miR-210 level before light irradiation. However, after the cells pre-treated with **TGP-210-Ppa** were exposed to red light irradiation to trigger the formation of $^1\text{O}_2$, the ligand-directed modification or degradation of pre-miR-210 became predominant. These results suggest that the enhanced suppression of miR-210 by **TGP-210-Ppa** depends on both its chimeric effects. Also, **TGP-210-Ppa** further reduced the intracellular level of miR-210 in a dose-dependent manner under light irradiation, with similar effects observed for pre-miR-210 (Fig. S10, ESI†). Consequently, **TGP-210-Ppa** led to a more potent inhibition of miR-210 biogenesis after photo-irradiation. Moreover, non-target miRNAs, including miR-21, miR-24 and miR-192, were not modulated by **TGP-210-Ppa** even after light irradiation (Fig. 3d), confirming a ligand-directed selective inhibition of miR-210.

Finally, we investigated whether the ligand-directed photo-regulation of pre-miR-210 was able to further regulate relevant genes down-stream of miR-210. It has been well established that the overexpression of miR-210 promotes cancer cell growth by inhibiting the glycerol-3-phosphate dehydrogenase 1-like (GPD1L)-hypoxia inducible factor 1-alpha (HIF-1 α) pathway (Fig. 4a).³⁴ Therefore we examined the potential of **TGP-210-Ppa** for photo-regulating the expression level of GPD1L and HIF-1 α . The results shown in Fig. 4b and c confirmed our assumption that **TGP-210-Ppa** in MDA-MB-231 cells upon

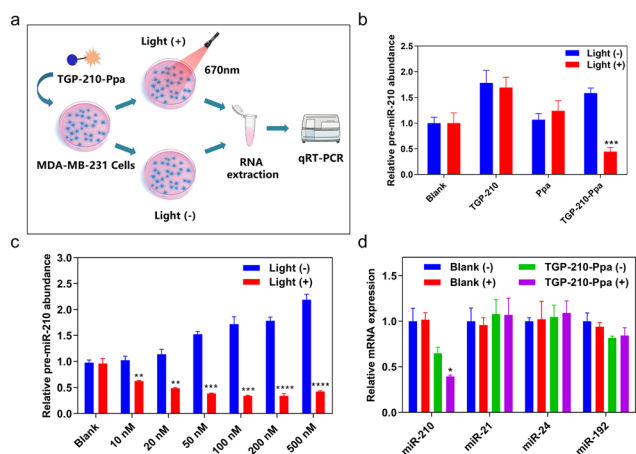


Fig. 3 (a) Schematic illustration of the protocol to evaluate the efficacy of the ligand-directed red-light regulation of intracellular pre-miR-210. (b) qRT-PCR analysis of pre-miR-210 in MDA-MB-231 cells pre-treated with **TGP-210** (500 nM), Ppa (500 nM) or **TGP-210-Ppa** (500 nM) before and after red light irradiation. Cells were incubated with different compounds for 4 hours, the red light applied was 670 nm light with intensity set at 70 mW cm^{-2} and the irradiation time was 5 minutes. (c) Relative expression levels of pre-miR-210 in the different concentrations of **TGP-210-Ppa** treated groups with or without light. (d) Treatment of MDA-MB-231 cells with **TGP-210-Ppa** (500 nM) for 4 h and 670 nm irradiation for 5 min and 24 h later, qRT-PCR analysis of miR-210, miR-21, miR-24 and miR-192. Data are shown as mean \pm SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.



Fig. 4 (a) Schematic illustration of the miR-210-GPD1L-HIF1 α regulatory pathway that might be photo-regulated in MDA-MB-231 cells treated by **TGP-210-Ppa**. (b) Quantitative analysis for GPD1L and (c) HIF-1 α is measured by qRT-PCR with or without light. (d) Relative viabilities of MDA-MB-231 cells incubated with **TGP-210**, Ppa or **TGP-210-Ppa** for 4 h and 670 nm irradiation for 5 min at 24 h post incubation. Data are shown as mean \pm SEM ($n = 6$). *** $P < 0.001$.

photo-irradiation caused elevated expression of GPD1L and suppression of the HIF1 α gene. MTT assay showed that **TGP-210-Ppa** with light irradiation elicited 52% cell apoptosis (Fig. 4d). Therefore, the ligand-directed photo-degradation of pre-miR-210 by **TGP-210-Ppa** could partially reverse the miR-210-involved signaling pathway to induce cancer apoptosis.

In summary, we reported a novel bifunctional chimera for photo-oxidation and degradation of target pre-miRNA that is related to cancer. **TGP-210-Ppa** that integrated the small-molecule ligand to the oncogenic pre-miR-210 with the photosensitizer Ppa was prepared, which demonstrated high efficacy for ligand-directed red-light degradation of intracellular pre-miR-210. **TGP-210-Ppa** could efficiently produce $^1\text{O}_2$ upon red-light irradiation, which induced the oxidization of rG into 8-oxo-rG in pre-miR-210 and further degradation of intracellular pre-miR-210. Using **TGP-210-Ppa** and red-light irradiation, we demonstrated the possibility of reversing oncogenic signaling pathways downstream of miR-210 to promote cancer cell apoptosis. As more small-molecule ligands are being reported to bind with specific RNA targets, this proof-of-concept work holds promise to develop various photo-regulatable bifunctional chimeras for spatial and temporal degradation of disease-related precursor microRNAs. Further efforts to address the limit of this system for *in vivo* applications due to the penetration ability of red light into deep tissue are now underway in our group.

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Conflicts of interest

There are no conflicts to declare.

References

- 1 A. Abi, N. Farahani, G. Molavi and S. M. Gheibi Hayat, *Cancer Gene Ther.*, 2020, **27**, 280–293.
- 2 M. Garofalo, G. D. Leva and C. M. Croce, *Curr. Pharm. Des.*, 2014, **20**, 5328–5335.
- 3 M. Fan, M. Shan, X. Lan, X. Fang, D. Song, H. Luo and D. Wu, *Front. Pharmacol.*, 2022, **13**, 1033017.
- 4 S. T. Crooke, J. L. Witztum, C. F. Bennett and B. F. Baker, *Cell Metab.*, 2018, **27**, 714–739.
- 5 T. Felicetti and G. Manfroni, *Future Med. Chem.*, 2021, **13**, 1245–1248.
- 6 A. Murata, T. Fukuzumi, S. Umemoto and K. Nakatani, *Bioorg. Med. Chem. Lett.*, 2013, **23**, 252–255.
- 7 D. D. Vo, C. Staedel, L. Zehnacker, R. Benhida, F. Darfeuille and M. Duca, *ACS Chem. Biol.*, 2014, **9**, 711–721.
- 8 D. D. Vo, T. P. Tran, C. Staedel, R. Benhida, F. Darfeuille, A. Di Giorgio and M. Duca, *Chemistry*, 2016, **22**, 5350–5362.
- 9 N. Ankenbruck, R. Kumbhare, Y. Naro, M. Thomas, L. Gardner, C. Emanuelson and A. Deiters, *Bioorg. Med. Chem.*, 2019, **27**, 3735–3743.
- 10 J. L. Childs-Disney, X. Yang, Q. M. R. Gibaut, Y. Tong, R. T. Batey and M. D. Disney, *Nat. Rev. Drug Discovery*, 2022, **21**, 736–762.
- 11 P. Zhang, X. Liu, D. Abegg, T. Tanaka, Y. Tong, R. I. Benhamou, J. Baisden, G. Crynen, S. M. Meyer, M. D. Cameron, A. K. Chatterjee, A. Adibekian, J. L. Childs-Disney and M. D. Disney, *J. Am. Chem. Soc.*, 2021, **143**, 13044–13055.
- 12 S. M. Meyer, T. Tanaka, P. R. A. Zanon, J. T. Baisden, D. Abegg, X. Yang, Y. Akahori, Z. Alshakarchi, M. D. Cameron, A. Adibekian and M. D. Disney, *J. Am. Chem. Soc.*, 2022, **144**, 21096–21102.
- 13 Y. Tong, Q. M. R. Gibaut, W. Rouse, J. L. Childs-Disney, B. M. Suresh, D. Abegg, S. Choudhary, Y. Akahori, A. Adibekian, W. N. Moss and M. D. Disney, *J. Am. Chem. Soc.*, 2022, **144**, 11620–11625.
- 14 Y. Li and M. D. Disney, *ACS Chem. Biol.*, 2018, **13**, 3065–3071.
- 15 M. G. Costales, B. Suresh, K. Vishnu and M. D. Disney, *Cell Chem. Biol.*, 2019, **26**, 1180–1186.e1185.
- 16 X. Liu, H. S. Haniff, J. L. Childs-Disney, A. Shuster, H. Aikawa, A. Adibekian and M. D. Disney, *J. Am. Chem. Soc.*, 2020, **142**, 6970–6982.
- 17 P. Di Mascio, G. R. Martinez, S. Miyamoto, G. E. Ronsein, M. H. G. Medeiros and J. Cadet, *Chem. Rev.*, 2019, **119**, 2043–2086.
- 18 H. Sies, C. Berndt and D. P. Jones, *Annu. Rev. Biochem.*, 2017, **86**, 715–748.
- 19 W. K. Martins, N. F. Santos, C. S. Rocha, I. O. L. Bacellar, T. M. Tsubone, A. C. Viotto, A. Y. Matsukuma, A. B. P. Abrantes, P. Siani, L. G. Dias and M. S. Baptista, *Autophagy*, 2019, **15**, 259–279.
- 20 K. Jakubczyk, K. Dec, J. Kaldunska, D. Kawczuga, J. Kochman and K. Janda, *Pol. Merkurys Lek.*, 2020, **48**, 124–127.
- 21 J. Shi, D. Wang, Y. Ma, J. Liu, Y. Li, R. Reza, Z. Zhang, J. Liu and K. Zhang, *Small*, 2021, **17**, e2104722.
- 22 Y. Jiao, Y. Gao, J. Wang, H. An, Y. X. Li and X. Zhang, *Int. J. Pharm.*, 2022, **621**, 121805.
- 23 J. Wang, H. He, X. Xu, X. Wang, Y. Chen and L. Yin, *Biomaterials*, 2018, **171**, 72–82.
- 24 S. Nath and K. Moore, *Bio-Protoc.*, 2019, **9**, e3314.
- 25 K. Takemoto, *Nihon Yakurigaku Zasshi*, 2022, **157**, 238–243.
- 26 K. Miura, Y. Wen, M. Tsushima and H. Nakamura, *ACS Omega*, 2022, **7**, 34685–34692.
- 27 J. Ge, Q. Jia, W. Liu, M. Lan, B. Zhou, L. Guo, H. Zhou, H. Zhang, Y. Wang, Y. Gu, X. Meng and P. Wang, *Adv. Healthcare Mater.*, 2016, **5**, 665–675.
- 28 C. Fimognari, *Oxid. Med. Cell. Longevity*, 2015, **2015**, 358713.
- 29 A. M. Fleming, O. Alshykhly, J. Zhu, J. G. Muller and C. J. Burrows, *Chem. Res. Toxicol.*, 2015, **28**, 1292–1300.
- 30 P. I. Moreira, A. Nunomura, M. Nakamura, A. Takeda, J. C. Shenk, G. Aliev, M. A. Smith and G. Perry, *Free Radical Biol. Med.*, 2008, **44**, 1493–1505.
- 31 W. Martinet, G. R. de Meyer, A. G. Herman and M. M. Kockx, *Eur. J. Clin. Invest.*, 2004, **34**, 323–327.
- 32 M. H. Moon, T. A. Hilimire, A. M. Sanders and J. S. Schneekloth, Jr., *Biochemistry*, 2018, **57**, 4638–4643.
- 33 M. G. Costales, C. L. Haga, S. P. Velagapudi, J. L. Childs-Disney, D. G. Phinney and M. D. Disney, *J. Am. Chem. Soc.*, 2017, **139**, 3446–3455.
- 34 S. Grosso, J. Doyen, S. K. Parks, T. Bertero, A. Paye, B. Cardinaud, P. Gounon, S. Lacas-Gervais, A. Noel, J. Pouyssegur, P. Barbry, N. M. Mazure and B. Mari, *Cell Death Dis.*, 2013, **4**, e544.