Synthesis of enantiopure $^{18}$F-trifluoromethyl cysteine as a structure-mimetic amino acid tracer for glioma imaging

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Abstract

Although $^{11}$C-labelled sulfur-containing amino acids (SAAs) including L-methyl-$[^{11}$C]methionine and S-$[^{11}$C]-methyl-L-cysteine, are attractive tracers for glioma positron emission tomography (PET) imaging, their applications are limited by the short half-life of the radionuclide $^{11}$C ($t_{1/2} = 20.4$ min). However, development of $^{18}$F-labelled SAAs ($^{18}$F, $t_{1/2} = 109.8$ min) without significant structural changes or relying on prosthetic groups remains to be a great challenge due to the absence of adequate space for chemical modification.

Methods: We herein present $^{18}$F-trifluoromethylated D- and L-cysteines which were designed by replacing the methyl group with $^{18}$F-trifluoromethyl group using a structure-based bioisosterism strategy. These two enantiomers were synthesized stereoselectively from serine-derived cyclic sulfamidates via a nucleophilic $^{18}$F-trifluoromethylthiolation reaction followed by a deprotection reaction. Furthermore, we conducted preliminary in vitro and in vivo studies to investigate the feasibility of using $^{18}$F-trifluoromethylated cysteines as PET tracers for glioma imaging.

Results: The two-step radiosynthesis provided the desired products in excellent enantiopurity (ee > 99%) with 14% ± 3% of radiochemical yield. In vitro cell study demonstrated that both enantiomers were taken up efficiently by C6 tumor cells and were mainly transported by systems L and ASC. Among them, the D-enantiomer exhibited relatively good stability and high tumor-specific accumulation in the animal studies.

Conclusion: Our findings indicate that $^{18}$F-trifluoromethylated D-cysteine, a new SAA tracer, may be a potential candidate for glioma imaging. Taken together, our study represents a first step toward developing $^{18}$F-trifluoromethylated cysteines as structure-mimetic tracers for PET tumor imaging.

Key words: Positron emission tomography, $^{18}$F-trifluoromethylthiolation, $^{18}$F-trifluoromethylated cysteine, $^{18}$F-labelled sulfur-containing amino acid, glioma imaging

Introduction

Amino acids (AAs) enter cells via transport mediated by specific plasmatic membrane proteins [1, 2], also known as AA transporters that are highly up-regulated in various malignant tumors in comparison to normal tissues (e.g., systems L, ASC, and A) [3-9]. Targeting the elevated expression of AA transporters is an effective way to design the radiolabelled AAs as tumor-specific imaging tracers. No surprise, positron-labelled AAs have been an important class of radiopharmaceuticals for positron emission tomography (PET) imaging of cancer (e.g., prostate, breast, and brain cancer) [10-17].
Initial applications and studies mainly focused on the naturally occurring AAs, because carbon-11 ($^{11}$C) can be easily incorporated into AAs without any effects on biological properties [18-21]. Sulfur-containing AAs (SAAs) play many physiological and metabolic roles in living systems, such as protein synthesis, methylation of DNA, and biosynthesis of glutathione [22]. L-methyl-[$^{11}$C]methionine ([$^{11}$C]MET, Figure S1A), an essential SAA labelled with $^{11}$C, has been extensively used for brain tumor imaging [23-27]. Compared with clinically used 2-[18F]fluoro-2-deoxy-D-glucose ([18F]FDG), [$^{11}$C]MET accumulates preferentially in tumor cells but poorly in normal brain cells, thus providing a higher sensitivity to detect brain tumors [26, 28, 29]. However, [$^{11}$C]MET is taken up not only by tumors but also by other inflammatory lesions, leading to low tumor specificity [30-33]; additionally, it is susceptible to in vivo metabolism [34], complicating kinetic analysis. To address these deficiencies, S-[$^{11}$C]-methyl-L-cysteine $^{1L}$ (S-[$^{11}$C]CH$_3$-L-CYS) and S-[$^{11}$C]-methyl-D-cysteine $^{1D}$ (S-[$^{11}$C]CH$_3$-D-CYS), a pair of $^{11}$C-labelled S-methylcysteine enantiomers (Figure 1A and Figure S1B), were successively developed via $^{11}$C-isotopic substitution in our previous studies [35-38]. Preliminary studies indicated that the tracers were superior to [18F]FDG and [$^{11}$C]MET in the differentiation of tumor from inflammation [35, 36, 38-40]. Nevertheless, the short half-life of $^{11}$C ($t_{1/2} = 20.4$ min) restricts the widespread application of these tracers, resulting in an urgent demand for $^{18}$F-labelled SAA tracers ($^{18}$F, $t_{1/2} = 109.8$ min).

To date, most previous studies on the $^{18}$F-labelled SAA tracers (Figure S1B) have concentrated on molecular scaffolds which can be readily radiolabelled by linking with a prosthetic group, such as S-(2-[18F]fluoroethyl)-L-homocysteine [41, 42], S-(3-[18F]fluoropropyl)-L-homocysteine [43], S-(3-[18F]fluoropropyl)-D-homocysteine [43, 44]. For the structure-sensitive SAA molecules, however, even minor side-chain alterations caused by the prosthetic groups (S-ethyl and S-propyl) may lead to significant changes in biological properties. More recently, to avoid affecting the biological activity, $^{18}$F-B-MET (a methionine boramino acid derivative; Figure S1B) was developed as a potential substitute of [$^{11}$C]MET by isoteric substitution of carboxylate (-CO$_2$) group with trifluoroborate (-BF$_3$) group [45]. $^{18}$F-B-MET shared the same AA transport systems with [$^{11}$C]MET owing to the nearly identical charge distribution patterns. But these groups (-BF$_3$ and -CO$_2$) differ considerably in chemical structure and properties, which may cause potential differences in metabolism of the tracers in vivo. Therefore, despite these undeniable successes, the development of $^{18}$F-labelled SAA tracers without significant structural changes or relying on prosthetic groups remains to be a great challenge, highlighting the importance of research on a structure-mimetic tracer.

Trifluoromethyl (-CF$_3$), the smallest symmetrical multi-fluorine group, has captured intense attention in the fields of chemistry and pharmacy, because of its ability to increase chemical and metabolic stability, to improve bioavailability and lipophilicity, and to enhance binding selectivity [46-50]. Given these advantages of -CF$_3$ and our interest in $^{18}$F-labelled SAA tracers, in this work, we aimed to develop a couple of $^{18}$F-trifluoromethylated cysteine enantiomers for PET imaging of glioma. As shown in Figure 1.
According to the chiral radio-HPLC analysis, almost no racemization was detected during the synthesis of 2L and 2D in acidic conditions. 2L and 2D had log P values of -2.75 and -2.22, respectively, and were > 95% stable in PBS at 37 °C for up to 2 hours (Figure S5). According to the chiral radio-HPLC analysis, almost no racemization was detected during the synthesis of 2L and 2D (optical purity: ee > 99%; Figure 2 and Figure S4), which forcefully confirmed the feasibility of this nucleophilic 18F-trifluoromethylthiolation protocol (Scheme S2) for synthesizing enantiopure 18F-trifluoromethylated cysteines.

**Results and Discussion**

**Radiochemistry**

Although the development of the 18F-trifluoromethylated SAA tracers is conceptually straightforward, it is actually quite challenging due to the difficulty of introducing fluorine-18 into the radiolabelled -SCF3 group. The most efficient synthetic routes toward non-labelled trifluoromethylated SAAs involve direct trifluoromethylation of thiols using electrophilic trifluoromethyllating reagents, such as the Togni’s [51, 52] and Umemoto’s [53] reagents. However, until recently, only one such radiolabelled reagent (18F-Umemoto’s reagent) was successfully developed for electrophilic 18F-trifluoromethylthiolation [54]. In addition, Liang and Xiao reported a nucleophilic 18F-trifluoromethylthiolation of α-bromo carbonyl compounds and aliphatic halides with difluorocarbene (generated from Ph3P+CF2CO2-; PDFA) in the presence of 18F-fluoride and elemental sulfur (S8) [55, 56]. Cahard and Ma recently developed a straightforward method for the synthesis of β- and γ-SCF3 α-AA derivatives through nucleophilic trifluoromethylthiolation of cyclic sulfamidates [57]. Moreover, serine-derived cyclic sulfamidates have been widely used as configurationally stable chiral building blocks for the synthesis of enantiopure β-substituted α-AAs [57-59]. Inspired by these studies, we envisioned that the 18F-trifluoromethylated SAAs 2L and 2D could be synthesized stereoselectively from serine-derived cyclic sulfamidates via a nucleophilic 18F-trifluoromethylthiolation reaction followed by a deprotection reaction.

The initial step in our work was to synthesize the cyclic sulfamidates 3L and 3D via a four-step reaction (Scheme S1), according to the reported methods [12, 58, 60-62]. With the desired cyclic-sulfamidates in hand, we set out to optimize the reaction conditions (Table S1) and to explore the synthesis of 2L and 2D. As shown in Scheme 1, the 18F-trifluoromethylthiolation of cyclic-sulfamidates 3L and 3D (2 mg, 6 μmol) with PDFA (1.5 mg, 6 μmol) and S8 (3.0 mg, 12 μmol) in the presence of heating-block-dried K222/K18F was carried out at 70 °C for 5 min to give the radiolabelled intermediates 4L and 4D which were subsequently purified by the C18 cartridge and eluted with ethanol. Then, the solution was evaporated and hydrolyzed in 4N HCl aq. at 90 °C for 10 min [61, 62]. Finally, the desired products 2L and 2D were neutralized (pH ≈ 6) and isolated using solid phase extraction to obtain 14% ± 3% RCY (n = 6) in 35 min. The radiochemical purity was higher than 98%, as determined by radio-TLC (Figure S2-3) [63]. Similar to a previous report about the synthesis of non-radiolabelled L-trifluoromethylcysteine [64], the harsh hydrolysis conditions failed to lead to a β-elimination side reaction, suggesting a good stability of 2L and 2D in acidic conditions. 2L and 2D had log P values of -2.75 and -2.22, respectively, and were > 95% stable in PBS at 37 °C for up to 2 hours (Figure S5). According to the chiral radio-HPLC analysis, almost no racemization was detected during the synthesis of 2L and 2D (optical purity: ee > 99%; Figure 2 and Figure S4), which forcefully confirmed the feasibility of this nucleophilic 18F-trifluoromethylthiolation protocol (Scheme S2) for synthesizing enantiopure 18F-trifluoromethylated cysteines.

![Figure 1A](http://www.thno.org)

**Figure 2.** Chiral radio-HPLC analysis of 2L and 2D (test tube method).
In vitro cell research

Encouraged by the successful synthesis of 2L and 2D, we conducted in vitro cell uptake study to explore the specificity of each enantiomer. As shown in Figure 3A, the uptake of 2L in C6 cells increased steadily in a time-dependent manner, and the maximal value (4.33% uptake/100 μg protein) appeared at the 120 min. 2D rapidly accumulated in the cells within a short time and reached a maximum of about 6.34% uptake/100 μg protein after incubation for 20 min, but then declined slowly afterward. One possible reason for the above situation was that 2D was being transported into/out of the C6 cells at a higher rate compared with 2L. Thus, the uptake of 2L was higher than that of 2D in C6 cells after approximately 60 min, and the uptake difference was gradually enlarged with the prolonged incubation time. In vitro cell uptake studies indicated that cysteines functionalized with an [18F]CF3 moiety could be taken up efficiently by C6 cells, but there was an obvious distinction between different chiral isomers on the cellular uptake efficiency.

Although mammalian cells generally tend to employ L-enantiomer for the biological basic needs, both the enantiomers of AAs can be transported [44]. In order to investigate the uptake mechanism of each enantiomer, a competitive inhibition study was performed using C6 glioma cells in the presence of AA transporter inhibitors (Figure 3B-C). After 15 min of incubation in choline chloride solution (-Na+), the cellular uptake of 2L and 2D was obviously decreased by BCH (2-amino-2-norbornanecarboxylic acid), a classical inhibitor for system L transporters. Additionally, the transportation of 2L and 2D in saline solution (+Na+) was effectively blocked by L-serine (Ser), a non-specific inhibitor for system ASC transporters [36, 65]. By contrast, MeAIB (2-aminoisobutyric acid), a system A inhibitor, exerted almost no significant effect on the transportation of 2L and 2D into the cells in either choline chloride or saline solution. Similar to 14C-methyl-cysteines 1L and 1D [35, 36], we found that the cellular uptake of both enantiomers of [18F]trifluoromethylated cysteine in C6 cells mainly relied on the systems L and ASC; however, the system A did not contribute to the radioactive accumulation. Remarkably, the cellular uptake of 2L was significantly suppressed by L-MCYS (S-methyl-L-cysteine) in both choline chloride and saline solution (Figure 3B), strongly suggesting that L-MCYS and its mimic 2L shared the same AA transport systems.

Next, we examined the extent of protein incorporation of each enantiomer in C6 tumor cells, according to the similar reported method [66]. After precipitation with trichloroacetic acid (TCA), the protein incorporation of 2L in C6 cells was 0.6% and 4.5% at 30 and 120 min incubation times (Figure 3D), respectively. Thus, there was almost no incorporation of 2L into protein. In comparison, a markedly higher percentage of 2D incorporated into protein, with about 5% and 13% at 30 and 120 min, respectively, implying that there were some interactions between 2D and intracellular macromolecules (perhaps enzymes). Overall, the in vitro cell studies fully demonstrated that cellular uptake of [18F]-trifluoromethylated cysteines was mainly associated with their AA transport systems across the cell membrane rather than with the protein incorporation.

In vivo biodistribution studies

To explore the in vivo biodistribution of each enantiomer, we subsequently performed the studies by dissection on normal Kunming mice (n = 4 per group). Surprisingly, a rapid and progressive accumulation of radioactivity was observed in the bone from 5 to 90 min after injection of 2L (Figure 4A). But for 2D, the bone uptake only slightly increased over time (Figure 4B), suggesting a slow defluorination or a bone marrow uptake. Even though both 2L and 2D were stable in vitro, there was a marked difference in stability between the two enantiomers in vivo. One reasonable explanation is that 2L might serve as a preferential substrate for cysteine S-conjugate β-lyases and underwent an enzyme-catalyzed β-elimination reaction [67, 68]. Structurally, 2D is also a cysteine S-conjugate but showed relatively good in vivo stability, presumably because the β-elimination reaction proceeded with high L-stereoselectivity. On the basis of these analyses, a possible mechanism was proposed to explain the surprising in vivo instability of 2L. As illustrated in Scheme 2, the deprotonated base (B-) abstracts a proton from 2L and initiates transaldimination of pyridoxal 5'-phosphate (PLP)-imine with the deprotonated α-amino group to form the 2L-PLP Schiff base [69, 70]. The Schiff base is then α-deprotonated by the ε-amino group of the lysine residue to give a quinoid intermediate [71, 72]. Subsequent elimination of [18F]trifluoromethanethiol ([18F]CF3SH further decomposes to release 18F-fluoride; please see the green box in Scheme 2) from the β-carbon position produces a ketimine intermediate which is finally hydrolyzed to afford PLP-imine, pyruvate and ammonium [69, 73].

In addition, biodistribution studies by dissection indicated that 2D was primarily excreted via the kidneys (urinary system) and to a minor extent via the hepatic route. Fast washout of radioactivity from the main tissues and organs (e.g., blood, heart, lung,
pancreas, and stomach) was observed during the entire experimental process (Figure 4B), revealing that 2D has advantages of rapid in vivo clearance. Similar to 1L and 1D [35, 36, 39], low accumulation of 2D in the brain was found in the biodistribution data, which could be considered as an advantage or a disadvantage. It was an advantage because the tracer with low brain uptake would contribute to providing a low background activity for PET imaging of brain tumors. On the other hand, it could also be a disadvantage for the tracer, as the uptake in any cranial tumor would be low due to a low availability of the tracer after transport through the blood-brain barrier. Moreover, we also performed a comparison between [18F]FDG and 2D in Kunming mice (n = 4 per group) with turpentine-induced acute inflammation. The preliminary results (data obtained by dissection) showed that 2D had significantly lower inflammation/muscle and inflammation/blood ratios than [18F]FDG at 60 min post-injection (Table S2), which was similar to our previous report on 1D [40].

Figure 3. (A) Time-dependent cell uptake assays with 2L and 2D in C6 cells; (B) Competitive inhibition of C6 cell uptake of S-[18F]CF3-L-CYS 2L after co-incubation with each inhibitor or L-MCYS for 15 min; (C) Competitive inhibition of C6 cell uptake of S-[18F]CF3-D-CYS 2D after co-incubation with each inhibitor for 15 min; (D) The comparison of protein incorporation of 2L and 2D in C6 tumor cell line after incubation for 30 min and 120 min.

Figure 4. (A) The biodistribution of 2L in normal Kunming mice at 5, 30, 60 and 90 min post-injection; (B) The biodistribution of 2D in normal Kunming mice at 5, 30, 60 and 90 min post-injection.
**Scheme 2.** Possible mechanism of cysteine S-conjugate β-lyases catalyzed β-elimination of S-[18F]CF3-L-CYS 2L. PLP, the biologically active cofactor, is bound to the enzyme (Enz) at the ε-amino group of the lysine residue.

**In vivo PET imaging of S-[18F]CF3-D-CYS**

The promising results from in vitro cell uptake studies and in vivo biodistribution studies, such as specific tumor-targeting properties and favourable pharmacokinetic characteristics, inspired us to further investigate the feasibility of 2D as an amino acid tracer for glioma PET imaging. As shown in Figure 5A, the D-enantiomer selectively accumulated in C6 tumor tissues to give a good tumor-to-background contrast, which was predominantly cleared by renal excretion with moderate liver accumulation. The average uptake values of 2D in the tumor were 3.81 ± 0.23, 3.74 ± 0.18, 3.56 ± 0.15% ID/g (n = 3) at 45, 60 and 75 min after injection, respectively. Compared with [18F]FDG, 2D exhibited relative less tumor radioactivity accumulation but much lower uptake in most major organs (except of pancreas, kidney and bladder), particularly in normal brain tissue (Figure 5A-B). Hence, the tumor-to-brain uptake ratio of 2D was substantially higher than that of [18F]FDG (Figure 5C). In addition, a slightly high 2D uptake was observed in the muscle tissues, which might restrict the application of the D-enantiomer in regions beyond the brain.

To further determine the distribution patterns of 2D, a 90 min dynamic micro-PET scan was performed in other C6-bearing mice (Figure S6). The time-activity curves were obtained from dynamic images after drawing regions of interest (Figure 6A). Relative uptake ratios of tumor-to-brain, tumor-to-muscle and tumor-to-bone at different time points were then calculated and illustrated in Figure 6B. During the first 30 min, 2D reached its maximum uptake value and exhibited a long-term retention in the C6 tumor, then declined slowly. The highest tumor-to-brain uptake ratio of 14.70 was achieved at 15 min after injection of 2D. It was also noteworthy that the bone uptake increased slightly as time went on, which was consistent with the results of the biodistribution studies. Even so, several competing factors should be considered synthetically in the process of defining PET images, such as tumor tissue uptake, in vivo defluorination or bone marrow accumulation, and pharmacokinetic characteristics [74]. Additional investigations are warranted in the future to ascertain in vivo metabolic fate of the [18F]-trifluoromethylated cysteines as well as their stability in vitro in the presence of the cysteine S-conjugate β-lyases. Taken together, these results
showed that S-[18F]CF3-D-CYS 2D, an [18F]CF3-functionalized SAA tracer, might be a potential candidate for glioma imaging.

**Conclusions**

In conclusion, we have successfully designed and synthesized a couple of 18F-trifluoromethylated cysteine enantiomers (2L and 2D) according to a structure-based bioisosterism strategy. In vitro study indicated that cellular uptake of the two enantiomers was primarily associated with AA transport systems L and ASC. Notably, in vivo biodistribution and PET imaging studies demonstrated that 2D was characterized with relatively good stability and high tumor-specific accumulation. Our results suggest that 18F-trifluoromethylated D-cysteine, a new SAA tracer, may be a potential candidate for PET imaging of glioma. To the best of our knowledge, this is the first study to synthesize enantiopure 18F-trifluoromethylated cysteines and to evaluate their feasibility as “structure-mimetic” AA tracers for tumor imaging. Also, a more detailed biological evaluation is underway.

**Materials and Methods**

**Radiochemistry**

18F-fluoride was trapped on a QMA light cartridge and subsequently eluted by a H2O/CH3CN (1:9, 1.0 mL) mixed solution of K2CO3 (3 mg) and K2.2.2 (13 mg) into a sealed penicillin bottle (10 mL). The K2.2.2/K18F solution was evaporated at 95 °C for 10 min under a N2 flow and resolubilized in anhydrous CH3CN (10 mL). The resulting solution of K2.2.2/K18F in anhydrous CH3CN was entirely transferred into a 10 mL volumetric penicillin bottle (sealed by a rubber cap) containing the cyclic-sulfamidate precursor (3L or 3D; 2 mg, 6 μmol), PDFA (1.5 mg, 6 μmol) and S8 (3.0 mg, 12 μmol). The nucleophilic 18F-trifluoromethylthiolation reaction was carried out...
at 70 °C for 5 min without electromagnetic stirring. After the reaction completed, the reaction mixture was diluted by 5% AcOH aqueous solution (10 mL) and passed through a C18 plus short cartridge. After washed by sterilized water (10 mL), the \(^{18}\text{F}\)-labelled intermediate \(4L\) or \(4D\) was eluted from C18 cartridge by ethanol (1.5 mL) into another sealed penicillin bottle. The solvent was removed by evaporation at 85 °C for 5 min under a \(\text{N}_2\) flow. 4 N HCl aq. (0.8 mL) was added to the residue and heated for 10 min at 90 °C. Finally, the product (\(2L\) or \(2D\)) was purified by passing serially through an AG 11 A8 ion retardation cartridge, a C18 plus short cartridge, and a sterile Millipore 0.22 μm filter with 0.9% NaCl aq. solution (2 mL) into a final product vial (pH = 6).

A detailed descriptions of all experimental procedures, including organic chemistry synthesis, radiochemistry synthesis, and in vitro and in vivo biological evaluation experiments, can be found in the Supplementary Materials.

**Abbreviations**

AAs: Amino acids; PET: Positron emission tomography; SAAs: Sulfur-containing amino acids; DNA: Deoxyribonucleic acid; \([^{11}\text{C}]\text{MET}\): L-methyl-\([^{11}\text{C}]\)methionine; \([^{18}\text{F}]\text{FDG}\): 2-\([^{18}\text{F}]\)fluoro-2-deoxy-D-glucose; S-\([^{11}\text{C}]\)CH\(_3\)+L-CYS; S-\([^{11}\text{C}]\)methyl-L-cysteine; S-\([^{11}\text{C}]\)CH\(_3\)-D-CYS; S-\([^{11}\text{C}]\)methyl-D-cysteine; MEP: Molecular electrostatic potential; S-\(\text{CF}_3\)-L-CYS: S-trifluoromethyl-L-cysteine; S-\(\text{CF}_3\)-D-CYS: S-trifluoromethyl-D-cysteine; -\(\text{CO}_2\): Carboxylate group; -\(\text{BF}_3\): Trifluoroborate group; -\(\text{CF}_3\): Trifluoromethyl group; -\(\text{CH}_3\): Methyl group; -SC\(_3\): Trifluoromethyl group; PDFA: Difluoro-methylene phosphobetaine (\(\text{Ph}_3\text{P}^+\text{CF}_2\text{CO}_2^-\)); S\(_8\): Elemental sulfur; aq.: Aqueous; RCY: Radiochemical yield; TLC: Thin-layer chromatography; HPLC: High performance liquid chromatography; BCH: 2-amino-2-norbornanecarboxylic acid; Ser: L-serine; MeAIB: 2-aminoisobutyric acid; L-MCYS: S-methyl-L-cysteine; TCA: Trichloroacetic acid; B-: Deprotonated base; PLP: Pyridoxal 5′-phosphate; \([^{18}\text{F}]\)CF\(_3\)SH: \([^{18}\text{F}]\)Trifluoromethanethiol; Enz: Enzyme.

**Supplementary Material**

Supplementary information, figures and tables. http://www.thno.org/v09p1144s1.pdf

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**Author Contributions**

S. Liu designed the study, synthesized the compounds and wrote the original manuscript. S. Liu, H. Ma, Z. Zhang, L. Lin, and G. Yuan conducted the cell and animal experiments. X. Tang, D. Nie, and S. Jiang discussed the results and analysed the data. G. Yang supervised the studies in synthetic chemistry, discussed the results and revised the paper. G. Tang supervised the project, discussed the results, analysed the data and revised the paper. All authors read and approved the manuscript.

**Competing Interests**

The authors have declared that no competing interest exists.

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