**Polypod-like structured guanine-rich oligonucleotide aptamer as a selective and cytotoxic nanostructured DNA to cancer cells**

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**ABSTRACT**

Guanine-rich oligonucleotide (GRO) can be developed as an effective anticancer agent owing to its high selectivity, affinity and antiproliferative activity in cancer cells. In this study, to increase the potency of GRO29A, a 29-mer GRO aptamer against nucleolin, an overexpressed protein in cancer cells, GRO29A was incorporated into three or six pods of polypod-like structured DNA (polypodna), tripodna or hexapodna, respectively. The polypod-like structured GROs, tri-G3, consisting of one tripodna and three GRO29A, or hexa-G1, hexa-G3 or hexa-G6, each of which comprises one hexapodna and one, three or six GRO29A, respectively, were designed. Tri-G3, hexa-G1 and hexa-G3 were prepared in high yield, except for hexa-G6. Polypod-like structured GROs had quadruplex structures under physiological salt conditions, and degraded at a slower rate in buffer containing serum. Cellular interaction experiments using fluorescently labelled DNA samples showed that the uptake of hexa-G3 by nucleolin-positive MCF-7 cells was more than 2-fold higher than GRO29A, and the interaction was increasingly dependent on the number of GRO29A in the structures. Hexa-G3 inhibited the proliferation of MCF-7 cells in more than 40%, but not of CHO cells. These results indicate that polypod-like structured GROs are useful DNA aptamers with high selectivity and cytotoxicity against nucleolin-positive cancer cells.

**Introduction**

Aptamers are single-stranded oligonucleotides that bind to target proteins with high selectivity and affinity [1]. Thus, aptamers have been widely used as a tool with targeting functions in research fields such as cellular analysis, imaging and drug delivery [2,3]. Reportedly, guanine-rich oligonucleotide (GRO) aptamers have high selectivity, affinity and antiproliferative activity in cancer cells. AS1411, which was formerly developed as AGRO100 by Aptamera, is a 26-mer GRO with a phosphodiester backbone, demonstrating affinity and cytotoxicity against nucleolin-positive cancer cells [4]. Under physiological conditions, AS1411 forms a four-stranded or ‘quadruplex’ structure and specifically interacts with nucleolin on the cancer cell surface [5].

AS1411 specifically binds to nucleolin with high selectivity and affinity [6,7]. Nucleolin normally locates in the nucleus of most types of cells, whereas it was found in high levels in the cytoplasm and surface of cancer cells [8-11]. Thus, AS1411 can selectively bind to cancer cells through the extranuclear nucleolin, but not to normal cells. By blocking several functions of nucleolin, such as assisting DNA repair in the nucleus, inhibition of apoptosis via stabilising Bcl-2 mRNA, and limiting oncogenic signalling via Rac1 activation, AS1411 exhibits antiproliferative effects on cancer cells [12-14]. Therefore, GRO aptamers such as AS1411 could be effective anticancer agents or devices for cancer therapy.

Recently, remarkable advances in the technology developing DNA-based nanostructures have permitted the designing of various DNA nanosystems [15]. Some studies reported that DNA nanostructures are useful tools for the delivery of functional nucleic acids, such as antisense oligonucleotides, small interfering RNA and immunostimulatory oligodeoxynucleotides (ODNs), to cells [16]. We have demonstrated that polypod-like structured DNA, or polypodna, significantly increases the potency of immunostimulatory DNA containing unmethylated cytosine-phosphate-guanine dinucleotides (CpG DNA) [17–19]. Furthermore, DNA dendrimer or DNA hydrogel, prepared by connecting polypodna as building blocks, dramatically increases the immunostimulatory activity of CpG DNA [20–22]. These results indicate that the biological or biopharmaceutical properties of nucleic acid drugs are dependent not only on their primary and secondary structures but also on their tertiary or three-dimensional structure. In addition, our previous study has reported that a DNA hydrogel loaded with the anticancer agent doxorubicin effectively inhibits tumour growth in mice [23]. Therefore, DNA-based systems can also be useful for the delivery of anticancer agents.

Despite these positive results, little information is available regarding the incorporation of GRO aptamers into DNA nanostructures to enhance their cytotoxic activity against cancer cells. Therefore, in the present study, polypod-like structured GRO aptamers were newly designed and developed. We selected...
GRO29A [6], an elongated AS1411 with three thymidines at the 5'-terminus, as a GRO aptamer to determine the structural properties and biological activity of polypod-like structured GROs.

Materials and methods

Chemicals

Dulbecco’s modified Eagle medium (DMEM) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Ham’s F-12 was obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Foetal bovine serum (FBS) was procured from Equitech-Bio Inc. (Kerrville, TX). Opti-modified Eagle’s medium (Opti-MEM) was purchased from Life Technologies (Rockville, MD). The 20-bp and 100-bp DNA ladders were purchased from Takara Bio Inc. (Shiga, Japan). All other chemicals were of the highest grade available and used without further purification.

Cell culture

The human breast tumour MCF-7 cell line and Chinese hamster ovary (CHO) cell line were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). MCF-7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 0.15% NaHCO3, 2-mM L-glutamine and 4500 mg/L glucose. CHO cells were cultured in Ham’s F-12 supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2-mM L-glutamine. All cells were cultured at 37 °C in a humidified atmosphere of 5% CO2, and then the cells were seeded on 48- or 96-well culture plates and cultured for 24 h before use.

ODNs

Phosphodiester ODNs were purchased from Integrated DNA Technologies, Inc. (Coralville, IA), and those labelled with Alexa Fluor 488 were purchased from JBioS (Saitama, Japan). The sequences of the ODNs are listed in Table S1. GRO29A, a 29-mer ODN, was selected as the GRO aptamer. To construct GRO assemblies, including polypod-like structured GROs, several ODNs with GRO29A at the 3'-terminal were designed, with one of them, ss-GRO29A, used as a single-stranded GRO. Two, three or six ODNs were used to construct double-stranded GRO, or ds-G2, tripod-like structured GRO, or tri-G3 and hexapod-like structured GRO, or hexa-G6, respectively, (Scheme 1). Additionally, other types of hexapod-like structured GROs, including hexa-G1 and hexa-G3, were also designed using ODNs with and without GRO29A; the number after G indicates the number of ODNs with GRO29A per assembly. Therefore, ss-GRO29A, ds-G2, tri-G3, hexa-G1, hexa-G3, hexa-G5 and hexa-G6 possessed 1, 2, 3, 1, 6 and 6 GRO29A sequences per assembly, respectively. As controls, several assemblies were prepared by replacing the guanines with cytosines in the sequence of GRO29A to obtain polypod-like structured cytosine-rich oligonucleotides (CROs) and other CRO assemblies. To examine the interaction with cells, the Alexa Fluor 488-labelled ODN was used to prepare fluorescently labelled DNA samples, hexa-G1, hexa-G3, hexa-G5 and hexa-C3. Alexa Fluor 488-labelled hexa-G5 was used as a surrogate for Alexa Fluor 488-labelled hexa-G6 as it was difficult to synthesise fluorescently labelled 65-mer ODN.

Preparation of GRO and CRO assemblies

As previously described, GRO and CRO assemblies were prepared by mixing equimolar amounts of corresponding ODNs [17]. Briefly, ODNs were dissolved in a buffer comprising 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetate acid (EDTA) (TE buffer; pH 8) to obtain stock solutions of each ODN at a concentration of 0.1 μM. Then, ODNs were mixed and the concentrations of potassium chloride, sodium chloride and magnesium chloride were adjusted to 150, 150 and 50 mM, respectively. The mixtures were then incubated at 95 °C for 5 min, 65 °C for 2 min and 62 °C for 1 min and were then slowly cooled to 4 °C using a thermal cycler. The products were analysed by 8% or 20% polyacrylamide gel electrophoresis (PAGE) at 200 V for 40 min or 90 min, respectively. The gel was stained with SYBR Gold (Thermo Fisher Scientific, Inc., Waltham, MA) and ethidium bromide (EtBr) (Nippon gene, Tokyo, Japan) and observed using the GelDoc XR System with Quantity One 1-D analysis software (Bio-Rad Laboratories, Tokyo, Japan).

Circular dichroism spectra

Circular dichroism (CD) spectra of DNA samples were recorded using a JASCO-820 type spectropolarimeter (JASCO, Tokyo, Japan) with a quartz cell of 1-cm path length at room temperature. After annealing, DNA samples were diluted with TE buffer containing 150 mM sodium chloride to a final DNA concentration of 4 μM. The spectra were measured at a wavelength of 200–300 nm.

Stability in FBS

DNA samples were incubated in 50% FBS diluted with phosphate-buffered saline (PBS) at a concentration of 100 μg/mL at 37 °C. After 0, 0.5, 1, 2 or 4 h of incubation, a 10 μL aliquot of the sample solution was transferred to plastic tubes and mixed with 20 μL of 0.5 M EDTA solution to stop the degradation and then stored at −20 °C until use. These samples were run on 8% or 20% PAGE, and the gel was stained with SYBR Gold or EtBr to confirm the formation of DNA assemblies. The density of the DNA bands was
quantitatively evaluated using Image Lab Software (Bio-Rad Laboratories).

**Antiproliferative activity against cancer cells**

MCF-7 or CHO cells on 96-well plates were seeded at a density of $1 \times 10^4$ cells/well or $5 \times 10^2$ cells/well, respectively, and incubated for 24 h at $37^\circ$C. The culture medium was replaced with fresh medium containing DNA samples for 72 h at $37^\circ$C. Then, 10 µL of the cell proliferation reagent WST-1 (Roche, Indianapolis, IN) was added to each well, and the cells were incubated for 1 h at $37^\circ$C. The absorbance was measured using a microplate reader at 450 nm to count the living cells.

**Interaction with cells**

MCF-7 cells were seeded on 96-well plates at a density of $5 \times 10^4$ cells/well and incubated for 24 h at $37^\circ$C. The culture medium was replaced with Opti-MEM containing Alexa Fluor 488-labelled DNA and incubated for 2 h at $37^\circ$C. Cells were then washed twice with 200 µL PBS and harvested using a trypsin-EDTA (0.25% trypsin and 0.038% EDTA) solution. Then, the fluorescence intensity of the cells was determined by flow cytometry (FACSCalibur, BD Biosciences, NJ) using the CellQuest software (version 3.1, BD Biosciences). For confocal microscopy experiments, MCF-7 cells or CHO cells were seeded on 13-mm diameter glass coverslips and incubated for 24 h at $37^\circ$C. After the addition of Alexa Fluor 488-labelled DNA to cells in the same manner as described above, the cells were washed three times with PBS, fixed with 4% paraformaldehyde for 15 min, and washed again three times with PBS. Next, 60 nM 4’,6-diamino-2-phenylindole was added to stain the nuclei, and the cells were washed three times with PBS after 10 min of incubation. Then, the coverslips were mounted on glass slides with SlowFade Gold (Life Technologies) and observed using a confocal microscope (FV1000D Olympus, Tokyo, Japan).

**Statistical analysis**

Differences were statistically evaluated by one-way analysis of variance followed by the Tukey-Kramer test for multiple comparisons. $p < .05$ was considered statistically significant and marked with an asterisk (*).

**Results**

**Formation of GRO and CRO assemblies**

The formation of GRO and CRO assemblies was confirmed by PAGE (Figure 1). GRO29A, ss-GRO29A, ss-CRO29A and ds-G2 possessed a major single band, and the migration distance was dependent on the base number per unit (Figure 1(A)). CRO29A was hardly stained probably due to low affinity to SYBR Gold. Figure 1(B) shows the PAGE analysis of GRO and CRO assemblies. Ds-G2, tri-G3, hexa-G1 and -G3 demonstrated a major single band and the migration distance was shorter with increasing molecular mass. Similar results were obtained for hexa-C1, -C3 or -C6 preparations, indicating that polypod-like structured GRO and CRO were successfully formed with high yields, except for hexa-G6. The band of hexa-G6 was paler than that of other assemblies, suggesting the low formation efficiency of hexa-G6.

**CD Spectra of GRO29A, CRO29A, GRO and CRO assemblies**

Figure 2 shows the CD spectra of the GRO29A, CRO29A, GRO and CRO assemblies. GRO29A demonstrated positive and negative peaks around 265 nm and 240 nm, respectively, (Figure 2(A)), which are typical peaks for the ‘parallel’ type tetramers like AS1411. CRO29A and ss-CRO29A reported positive and negative peaks around 280 nm and 240 nm, respectively. The GRO29A peak around 265 nm was shifted to a longer wavelength in ss-GRO29A. Figure 2(B) shows the CD spectra of ds-G2, tri-G3, hexa-G1, -G3 and -G6. All preparations had similar peaks to ss-GRO29A, suggesting that ds-G2 and polypod-like structured GROs contained quadruplex structures. In contrast, the waveforms of hexa-G1, -C3 and -C6 were similar to those of CRO29A and ss-CRO29A (Figure 2(C)).

**Stability of GRO29A, GRO or CRO assemblies in FBS**

Figure 3 presents the stability of GRO29A, GRO or CRO assemblies in 50% FBS. All DNA samples were degraded over time. The degradation rates of GRO29A, ss-GRO29A, ds-G2 and tri-G3 were slower than those of ss-CRO29A (Figure 3(A)). GRO29A was degraded the slowest, followed by ds-G2, ss-GRO29A and tri-G3, in this order. Figure 3(B) shows the degradation rate of hexapod-like GROs and CROs. Hexa-G1 was degraded faster than hexa-G3 and hexa-G6. The degradation rates of hexa-G1, -G3 and -G6 were slower than those of hexapod-like structured CROs.

**Interaction of GRO29A, CRO29A, GRO and CRO assemblies with MCF-7 and CHO cells**

Figure 4(A–D) presents the fluorescent images of MCF-7 cells after the addition of Alexa Fluor 488-labelled DNA samples. The fluorescence signal was distributed in punctate patterns in the cytoplasm and cell surface when Alexa Fluor 488-labelled GRO29A or hexa-G3 was added (Figure 4(A,B)), whereas the signals were hardly detected after the addition of Alexa Fluor 488-labelled CRO29A or hexa-C3 (Figure 4(C,D)). When CHO cells were treated with Alexa Fluor 488-labelled DNA samples, no significant fluorescent signals were detected (Figure 4(E–H)).

Figure 5 shows the mean fluorescence intensity (MFI) of MCF-7 cells after the addition of Alexa Fluor 488-labelled DNA samples.
Alexa Fluor 488-labelled hexa-G5 was used instead of Alexa Fluor 488-labelled hexa-G6 due to the difficulty to synthesise fluorescently labelled longer ODNs. The MFI values of cells after the addition of Alexa Fluor 488-labelled GRO29A or hexa-G3 increased in a concentration-dependent manner, and the MFI values of cells treated with Alexa Fluor 488-labelled hexa-G3 were significantly higher than those with Alexa Fluor 488-labelled GRO29A (Figure 5(A)). In contrast, the MFI values of cells treated with Alexa Fluor 488-labelled CRO29A or hexa-C3 were significantly lower than those with Alexa Fluor 488-labelled GRO29A. Figure 5(B) shows the MFI values of MCF-7 cells after the addition of Alexa Fluor 488-labelled GRO29A, hexa-G1, -G3 or -G5. The MFI value of cells after the addition of Alexa Fluor 488-labelled GRO29A was comparable to that of cells treated with Alexa Fluor 488-labelled hexa-G1, whereas the MFI values of cells with Alexa Fluor 488-labelled hexa-G3, or -G5 were much higher. In addition, the values were dependent on the number of GRO29A per assembly.

**Antiproliferative activity of GRO29A, CRO29A, GRO and CRO assemblies**

Figure 6(A) shows the number of MCF-7 cells after the addition of GRO29A, CRO29A, GRO or CRO assemblies. Ds-G2, tri-G3, hexa-G1, -G3 and -G6 significantly reduced the number of cells. Hexa-G3 exhibited the highest inhibition among the samples, despite all the GRO assemblies containing the same amount of ss-GRO29A. In contrast, CRO29A, hexa-C3 and -C6 hardly reduced the number of MCF-7 cells. The number of CHO cells was scarcely altered by the addition of any DNA sample (Figure 6(B)).

**Discussion**

GRO aptamers with guanine-quadruplex structures can be selectively bound to nucleolin proteins on cancer cell surfaces and can exhibit antiproliferative activity on cancer cells [24]. Therefore, GRO aptamers have been widely investigated as anticancer agents.
or devices to deliver pharmaceutical agents to cancer cells [25–28]. The integration of GRO aptamers into DNA nanostructures can be a useful approach to increase their anticancer activity against nucleolin-positive cancer cells. Reportedly, AS1411 integrated into tetrahedral DNA nanostructures selectively interacts with human cervical cancer cells or human alveolar basal epithelial cells and effectively inhibits cell proliferation [29,30]. However, there is little systematic information on the relationship between the structural and biological properties of GRO aptamers constructed into DNA nanostructures or GRO aptamers assembled with tertiary structures. Therefore, the present study aimed to elucidate the relationship using polypod-like structured DNA assemblies.

Polypodnas are nano-sized DNA structures with high structural stability. Our previous study using dynamic laser scattering elucidated that the sizes of tripodna, which constructed with three 36-, 60- or 90-mer ODNs, were 6.2, 8.9 or 12.1 nm, respectively, and the size of hexapodna, which constructed with six 36-mer ODN, was 8.2 nm [17]. Polypod-like structured GROs and CROs were prepared using 36- and 65-mer ODNs and, therefore, their sizes could be approximately 10 nm. On the other hand, the melting temperatures of tripodna and hexapodna, which was constructed with 36-mer ODN, were 54.6 and 44.8 °C, respectively, in the buffer containing 5 mM sodium chloride [17]. These results suggest that these DNA assemblies, including GRO assemblies, are structurally stable under physical salt conditions. The low formation efficiency of hex-G6 (Figure 1(B)) indicates that formation efficiency is dependent on the number of ss-GRO29A per assembly. This could be explained by the charge and steric hindrance on the hybridisation of ss-GRO29A to hexapodna. Hex-C6, which has six ss-CRO29A per assembly, could be prepared with high efficiency, suggesting that ss-GRO29A has a bulkier structure than ss-CRO29A, probably due to the formation of the G-quadruplex structure. Previously, we have reported that the formation yield of polypodna reduces when the terminal ends of polypodna are connected with more complicated DNAs [20]. In contrast, it has been reported that the formation yield of branched DNA structures increases with the increasing length of the ODNs used [31]. Furthermore, we have reported that the structural stability of polypodna preparations increases depending on the ODN length [17]. Therefore, hex-G6 could be obtained in high yield if longer ODNs are used to increase the stability of the hexapodna structure. However, from a pharmaceutical standpoint, longer ODNs may not be suitable for designing DNA aptamers in terms of cost and purity. Thus, further structural optimisation is necessary for polypod-like structured GROs, which would help overcome the limitations in their formation.

Reportedly, GRO aptamers demonstrate slow blood clearance after intravenous administration, as well as anticancer effects in tumour-bearing mice [32,33]. Additionally, some studies have reported that GROs were more stable than ODNs without quadruplex structures in the serum-containing medium because of the high nuclease resistance of such quadruplex structures [34–36]. We also observed that the GRO29A was more stable in FBS than ss-CRO29A (Figure 3(A)), which is in agreement with the findings reported in previous reports. The present study revealed that polypod-like structured GROs demonstrated greater stability than tripodna or hexapodna with no ss-GRO29A (Figure 3). This could be explained by the fact that all the 3’-ends of the polypodnas were protected from exonucleases by modification with GRO29A.
because DNA is mainly degraded from the 3′-end by exonucleases in biological fluids [37]. Our previous studies have reported that the polypodnas were easily degraded in mouse serum compared with double-stranded DNA [17,38]. Therefore, the GRO modification at the 3′-end could be a useful approach to improve the biological stability of DNA assemblies.

An important finding of the present study is that polypod-like structured GROs more effectively inhibited the proliferation of human breast tumour MCF-7 cells than GRO29A, and such activity was not observed in CHO cells (Figure 6). It has been reported that AS1411 selectively inhibits the proliferation of MCF-7 cells, but not CHO cells, which lacked nucleolin on their surfaces [14,39–41]. Furthermore, AS1411 interacts with the overexpression of nucleolin on the plasma membrane of MCF-7 cells, resulting in enhanced cellular uptake and anticancer activity [12,14,42,43]. The present study observed that hexapod-like structured GROs selectively interacted with MCF-7 cells, but not CHO cells (Figure 4), probably because of the expression of nucleolin on the cell surface. These results indicate that the polypod-like structured GROs can be developed as an effective anticancer agent selective to nucleolin-expressing cancer cells. In addition, the construction of GRO aptamers into polypodna structures could be a useful approach to increase the potential affinity and anticancer activity for effective and selective cancer therapy.

The present study showed the antiproliferative activity of polypod-like structured GROs as aptamer in human breast tumour MCF-7 cells (Figure 6). It was reported that AS1411 exhibited potent antiproliferative activity in some types of cancer cells including prostate, breast and lung cancer cells [4], and that AS1411 was also effective in some patients with acute myeloid leukaemia or metastatic renal cell carcinoma [24]. Therefore, polypod-like structured GROs can be also expected to be a useful anticancer agent or device against these types of nucleolin-positive cancer cells. In the present study, the antiproliferative experiments were performed using a serum-containing culture medium to promote cell proliferation during the experimental period of 72 h (Figure 6). On the other hand, the cellular interaction experiments were performed using Opti-MEM, a serum-free culture medium with limited nutrients (Figures 4 and 5). Opti-MEM is suitable for cellular uptake/interaction experiments and has been used in many publications because it is not necessary to pay attention to the interaction of samples with serum components.

The results of the present study showed a close correlation between the structural and biological properties of polypod-like structured GROs. The CD spectra data showed that the G-quadruplex structure of the GRO aptamer was maintained in the
polypod-like structured GROs (Figure 2). In addition, the PAGE analysis showed that polypod-like structured GROs were successfully formed as designed except for hexa-G6 (Figure 1). The interaction of hexapod-like structured GRO with MCF-7 cells increased as the number of GRO aptamers in a hexapodna unit increased (Figure 5(B)). These results indicate that the number or valency of the GRO aptamer per DNA assembly unit is an important factor for cellular uptake by nucleolin-positive cells. On the other hand, hexa-G6 exhibited a high antiproliferative activity despite low formation efficiency (Figures 1 and 6). Considering the amounts of GRO aptamer taken up by cells, this could be reasonable because incomplete hexa-G6 structures would have up to 5 GRO aptamers in a hexapodna unit. Thus, hexa-G6 could have more potent antiproliferative activity if the formation efficiency can be increased.

Reportedly, a multivalent DNA aptamer against CD30 receptors induced the oligomerization of target receptors, resulting in apoptosis of anaplastic large-cell lymphoma cells [44]. This report indicates that the biological activity of aptamers is affected by the properties of primary and secondary structures, as well as tertiary structures. The present study showed that the number or valency of the GRO aptamer in polypod-like structures was an important factor for cellular uptake by nucleolin-positive cells. Furthermore, it has been reported that incorporating AS1411 aptamers into DNA pyramids increases their uptake by nucleolin-expressing cells with increased GRO valency in DNA structures [29]. These results indicate that the biological activity of GRO aptamers is also affected by the tertiary structural properties of DNA assemblies. Our previous studies demonstrated that polypod-like structures increased the potency of CpG DNA in vivo [21–23]. Thus, it can be also expected that polypod-like structured GROs behave as an effective aptamer in vivo, although further studies are required to conclude their in vivo efficacy. Polypodna structures have been investigated as building blocks of several DNA structures, including DNA dendrimer and DNA hydrogel [20–22]. Our previous report has demonstrated that polypodna-based DNA hydrogels are an effective sustained system for the delivery of the anticancer agent doxorubicin to cancer cells [23]. Therefore, polypod-like structured GRO-based DNA hydrogels could be used as a targeted and sustained system to deliver anticancer agents to nucleolin-positive cancer cells for efficacious and safe cancer therapy.

**Conclusion**

We demonstrated that polypod-like structured GROs, except for hexa-G6, were efficiently constructed under physiological conditions and were stable in serum. Polypod-like structured GROs demonstrated an enhanced potent affinity and anticancer activity than GRO29A against nucleolin-positive cancer cells. These findings provide useful information on the utility of these nanosized DNA assemblies for development as safe and efficient devices or anticancer agents.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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