MDM2-Associated Clusterization-Triggered Emission and Apoptosis Induction Effectuated by a Theranostic Spiropolymer

Pai Liu,^[a,b,*] Weiqiang Fu,^[a,*] Peter Verwilt,^[b] Miae Won,^[b] Jinwoo Shin,^[b] Zhengxu Cai,^{*,[a]} Bin Tong,^[a] Jianbing Shi,^[a] Yuping Dong,^{*,[a]} and Jong Seung Kim^{*,[b]}

Abstract: In this study, heteroatom-containing spiropolymers were constructed in a facile manner by a catalyst-free multicomponent spiropolymerization route. **P1a2b** as the most potent of these spiropolymers, demonstrates cluster-triggered emission resulting from strong interactions with the MDM2 protein. By preventing the anti-apoptotic p53/MDM2 interaction, **P1a2b** triggers apoptosis in cancerous cells, while demonstrating a good biocompatibility and non-toxicity in non-cancerous cells. The combined results from solution and cell-based cluster-triggered emission studies, docking, protein expression experiments and cytotoxicity data strongly support the MDM2-binding hypothesis and indicate a potential application as a fluorescent cancer marker as well as therapeutic for this spiropolymer.

Due to their attractive three-dimensional shape and physicochemical properties, spiro compounds have received a lot of attention in drug design.^[1] Their shape perfectly lends them to occupy distinct pockets in protein receptors, without the entropic penalties associated with more flexible organic ligands, as well as allowing a better occupancy of deep hydrophobic pockets as a result of their non-coplanarity.^[2] Spiro-atom containing polymers, also known as spiropolymers, are characterized by good thermal and chemical stabilities as well as biocompatibility and have in recent years found a multitude of applications as functional materials for drug carriers as well as potent protein inhibitors.^[3] However, the complexity of monomers limits the current development of spiropolymers. Thus, it is imperative to develop new, simple, and efficient polymerization reactions for spiropolymers.

In recent years, our group has been working on the development of novel synthetic routes to spiropolymers, for example based on the catalyst-free one-pot multicomponent spiropolymerization (MCS) route using diisocyanides, acetylenedicarboxylate esters, and CO₂.^[4] In this work we expanded the scope of this synthetic methodology to include a MCS route based on diisocyanides, acetylenedicarboxylate esters and benzoyl isothiocyanate and studied their application as cancer theranostics.

Aggregation-induced emission (AIE), a process in which the inhibition of intramolecular mobility upon dye aggregation leads to a large rise in fluorescence emission efficiencies, has become the topic of intense investigations over the last decade.^[5] Interestingly, a related phenomenon was more recently observed for small non-conjugated molecules and polymers. Despite the absence of a traditional fluorophore, upon aggregation these essentially non-luminescent soluble compounds exhibit bright fluorescence at elevated concentrations and usually show a fluorescence profile where the emission wavelength is dependent on the excitation wavelength. This phenomenon, named clusterization-triggered emission (CTE), is believed to occur via through-space conjugation (TSC) of isolated π -electron clouds.^[5-6] As demonstrated in this work, the use of spiropolymer CTE holds great promise in the design and development of new generations of diagnostics and theranostics, where the absence of large flat and lipophilic surfaces, as generally is required for traditional fluorophores, can be avoided. Thus, a better biocompatibility could be anticipated.

p53 is a cancer suppressor protein, often referred to as the cellular master switch, due to its crucial role in controlling DNA repair and the induction of apoptosis when DNA damage becomes irreparable.^[2d, 7] Cellular p53 levels are normally maintained at low steady-state levels via interactions with MDM2, preventing nuclear localization of p53 and enabling proteolytic degradation as a result of ubiquitin ligation. As such, several human cancers are characterized by overexpression of MDM2 resulting in cancer cell survival.^[8] The MDM2-p53 interaction is maintained by several relatively deep pockets on the MDM2 surface, providing an appealing target for inhibitors of this interaction, liberating p53 and inducing apoptosis. Several scaffolds have been employed in the design of MDM2 inhibitors, most notably nutlins, a series of *cis*-imidazolines,^[9] as well as several spiro compounds.^[2e, 2f, 7]

In the current work, we investigated a series of 4,7-bis[alkyl(aryl)imino]-2-phenyl-3-oxa-6-thia-1-azaspiro[4.4]nona-1,8-dienes spiropolymers and assessed their potential for MDM2 inhibition as well as clusterization-triggered emission (CTE). Among the tested polymers, **P1a2b** demonstrates a remarkable cancer-specificity, with blue CTE observable in cancerous cell lines exclusively. Furthermore, this selectivity profile was confirmed to result from an *in vitro* MDM2 binding, causing the reactivation of p53, and associated increased ROS, ultimately leading to apoptosis in cancerous cell lines, without appreciable cytotoxicity in non-cancerous cells. Thus, **P1a2b** represents a great potential as a novel scaffold for cancer theranostics.

Several diisocyanides (1a–c, see SI) and acetylenedicarboxylate esters (2a–b, see SI) were reacted in the presence of benzoyl isothiocyanate using a mild one-pot catalyst-free polymerization method, as depicted in Scheme 1. The optimized synthetic procedures and characterization of these new polymers are reported in the SI (Table S1–S5 and Figure S1–S19). All spiropolymers exhibited a good solubility in common organic solvents, such as DCM, CHCl₃, THF, DMF and DMSO. Among

[a] P. Liu,^[*] Dr. W. Fu,^[*] Dr. Z. Cai, Prof. B. Tong, Dr. J. Shi, Prof. Y. Dong
Beijing Key Laboratory of Construction Tailorable Advanced Functional Materials and Green Applications
School of Materials Science and Engineering
Beijing Institute of Technology
Beijing 100081 (China)
E-mail: caizx@bit.edu.cn; chdongyp@bit.edu.cn

[b] P. Liu,^[*] Dr. P. Verwilt, Dr. M. Won, J. Shin, Prof. J. S. Kim
Department of Chemistry
Korea University
Seoul 02841 (Korea)
E-mail: jongskim@korea.ac.kr

[*] These authors contributed equally to this work

Supporting information and the ORCID identification numbers for the authors of this article can be found under:
<https://doi.org/10.1002/anie.xxxxxxxx>.

Scheme 1. The proposed catalyst-free polymerization mechanism and plausible MDM2-sensing mode of action.

these, **P1a2b** exhibited the highest structural similarity with known inhibitors of the MDM2-p53 interaction and thus this polymer was selected for subsequent experiments.

P1a2b exhibited an absorption peak at ~280 nm in THF (Figure S20), corresponding to the $n \rightarrow \pi^*$ transition of the carbonyl groups. Despite the absence of a traditional fluorophore, **P1a2b** solutions exhibited a weak blue emission in diluted solutions (10^{-6} M), while a concentration dependent fluorescence increase was seen, with bright fluorescence at 10^{-3} M and an associated concentration-dependent bathochromic shift (Figure 1A). These observations clearly suggest that **P1a2b** undergoes clustering-triggered emission (CTE)^[6a] with bathochromic shifts at higher concentrations due to increased chain collapse and aggregation at higher concentrations. Using excitations in the 320 to 440 nm range, **P1a2b** was further demonstrated to exhibit an excitation wavelength-dependent emission wavelength (Figure 1B), with gradual bathochromic shifts at longer excitation wavelengths, suggesting the presence of several distinct aggregated structures.^[10]

To examine the effect of aggregation on the CTE intensity, the fluorescence intensity, maximum emission wavelength and transmission spectra of **P1a2b** was determined in binary DMSO/PBS mixtures (Figure 1C, D and S21). As can be clearly seen, with PBS fractions exceeding 50%, a dramatic increase in fluorescence intensity and a bathochromic shift in fluorescence wavelength was observed. Transmission spectra also verified the formation of aggregation. In these kinds of clusters, TSE, namely overlap of π and rich electronic atoms (n) extends the conjugation and meanwhile rigidify the structure thus offering brighter emission. Compared to **P1a2b**, fluorescence property of monomer were tested and it didn't perform obvious CTE property (Figure S22-S25), which was due to the loose structure.

When added to HepG2 cells, a clear dependency of the fluorescence on the average molecular weight of the **P1a2b** polymer could be discerned. As can be seen in Figure S26, the brighter blue fluorescence for the high molecular weight polymers,

at similar constituting monomer concentrations, results from a greater propensity for the high molecular weight polymers to form large aggregates, with DLS indicating particle sizes of 478 nm to 1865 nm for polymers with an M_w of 1500 to 6500 Da, respectively (Figure S27).

Figure 1. (A) Fluorescence spectra of **P1a2b** with different concentrations ($\lambda_{\text{ex}} = 410$ nm). (B) Fluorescence spectra of **P1a2b** (10^{-4} M) with different excitation wavelengths. (C) Fluorescence spectra of **P1a2b** in DMSO/PBS mixtures ($\lambda_{\text{ex}} = 410$ nm). (D) Aggregation induced emission properties of **P1a2b** (10^{-4} M) in DMSO/PBS with different PBS fractions ($\lambda_{\text{ex}} = 410$ nm).

Importantly, the retention rate, determined as a fraction of the absorbance of the initial dose, is similar across several cell lines and no statistically significant difference was observed between cancerous and non-cancerous cell lines (Figure 2A). Nonetheless, the fluorescence in cancerous HeLa cells was significantly higher than that in co-cultured non-cancerous NIH3T3 cells (Figure 2L-N). Similarly, all cancerous cells incubated with **P1a2b** exhibited relatively bright fluorescence (Figure 2B-H and Figure S28), while non-cancerous cell lines exhibited only very faint fluorescence (Figure 2I-K and Figure S29). Intrigued by the potential of **P1a2b** for the discrimination of cancerous and non-cancerous cells, we sought to rationalize this behavior. As mentioned above, **P1a2b** was selected on the basis of its structural similarity to known MDM2-p53 interaction inhibitors and this prompted us to investigate if a direct interaction between **P1a2b** and MDM2 would be possible.

Figure 2. (A) The retention rate of different cell lines with **P1a2b** (10^{-5} M). (B)-(K) The CLSM images of different cell lines. (L) The DAPI channel, (M) Bright channel and (N) Merge of CLSM imaging of HeLa and NIH 3T3 cells co-cultured with **P1a2b** (10^{-5} M) ($\lambda_{\text{ex}} = 405$ nm).

Figure 3. (A) Relative fluorescence intensity of **P1a2b** treated different concentrations of MDM2 with and without after being pre-incubated with nutlin-3 for 2 h ($\lambda_{\text{ex}} = 380 \text{ nm}$, $\lambda_{\text{em}} = 470 \text{ nm}$); (B) The CLSM imaging of HepG2 cells with **P1a2b** (10^{-5} M) but no nutlin-3 pre-incubation. (C) HepG2 cells with 5 μM nutlin-3 pre-incubation and **P1a2b**. (D) HepG2 cells with 10 μM nutlin-3 pre-incubation and **P1a2b** ($\lambda_{\text{ex}} = 405 \text{ nm}$, pre-incubation time was 24h). (E) The average fluorescence intensity of Fig.3 (B), (C) and (D). (F) The expression levels of MDM2, p53, p21 and β -actin proteins tested by western blotting. (G) Docking study between **P1a2b** (trimer) and MDM2 protein (1YCR). (H), (I), (J) The detail images of binding site in three hydrophobic pockets on the MDM2 protein surface. (K) Fluorescence imaging of **P1a2b** in HepG2 cells. (L) Alexa Fluor® 546-linked MDM2 antibody imaging in HepG2 cells. (M) Merged image of panels K and L.

As can be seen in Figure 3A, the addition of increasing concentrations of MDM2 to **P1a2b** in solution results in an increased CTE fluorescence. However, preincubation of MDM2 with nutlin-3, a well-known MDM2 inhibitor,^[11] clearly precludes the MDM2-induced fluorescence enhancement, strongly suggesting a direct competition for the binding pockets on the MDM2 protein.

Additionally, the addition of **P1a2b** to nutlin3-loaded HepG2 cells also reduced the cellular fluorescence in a nutlin3 dose-dependent fashion (Figure 3B-E), supporting the notion of direct competition between **P1a2b** and nutlin-3. A co-localization experiment of **P1a2b** with Alexa Fluor® 546-labeled MDM2 antibody also revealed a near-perfect overlap in both HepG2 cells (Figure 3K-M) and A549 cells (Figure S30), indicative of a direct interaction with MDM2. Western blotting of HepG2 cells incubated with nutlin-3 clearly demonstrated a dose-dependent increase in p21, p53 and MDM2 protein concentrations, and these results were mirrored with **P1a2b**. Taken together, these results clearly point towards MDM2 as the cellular target for **P1a2b** (Figure 3F).

MDM2 exhibits strong interactions with various organic heterocyclic inhibitors, mainly via relatively deep hydrophobic pockets adjacent Phe19, Trp23, and Leu26. Docking experiments of a trimeric model system for **P1a2b** were carried out and

revealed that the side-chains, as well as the spiro-core itself can bind to the protein structure, with a predicted binding affinity (Autodock scoring function) of -6.29 kcal/mol , indicative of a stable interaction between MDM2 and **P1a2b** (Figure 3G-J).

As indicated by western-blotting (Figure 3F), the strong interaction of **P1a2b** with MDM2 releases and activates the MDM2-bound p53 and p21. The expression of p53 treated by **P1a2b** is higher than that treated by monomer or oligomer, which means better therapy (Figure S31). P53 activation in cancer cells is associated with significant increases in intracellular reactive oxygen species (ROS) and we confirmed that the addition of **P1a2b** to HepG2 cells gave a significant increase in intracellular ROS,^[12] but the commercial dye DAPI did not. (Figure 4A-F and Figure S32).

Finally, we investigated the effect of **P1a2b** incubation in several cancerous and non-cancerous cell lines, and this revealed a clear difference between **P1a2b** toxicity in cancerous cells and non-cancerous cells, most likely as a result of the increased p53-mediated oxidative stress. Thus, the differential level of p53 in cancerous and non-cancerous cells is also reflected in the varied susceptibility to apoptosis upon **P1a2b** treatment.

Figure 4. (A),(B),(C) The CLSM imaging of HepG2 cells with DCFH/DA; (D),(E),(F) with **P1a2b** (10^{-5} M) pre-incubation for 30 min then DCFH/DA was added ($\lambda_{\text{ex}} = 405$ nm (blue), $\lambda_{\text{ex}} = 485$ (green)). (G) The summary data of cell apoptosis with **P1a2b** (2.5×10^{-5} M) for 24, 48 and 72 h in different cell lines.

In summary, spiropolymers were prepared by a catalyst-free MCS route. The target spiropolymers contained 4,7-bis[alkyl(aryl)imino]-2-phenyl-3-oxa-6-thia-1-azaspiro[4.4]nona-1,8-dienes as the spirostructural unit in the main chain. The features of this MCS included no catalyst, simple operation, high atom economy, and convenient monomers, satisfying the requirements for “green” chemistry. Notably, polymer **P1a2b** showed CTE properties and exhibited strong fluorescence in cancer cells, which can be used to discriminate cancerous and non-cancerous cells. Furthermore, the addition of the polymer induced significant degrees of apoptosis in cancerous cells only, making this scaffold and interesting new cancer theranostic. The mode of action of **P1a2b** was determined to be a result of MDM2 binding, resulting in the liberation and activation of p53, as confirmed by solution experiments, cell imaging, and Western Blotting. Docking experiments were carried out to illuminate potential interactions at the molecular level. Based on the docking results further derivatives are being prepared in our laboratory to increase the affinity of the polymer for MDM2 by modifying the structures to include larger lipophilic side chains and introduce potential hydrogen-bonding interactions.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Grants 21490574, 21474009 and 51673024), and a CRI project (No. 2018R1A3B1052702, JSK) of the National Research Foundation of Korea (NRF) and the Basic Science Research Program (2017R1D1A1B03030062, M.W.) funded by the Ministry of Education.

Keywords: Multicomponent Spiropolymerization • MDM2 inhibitor • Cell apoptosis • Clusterization-Triggered Emission • Theranostics

- [1] a) R. Ramon, *Chem. Soc. Rev.* **2012**, *41*, 1060-1074; b) A. Ding, M. Meazza, H. Guo, J. W. Yang, R. Rios, *Chem. Soc. Rev.* **2018**, *47*, 5946-5996.
- [2] a) Q. Wei, L. Mei, Y. Yang, H. Ma, H. Chen, H. Zhang, J. Zhou, *Bioorg. Med. Chem.* **2018**, *26*, 3866-3874; b) R. C. Nunes, C. J. A. Ribeiro, A. Monteiro, C. M. P. Rodrigues, J. D. Amaral, M. M. M. Santos, *Eur. J. Med. Chem.* **2017**, *139*, 168-179; c) R. G. Gouveia, A. G. Ribeiro, M. Segundo, J. F. de Oliveira, M. de Lima, T. R. C. de Lima Souza, S. M. V. de Almeida, R. O. de Moura, *Bioorgan. Med. Chem.* **2018**, *26*, 5911-5921; d) H. Ibrahim, W. Eldehna, A. L. Fallacara, E. R. Ahmed, H. Ghabbour, M. Elaasser, M. Botta, S. Abou-Seri, H. Abdel-Aziz, *Fut. Med. Chem.* **2018**, *10*, 2771-2789; e) K. Ding, Y. Lu, Z. Nikolovska-Coleska, G. Wang, S. Qiu, S. Shangary, W. Gao, D. Qin, J. Stuckey, K. Krajewski, P. P. Roller, S. Wang, *J. Med. Chem.* **2006**, *49*, 3432-3435; f) U. F. Mansoor, A. R. Angeles, C. Dai, L. Yang, D. Vitharana, A. D. Basso, K. Gray, H. Tang, M. Liu, L. Liang, *Bioorg. Med. Chem.* **2015**, *23*, 2424-2434.
- [3] a) C. Alexander, K. M. Shakesheff, *Adv. Mater.* **2010**, *18*, 3321-3328; b) A. Kumar, G. Gupta, A. K. Bishnoi, R. Saxena, K. S. Saini, R. Konwar, S. Kumar, A. Dwivedi, *Bioorg. Med. Chem.* **2015**, *23*, 839-848; c) J. Khazir, P. P. Singh, D. M. Reddy, I. Hyder, S. Shafi, S. D. Sawant, G. Chashoo, A. Mahajan, M. S. Alam, A. K. Saxena, S. Arvinda, B. D. Gupta, H. M. Kumar, *Eur. J. Med. Chem.* **2013**, *63*, 279-289.
- [4] W. Fu, L. Dong, J. Shi, B. Tong, Z. Cai, J. Zhi, Y. Dong, *Polym. Chem.* **2018**, *9*, 5543-5550.
- [5] a) Z. He, C. Ke, B. Z. Tang, *ACS Omega* **2018**, *3*, 3267-3277; b) H. Wang, E. Zhao, J. W. Y. Lam, B. Z. Tang, *Mater. Today*, **2015**, *18*, 365-377.
- [6] a) H. Zhang, X. Zheng, N. Xie, Z. He, J. Liu, N. L. C. Leung, Y. Niu, X. Huang, K. S. Wong, R. T. K. Kwok, H. H. Y. Sung, I. D. Williams, A. Qin, J. W. Y. Lam, B. Z. Tang, *J. Am. Chem. Soc.* **2017**, *139*, 16264-16272; b) B. Sun, B. Zhao, D. Wang, Y. Wang, Q. Tang, S. Zhu, B. Yang, H. Sun, *Nanoscale*, **2016**, *8*, 9837-9841; c) R. Ye, Y. Liu, H. Zhang, H. Su, Y. Zhang, L. Xu, R. Hu, R. T. K. Kwok, K. S. Wong, J. W. Y. Lam, W. A. Goddard, B. Z. Tang, *Polym. Chem.* **2017**, *8*, 1722-1727; d) Y. Du, H. Yan, W. Huang, F. Chai, S. Niu, *ACS Sustain. Chem. Eng.* **2017**, *5*, 6139-6147.
- [7] R. C. Nunes, C. J. A. Ribeiro, A. Monteiro, C. M. P. Rodrigues, J. D. Amaral, M. M. M. Santos, *Eur. J. Med. Chem.* **2017**, *139*, 168-179.
- [8] a) M. C. Yang, C. Peng, H. Huang, L. Yang, X. H. He, W. Huang, H. L. Cui, G. He, B. Han, *Org. Lett.* **2017**, *19*, 6752-6755; b) S. V. Giofre, S. Cirmi, R. Mancuso, F. Nicolo, G. Lanza, L. Legnani, A. Campisi, M. A. Chiacchio, M. Navarra, B. Gabriele, R. Romeo, *Beilstein J. Org. Chem.* **2016**, *12*, 2793-2807.
- [9] L. T. Vassilev, B. T. Vu, G. Bradford, C. Daisy, P. Frank, F. Zoran, K. Norman, K. Ursula, L. Christine, K. Christian, *Science*, **2004**, *303*, 844-848.
- [10] S. Cong, W. Nan, H. Zhuo, Y. Shao, H. Wang, *J. Mater. Chem. C*, **2017**, *5*, 8082-8090.
- [11] a) M. N. Nguyen, N. Sen, M. Lin, T. L. Joseph, C. Vaz, V. Tanavde, L. Way, T. Hupp, C. S. Verma, M. S. Madhusudhan, *J. Chem. Inf. Model.* **2019**, *59*, 1529-1546; b) A. Vaupel, G. Bold, A. De Pover, T. Stachyra-Valat, J. H. Lisztwan, J. Kallen, K. Masuya, P. Furet, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 2110-2114; c) N. Estrada-Ortiz, C. G. Neochoritis, A. Domling, *ChemMedChem*, **2016**, *11*, 757-772; d) Z. Liu, Z. Miao, J. Li, K. Fang, C. Zhuang, L. Du, C. Sheng, M. Li, *Chem. Bio. Drug. Des.* **2015**, *85*, 411-417.
- [12] B. Liu, Y. Chen, D. K. St Clair, *Free Radic. Biol. Med.*, **2008**, *44*, 1529-1535.

RESEARCH ARTICLE

Entry for the Table of Contents (Please choose one layout)

RESEARCH ARTICLE

Heteroatom-containing spiropolymer **P1a2b** demonstrates cluster-triggered emission resulting from strong interactions with the MDM2 protein. By preventing the anti-apoptotic p53/MDM2 interaction, **P1a2b** triggers apoptosis in cancerous cells, while demonstrating non-toxicity in non-cancerous cells.

P. Liu, W. Fu, P. Verwilst, M. Won, J. Shin, Z. Cai,* B. Tong, J. Shi, Y. Dong,* J. S. Kim*

Page No. – Page No.

MDM2-Associated Clusterization-Triggered Emission and Apoptosis Induction Effectuated by a Theranostic Spiropolymer