


 Cite this: *RSC Adv.*, 2024, 14, 13336

# Inhibition of survivin by 2'-O-methyl phosphorothioate-modified steric-blocking antisense oligonucleotides<sup>†</sup>

 Yalin Li,<sup>‡a</sup> Suxiang Chen,<sup>‡\*bc</sup> Kamal Rahimizadeh,<sup>bc</sup> Zhen Zhang<sup>a</sup> and Rakesh N. Veedu<sup>id\*bc</sup>

Chemically modified antisense oligonucleotide (ASO) has been established as a successful therapeutic strategy for treating various human diseases. To date, ten ASO drugs, which are capable of either inducing mRNA degradation via RNase H recruitment (fomivirsen, mipomersen, inotersen, volanesorsen and tofersen) or splice modulation (eteplirsen, nusinersen, golodirsen, viltolarsen and casimersen), have been approved by the regulatory agencies for market entry. Nonetheless, none of these approved drugs are prescribed as cancer therapy. Towards this, we have developed steric-blocking ASOs targeting *BIRC5* – a well-validated oncogene. Initial screening was performed by transfection of HepG2 cells with seven *BIRC5* exon-2 targeting, uniformly 2'-OMe-PS modified ASOs at 400 nM respectively, leading to the identification of two best-performing candidates ASO-2 and ASO-7 in reducing the production of *BIRC5* mRNA. Subsequent dose–response assay was conducted via transfection of HepG2 cells by different concentrations (400, 200, 100, 50, 25 nM) of ASO-2 and ASO-7 respectively, showing that both ASOs consistently and efficiently inhibited *BIRC5* mRNA expression in a dose-dependent manner. Furthermore, western blot analysis confirmed that ASO-7 could significantly repress survivin production on protein level. Based on our preliminary results, we believe that ASO-7 could be a useful *BIRC5* inhibitor for both research purpose and therapeutic development.

 Received 13th March 2024  
 Accepted 15th April 2024

DOI: 10.1039/d4ra01925c

[rsc.li/rsc-advances](http://rsc.li/rsc-advances)

## Introduction

Antisense oligonucleotides (ASOs) are synthetic single-stranded nucleic acid molecules that can selectively modulate the expression of specific genes.<sup>1,2</sup> The designation “antisense” derives from the complementary nature of these molecules to the mRNA and/or pre-mRNA sequence(s) of the target gene, facilitating RNA binding. The two main mechanisms associated with ASO action are RNA degradation mediated by Ribonuclease H enzyme (RNase H) and modulation of RNA function by steric blockage.<sup>3–6</sup> In contrast to the RNase H-dependent ASOs, steric-blocking ASOs do not recruit RNase H enzyme but simply act as a steric hindrance that could interfere with RNA function, through avoiding ribosomal subunit binding or stalling ribosomes to arrest translation, blocking the upstream open

reading frame (uORF), blocking the binding of splicing factors to modulate splicing, or binding to 3' untranslated region (3'UTR) to modulate polyadenylation.<sup>1,7,8</sup> To date, ten ASO drugs have been approved by the U.S. Food and Drug Administration (FDA) and/or the European Medicines Agency (EMA). Among them, there are five RNase H-dependent ASOs (fomivirsen, mipomersen, volanesorsen, inotersen, tofersen) and five steric-blocking ASOs (nusinersen, eteplirsen, golodirsen, viltolarsen, casimersen).<sup>7</sup> Nonetheless, none of them were approved for cancer therapy, demonstrating the need to develop novel ASO therapeutics for tackling cancer.

Survivin, also termed as baculoviral inhibitor of apoptosis repeat-containing 5 (*BIRC5*), belongs to the inhibitor of apoptosis (IAP) protein family, playing a pivotal role in preventing programmed cell death and regulating cell division. Its increased presence in various cancers makes it a noteworthy therapeutic target, as inhibiting survivin could potentially induce cancer cell apoptosis and hinder uncontrolled proliferation.<sup>9,10</sup> The development of *BIRC5* inhibitors involves diverse strategies such as small molecules, peptides, and ASOs. Small molecule-based inhibitors aim to disrupt the function of survivin, peptides interfere with critical protein–protein interactions, while ASOs directly target *BIRC5* mRNA, thus impeding its expression.<sup>11,12</sup> By designing ASOs that are complementary to *BIRC5* mRNA and/or pre-mRNA, a selective inhibition of

<sup>a</sup>School of Food and Biological Engineering, Henan University of Animal Husbandry and Economy, Zhengzhou 450018, China

<sup>b</sup>Centre for Molecular Medicine and Innovative Therapeutics, Health Futures Institute, Murdoch University, Murdoch 6150, Australia. E-mail: Suxiang.Chen@murdoch.edu.au; R.Veedu@murdoch.edu.au

<sup>c</sup>Precision Nucleic Acid Therapeutics, Perron Institute for Neurological and Translational Science, Nedlands, WA 6009, Australia

<sup>†</sup> Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d4ra01925c>
<sup>‡</sup> These authors contribute equally.


survivin production can be achieved. This targeted approach offers potential advantages, minimizing off-target effects commonly associated with other strategies. The pursuit of ASO-based inhibitors against BIRC5 represents an encouraging path in the quest for cancer research and treatment.

Since 1999, different BIRC5-targeting ASOs have been designed and identified for research purposes and therapeutic development.<sup>13–17</sup> All these ASOs are dependent on RNase H-mediated mRNA degradation due to their central or uniform DNA sequences. Among them, 18mer 2'-O-methoxyethyl phosphorothioate (2'-MOE-PS) gapmer named ISIS 23722 (or LY2181308) and 16mer locked nucleic acid (LNA)-PS gapmer named SPC 3042 (or EZN-3042) have entered clinical trials but resulted in failure,<sup>18,19</sup> which may be due to chemical modification-induced toxicity and lack of efficacy.<sup>12</sup> Our lab has been working on developing chemically modified steric-blocking ASOs.<sup>20</sup> To this end, instead of continuing endeavour to develop RNase H-dependent oligonucleotides, we have designed and screened seven fully 2'-O-methyl (2'-OMe)-PS (Fig. 1) modified steric-blocking ASOs targeting BIRC5 (Table 1, Fig. S1†). Among them, ASO-7 was identified as the lead candidate in reducing the expression of *BIRC5* and can be chemically optimized in future studies.

## Experimental

### Antisense oligonucleotides

Uniformly 2'-OMe-PS modified ASOs (Table 1, Fig. S1†) were designed to be complementary to the acceptor site, donor sites, and other regions of *BIRC5* exon-2. ASOs were synthesized by Integrated Biotech Solutions (IBSBIO). Nomenclature of ASOs was used as “prefix: (gene name) + suffix: (exon, type of splice site, ASO coordinates)”. For example, BIRC5 E2A (+5 + 29) represents an ASO sequence targeting *BIRC5* gene, acceptor site of exon-2 and the coordinates are (+5 + 29). The standard control ASO sequence (No. 8, Table 1) targets an intronic mutation of human  $\beta$ -globin gene causing  $\beta$ -thalassemia, and this sequence has been widely used as a negative control in the field of ASO.

### Cell culture and transfection of ASOs

Human hepatoma cell line HepG2 was gifted by Dr Fengqiu Zhang (Henan key laboratory of ion beam bioengineering,

Zhengzhou University) and cultured in Dulbecco's modified Eagle's medium (DMEM) (BosterBio) with 10% fetal bovine serum (FBS) (Vivacell) in a humidified atmosphere (37 °C, 5% CO<sub>2</sub>) in an incubator. Cells were maintained to reach 70–90% confluency, and then seeded into a 12-well plate (100 000 cells per well) for transcript analysis or 6-well plate (300 000 cells per well) for protein analysis prior to transfection. A day after seeding, ASOs were transfected to cells using Lipofectin reagent (Thermo Fisher Scientific) following the manufacturer's instructions. In untreated groups, cells were treated by Lipofectin reagent only. For transcript analysis, cells were lysed 24 hours after transfection for subsequent RNA extraction, reverse transcription, and polymerase chain reaction (PCR). For protein analysis, cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (Solarbio) 48 hours after transfection for subsequent western blotting.

### RNA extraction, reverse transcription, PCR, and sequencing

Total RNA was extracted from cell lysate using RNAiso Plus (Takara) following the manufacturer's instructions. cDNA synthesis was performed using 2  $\mu$ g of total RNA and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Solarbio) following manufacturer's instructions. The *BIRC5* and *GAPDH* (used as loading control) transcripts were amplified by Taq 2 $\times$  Master Mix (CWBio) following the manufacturer's protocol. The primer sets (synthesized by Sangon Biotech) used for PCR were listed in Table S1.† The PCR conditions for both *BIRC5* and *GAPDH* were 94 °C for 2 minutes followed by 35 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds and 72 °C for 30 seconds. PCR products were separated by electrophoresis in 2% agarose gel (Biowest), stained with SuperRed nucleic acid gel stain (Biosharp), destained by water, and then visualized by Amersham Imager 600 gel documentation system (GE Healthcare Bio Sciences AB). The *BIRC5* PCR products were also sent for sequencing (performed by Sangon Biotech) to confirm that they are the correct products.

### Western blotting

Western blotting, as a smart strategy for enabling the detection of protein of interest,<sup>21</sup> has been employed for the lead ASO candidate (ASO-7) to evaluate its capability in inhibiting the expression of *BIRC5* on protein level. To ensure equal loading of protein, bicinchoninic acid (BCA) assay (BCA protein assay kit, Solarbio) was performed to determine the total protein concentration of each sample following the manufacturer's instructions. Proteins in samples were separated by polyacrylamide gel electrophoresis (PAGE) using precast gels (MeilunBio). Proteins were then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) in a Tris-glycine-methanol transfer buffer. The membrane was blocked in 1 $\times$  Tris buffered saline with Tween 20 (TBST) with 5% bovine serum albumin (BSA) (Solarbio) for 1 hour, and then incubated with primary antibodies (anti-BIRC5: Cell Signalling Technology, anti- $\beta$ -actin: Bioss) overnight at 4 °C. After washing the membrane with 1 $\times$  TBST three times for 10 minutes each, the membrane was incubated with secondary antibody [goat anti-

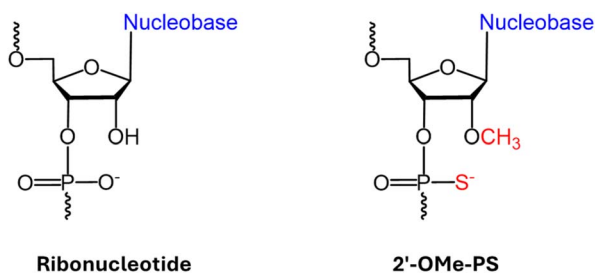


Fig. 1 Structure of ribonucleotide and 2'-OMe-PS analogue. 2'-OMe-PS: 2'-O-methyl phosphorothioate.



Table 1 Uniformly 2'-OMe-PS modified 25mer ASOs targeting exon-2 of BIRC5 and the negative control ASO

No.	ASO name	ASO sequence (5'-3' direction)
1	BIRC5 E2A (-20 + 5)	GCC AUC UGC AAG GGA CAG CAC AGC U
2	BIRC5 E2A (+5 + 29)	GGG CAG UGG AUG AAG CCA GCC UCG G
3	BIRC5 E2A (+14 + 38)	UUC UCA GUG GGG CAG UGG AUG AAG C
4	BIRC5 E2A (+46 + 70)	AGC AGA AGA AAC ACU GGG CCA AGU C
5	BIRC5 E2A (+54 + 78)	CUC CUU GAA GCA GAA GAA ACA CUG G
6	BIRC5 E2A (+69 + 93)	CUC CCA GCC UUC CAG CUC CUU GAA G
7	BIRC5 E2A (+86 + 110)	AUG GGG UCG UCA UCU GGC UCC CAG C
8	Negative control ASO	CCU CUU ACC UCA GUU ACA AUU UAU A

rabbit immunoglobulin G (IgG), conjugated with horseradish peroxidase (HRP)] (CWbio) for 2 hours. After washing the membrane three times (1× TBST, 10 minutes), enhanced electrochemiluminescence (eECL) western blot kit (CWbio) was used to detect the antibodies according to the manufacturer's instructions. Finally, the blots were visualized using Amersham Imager 600 gel documentation system (GE Healthcare Bio Sciences AB).

### Densitometry

To measure the intensity of bands (PCR products) and blots (proteins) from agarose gel and PVDF membrane, respectively, densitometry was performed using ImageJ software.<sup>22</sup> The intensity of BIRC5 bands/blots in different ASO-treated samples was measured and normalized to their corresponding GAPDH bands or  $\beta$ -actin blots, before being compared to the band/blot intensity of the untreated samples. The amount of BIRC5 transcript/protein in ASO-treated samples was expressed as percentages of the amount in untreated samples, thereby indicating the activity of ASOs.

## Results and discussion

During the past 25 years, researchers have designed and screened a large number of ASO sequences across different exons of *BIRC5* mRNA to downregulate its expression. As a result, five ASOs were identified as lead compounds and published by different groups (Fig. S2†).<sup>13–17</sup> These ASOs have been utilized as research tools to elucidate the role of BIRC5 in cancers and also considered as potential anti-cancer therapeutics. While three of the ASOs, *i.e.*, the 20mer ASO against survivin developed by Sun *et al.*, 21mer aODN-Surv developed by Coma *et al.*, and 20mer oligonucleotide 4003 developed by Olie *et al.* stayed on preclinical stage,<sup>15–17,23</sup> the other two ASOs (LY2181308 and EZN-3042) entered clinical stages.<sup>24–30</sup> However, the results of clinical investigations were disappointing, since LY2181308 did not improve the antitumor activity of docetaxel,<sup>27</sup> induced high incidence of side effects such as neutropenia and anemia,<sup>28</sup> and EZN-3042 showed toxicity that cannot be tolerated.<sup>30</sup>

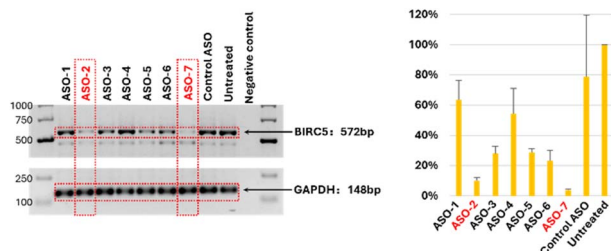
All the previously reported five ASO sequences are dependent on RNase H-mediated mRNA degradation due to their gapmer designs (MOE-DNA-MOE or LNA-DNA-LNA) or uniform DNA chemistry (with or without PS linkages) (Fig. S3†), since as few

as five consecutive deoxynucleotides in an ASO are enough for mRNA-ASO duplex to recruit RNase H.<sup>31–33</sup> Although nobody can really know the reasons that the previous BIRC5-ASOs failed to be developed as drugs for certain, there are two possibilities that could be considered. Firstly, sufficient and suitable chemical modification is required to protect DNA-based ASOs from ubiquitous nucleases *in vivo*, however, the ASO developed by Sun *et al.*, aODN-Surv, and oligo 4003 are merely fully or partly modified by PS, which may not be able to achieve sufficient nuclease stability. Secondly, while chemical modifications increase ASO stability, higher *in vivo* toxicity may occur due to the chemistry itself, or its difficulty to be eliminated from the body,<sup>12</sup> which may explain the safety issues of LY2181308 and EZN-3042.

Considering the lessons learned from the previous efforts in developing BIRC5-targeting ASOs, we designed and screened novel ASO sequences with uniform 2'-OMe-PS modification. Uniform modifications on the sugar moiety of nucleotides (such as 2'-OMe and 2'-MOE) do not allow recruitment of RNase H,<sup>34</sup> but act as a steric blocker that may lead to translational repression of the bound mRNA.<sup>4,8</sup> Therefore, uniformly sugar-modified steric-blocking ASOs are potentially an advantageous alternative to the conventional DNA or gapmer (containing a DNA gap) based RNase H-dependent BIRC5-ASOs, since full sugar modification may maximally optimize the drug-like properties of ASO. In fact, Aung-Htut *et al.* reported a useful approach to develop fully sugar-modified ASOs.<sup>35</sup> In short, cost-effective fully 2'-OMe-PS modified ASOs are designed and initially screened *in vitro*, leading to the identification of the lead ASO sequence, which is subsequently synthesized as phosphorodiamidate morpholino oligomer (PMO), a more preferable chemistry due to its excellent safety profile, for *in vivo* and clinical studies.<sup>35</sup> This systemic strategy for ASO development balances the efficacy, stability and safety of ASO, and it therefore lays the foundation of our BIRC5-ASO development.

Initial screening of seven fully 2'-OMe-PS modified BIRC5-ASOs (Table 1) was performed by transfecting HepG2 cells with 400 nM of each ASO, respectively. Reverse transcription PCR analysis showed that the BIRC5 specific ASOs could reduce the production of *BIRC5* mRNA to different extents (Fig. 2 and S4†), which was confirmed by sequencing PCR products (Fig. S5†). Among the candidates, ASO-2 and ASO-7 more efficiently and consistently knocked down *BIRC5* mRNA production than other oligonucleotides (Fig. 2 and S4†). Specifically,

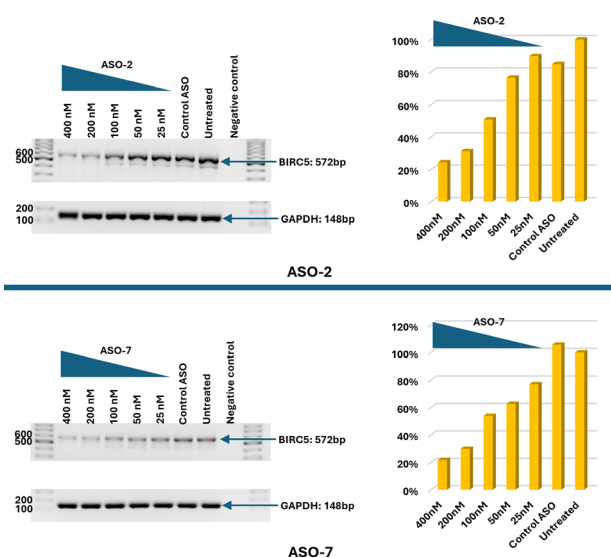




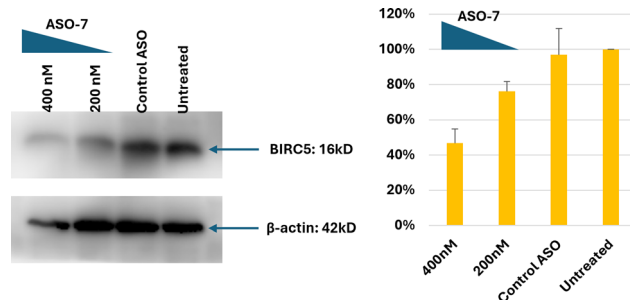
**Fig. 2** Reverse transcription PCR analysis of seven ASOs (400 nM) for *BIRC5* knockdown on mRNA level in HepG2 cells. 572bp: *BIRC5* mRNA; 148bp: *GAPDH* mRNA. The screening was repeated three times. Left panel: representative agarose gel image; right panel: densitometry analysis of *BIRC5* mRNA level. Original gel images and individual densitometry analysis are shown in ESI (Fig. S4†).

ASO-2 reduced on average ~90% of *BIRC5* mRNA production, while ASO-7 even decreased on average ~96% of the mRNA. As a result, ASO-7 was identified as the lead candidate.

To further verify the efficacy and consistency of ASO-2 and ASO-7 in *BIRC5* inhibition, dose-response assay was performed by transfecting HepG2 cells with the two ASOs at various concentrations. Both ASOs efficiently reduced the expression of *BIRC5* on mRNA level in a dose-dependent manner (Fig. 3 and S6†), indicating that the reduction is caused by sequence-dependent antisense effect instead of non-specific effect. Furthermore, at the lowest concentration (25 nM), ASO-7 induced higher percentage of *BIRC5* mRNA knockdown than ASO-2 (ASO-2: ~10%, ASO-7: ~23%), confirming that ASO-7 is the most preferable candidate for *BIRC5* inhibition. Thereafter, western blotting was performed to evaluate ASO-7 in down-regulating *BIRC5* expression on protein level. Notably, ASO-7 reduced on average ~50% of survivin production at 400 nM



**Fig. 3** Reverse transcription PCR analysis of the ASO-2 and ASO-7 for *BIRC5* knockdown on mRNA level in HepG2 cells. 572bp: *BIRC5* mRNA; 148bp: *GAPDH* mRNA. Left panel: agarose gel image; right panel: densitometry analysis of *BIRC5* mRNA level. Original gel images are shown in ESI (Fig. S6†).



**Fig. 4** Western blot analysis of the lead ASO sequence (ASO-7) for *BIRC5* knockdown on protein level in HepG2 cells. 16kD: survivin protein; 42 kD:  $\beta$ -actin protein. Left panel: representative membrane image; right panel: densitometry analysis of survivin protein level. Original membrane images are shown in ESI (Fig. S7†).

concentration (Fig. 4 and S7†). Collectively, these data confirmed that the ASO-7 could efficiently inhibit *BIRC5* expression on both mRNA and protein level, it is thereby identified as the lead sequence for further development in future studies.

## Conclusion and future perspectives

Herein, we report the identification of a fully 2'-OMe-PS modified ASO sequence (ASO-7) that can specifically and efficiently downregulate *BIRC5* expression, through designing and screening seven *BIRC5* specific ASO candidates. Based on the preliminary data, we believe that this steric-blocking ASO-based survivin inhibitor could be a useful tool for cancer-related research as well as potential therapeutic molecule. However, for therapeutic development, ASO-7 should be further optimized by replacing the 2'-OMe-PS chemistry with more preferable nucleotide analogues such as PMO and thiomorpholino oligonucleotide (TMO) (Fig. S8†), and future studies based on combinatorial administration of ASO-7 and front-line chemotherapy agents are needed to evaluate the potential role of ASO-7 as a chemosensitizer (sensitizing cancer cells to chemotherapy agents).

## Author contributions

Conceptualization, S. C.; experiments, Y. L., S. C., Z. Z.; original draft preparation, S. C., Y. L., K. R.; fund-raising, Y. L., R. N. V., Z. Z.; supervision, R. N. V. All authors have read and approved the manuscript.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

We acknowledge the fundings from PhD startups grant (provided by Henan University of Animal Husbandry and Economy), the College Key Research Project in Henan Province



(No. 24B350003), Science and Technology Research Project in Henan Province (No. 222102310212), training plan for young cadre teachers in universities of Henan Province (No. 2020GGJS257), Merit Award Scheme from the Western Australian Government's Department of Health, Perron Institute, and Murdoch University.

## References

- 1 J. H. Chan, S. Lim and W. S. Wong, *Clin. Exp. Pharmacol. Physiol.*, 2006, **33**, 533–540.
- 2 D. Di Fusco, V. Dinallo, I. Marafini, M. M. Figliuzzi, B. Romano and G. Monteleone, *Front. Pharmacol.*, 2019, **10**, 305.
- 3 B. T. Le, P. Raguraman, T. R. Kosbar, S. Fletcher, S. D. Wilton and R. N. Veedu, *Mol. Ther.–Nucleic Acids*, 2019, **14**, 142–157.
- 4 S. Chen, N. Sbhuh and R. N. Veedu, *Nucleic Acid Ther.*, 2021, **31**, 39–57.
- 5 P. Raguraman, A. A. Balachandran, S. Chen, S. D. Diermeier and R. N. Veedu, *Cancers*, 2021, **13**, 5555.
- 6 Y. Li, Y. Tan, R. Zhang, T. Wang, N. Na, T. Zheng, R. N. Veedu and S. Chen, *Kidney Dial.*, 2022, **2**, 16–37.
- 7 S. Chen, S. N. Heendeniya, B. T. Le, K. Rahimizadeh, N. Rabiee, Q. U. A. Zahra and R. N. Veedu, *BioDrugs*, 2024, **38**, 177–203.
- 8 X. H. Liang, J. G. Nichols, C. W. Hsu, T. A. Vickers and S. T. Crooke, *Nucleic Acids Res.*, 2019, **47**, 6900–6916.
- 9 P. K. Jaiswal, A. Goel and R. D. Mittal, *Indian J. Med. Res.*, 2015, **141**, 389–397.
- 10 S. P. Wheatley and D. C. Altieri, *J. Cell Sci.*, 2019, **132**, jcs223826.
- 11 X. Chen, N. Duan, C. Zhang and W. Zhang, *J. Cancer*, 2016, **7**, 314–323.
- 12 F. Li, I. Aljahdali, X. Ling and J. Exp, *Clin. Cancer Res.*, 2019, **38**, 368.
- 13 F. Li, E. J. Ackermann, C. F. Bennett, A. L. Rothermel, J. Plescia, S. Tognin, A. Villa, P. C. Marchisio and D. C. Altieri, *Nat. Cell Biol.*, 1999, **1**, 461–466.
- 14 J. B. Hansen, N. Fisker, M. Westergaard, L. S. Kjaerulff, H. F. Hansen, C. A. Thruue, C. Rosenbohm, M. Wissenbach, H. Orum and T. Koch, *Mol. Cancer Ther.*, 2008, **7**, 2736–2745.
- 15 R. A. Olie, A. P. Simões-Wüst, B. Baumann, S. H. Leech, D. Fabbro, R. A. Stahel and U. Zangemeister-Wittke, *Cancer Res.*, 2000, **60**, 2805–2809.
- 16 S. Coma, V. Noe, C. Lavarino, J. Adán, M. Rivas, M. López-Matas, R. Pagan, F. Mitjans, S. Vilaró, J. Piulats and C. J. Ciudad, *Oligonucleotides*, 2004, **14**, 100–113.
- 17 Y. Sun, R. Lin, J. Dai, D. Jin and S. Q. Wang, *Oligonucleotides*, 2006, **16**, 365–374.
- 18 P. Wiechno, B. G. Somer, B. Mellado, P. L. Chłosta, J. M. Cervera Grau, D. Castellano, C. Reuter, M. Stöckle, J. Kamradt, J. Pikiel, I. Durán, S. Wedel, S. Callies, V. André, K. Hurt, J. Brown, M. Lahn and B. Heinrich, *Eur. Urol.*, 2014, **65**, 516–520.
- 19 E. A. Raetz, D. Morrison, E. Romanos-Sirakis, P. Gaynon, R. Sposto, D. Bhojwani, B. C. Bostrom, P. Brown, E. Eckroth, J. Cassar, J. Malvar, A. Buchbinder and W. L. Carroll, *J. Pediatr. Hematol./Oncol.*, 2014, **36**, 458–463.
- 20 (a) M. Chakravarthy, S. Chen, T. Wang and R. N. Veedu, *Genes*, 2020, **11**, 667; (b) M. Chakravarthy and R. N. Veedu, *Genes*, 2019, **10**, 705; (c) B. T. Le, A. M. Adams, S. Fletcher, S. D. Wilton and R. N. Veedu, *Mol. Ther.–Nucleic Acids*, 2017, **9**, 155–161; (d) B. T. Le, S. Chen, M. Abramov, P. Herdewijn and R. N. Veedu, *Chem. Commun.*, 2016, **52**, 13467–13470; (e) B. T. Le, S. Chen and R. N. Veedu, *ACS Omega*, 2023, **8**, 48650–48661; (f) B. T. Le, S. Paul, K. Jastrzebska, H. Langer, M. H. Caruthers and R. N. Veedu, *Proc. Natl. Acad. Sci. U. S. A.*, 2022, **119**, e2207956119; (g) S. Chen, B. T. Le, M. Chakravarthy, T. R. Kosbar and R. N. Veedu, *Sci. Rep.*, 2019, **9**, 6078; (h) S. Chen, B. T. Le, K. Rahimizadeh, K. Shaikh, N. Mohal and R. N. Veedu, *Molecules*, 2016, **21**, 1582.
- 21 S. X. Chen, *Open Access Libr. J.*, 2022, **9**, 1–6.
- 22 J. Schindelin, C. T. Rueden, M. C. Hiner and K. W. Eliceiri, *Mol. Reprod. Dev.*, 2015, **82**, 518–529.
- 23 D. J. Dai, C. D. Lu, R. Y. Lai, J. M. Guo, H. Meng, W. S. Chen and J. Gu, *World J. Gastroenterol.*, 2005, **11**, 193–199.
- 24 D. C. Talbot, M. Ranson, J. Davies, M. Lahn, S. Callies, V. André, S. Kadam, M. Burgess, C. Slapak, A. L. Olsen, P. J. McHugh, J. S. de Bono, J. Matthews, A. Saleem and P. Price, *Clin. Cancer Res.*, 2010, **16**, 6150–6158.
- 25 M. Tanioka, H. Nokihara, N. Yamamoto, Y. Yamada, K. Yamada, Y. Goto, T. Fujimoto, R. Sekiguchi, K. Uenaka, S. Callies and T. Tamura, *Cancer Chemother. Pharmacol.*, 2011, **68**, 505–511.
- 26 W. G. Herrington, D. C. Talbot, M. M. Lahn, J. T. Brandt, S. Callies, R. Nagle, C. G. Winearls and I. S. Roberts, *Am. J. Kidney Dis.*, 2011, **57**, 300–303.
- 27 H. P. Erba, H. Sayar, M. Juckett, M. Lahn, V. Andre, S. Callies, S. Schmidt, S. Kadam, J. T. Brandt, D. Van Bockstaele and M. Andreeff, *Invest. New Drugs*, 2013, **31**, 1023–1034.
- 28 P. Wiechno, B. G. Somer, B. Mellado, P. L. Chłosta, J. M. Cervera-Grau, D. Castellano, C. Reuter, M. Stöckle, J. Kamradt, J. Pikiel, I. Durán, S. Wedel, S. Callies, V. André, K. Hurt, J. Brown, M. Lahn and B. Heinrich, *Eur. Urol.*, 2014, **65**, 516–520.
- 29 R. Natale, F. Blackhall, D. Kowalski, R. Ramlau, G. Bepler, F. Grossi, C. Lerchenmüller, M. Pinder-Schenck, J. Mezger, S. Danson, S. M. Gadgeel, Y. Summers, S. Callies, V. André, M. Das, M. Lahn and D. Talbot, *J. Thorac. Oncol.*, 2014, **9**, 1704–1708.
- 30 E. A. Raetz, D. Morrison, E. Romanos-Sirakis, P. Gaynon, R. Sposto, D. Bhojwani, B. C. Bostrom, P. Brown, E. Eckroth, J. Cassar, J. Malvar, A. Buchbinder and W. L. Carroll, *J. Pediatr. Hematol./Oncol.*, 2014, **36**, 458–463.
- 31 S. T. Crooke, B. F. Baker, R. M. Crooke and X. H. Liang, *Nat. Rev. Drug Discovery*, 2021, **20**, 427–453.
- 32 H. Wu, W. F. Lima, H. Zhang, A. Fan, H. Sun and S. T. Crooke, *J. Biol. Chem.*, 2004, **279**, 17181–17189.
- 33 X. Shen and D. R. Corey, *Nucleic Acids Res.*, 2018, **46**, 1584–1600.



- 34 K. H. Altmann, D. Fabbro, N. M. Dean, T. Geiger, B. P. Monia, M. Müller and P. Nicklin, *Biochem. Soc. Trans.*, 1996, **24**, 630–637.
- 35 M. T. Aung-Htut, C. S. McIntosh, K. A. Ham, I. L. Pitout, L. L. Flynn, K. Greer, S. Fletcher and S. D. Wilton, *Int. J. Mol. Sci.*, 2019, **20**, 5030.

