Fully Automated ^{89}Zr Labeling and Purification of Antibodies

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Dozens of monoclonal antibodies (mAbs) have been approved for clinical use, and hundreds more are under development. To support these developments and facilitate a personalized medicine approach, PET imaging and quantification of mAbs, after chelation with desferrioxamine B (DFO) and radiolabeling with ^{89}Zr, has become attractive. Also, the use of ^{89}Zr-mAbs in preclinical and clinical studies is expanding rapidly. Despite these rapid developments, ^{89}Zr radiolabeling is still performed manually. Therefore, we aimed to develop a simple, fully automated, good-manufacturing-practice (GMP)–compliant production procedure for the ^{89}Zr labeling of mAbs. Such procedures should increase the robustness and capacity of ^{89}Zr-mAb production while minimizing the radiation dose to the operator. Here, the procedures for fully automated ^{89}Zr-mAb production are described and applied to produce batches of ^{89}Zr-DFO-N-suc-cetuximab and ^{89}Zr-DFO-N-suc-rituximab suitable for clinical use. Both products had to meet the GMP-compliant quality standards with respect to yield, radiochemical purity, protein integrity, antigen binding, sterility, and endotoxin levels. **Methods:** Automated ^{89}Zr labeling of mAbs was developed on a Scintomics GRP 2V module and comprised the following steps: reagent transfer to the ^{89}Zr-containing reaction vial, mixing of the reagents followed by a 60-min reaction at room temperature to obtain optimal radiolabeling yields, and product purification using a PD-10 desalting column. **Results:** Radiochemical yields of ^{89}Zr-DFO-N-suc-cetuximab and ^{89}Zr-DFO-N-suc-rituximab were all more than 90% according to instant thin-layer chromatography. Isolated yields were 74.6% ± 2.0% and 62.6% ± 3.0% for ^{89}Zr-DFO-N-suc-cetuximab and ^{89}Zr-DFO-N-suc-rituximab, respectively, which are similar to isolated yields obtained using GMP protocols for manual ^{89}Zr labeling of mAbs. To meet the GMP-compliant quality standards, only the radiochemically pure fractions were collected from PD-10, resulting in a lower isolated yield than the radiochemical yield according to instant thin-layer chromatography. The radiochemical purity and protein integrity were more than 95% for both products, and the antigen binding was 95.6% ± 0.6% and 87.1% ± 2.2% for ^{89}Zr-DFO-N-suc-cetuximab and ^{89}Zr-DFO-N-suc-rituximab, respectively. The products were sterile, and the endotoxin levels were within acceptable limits, allowing future clinical production using this procedure. **Conclusion:** Procedures for fully automated GMP-compliant production of ^{89}Zr-mAbs were developed on a commercially available synthesis module, which also allows the GMP production of other radiolabeled mAbs.

Dozens of monoclonal antibodies (mAbs) have been approved to treat cancer and autoimmune diseases. Furthermore, hundreds are under preclinical and clinical development (1–5). Radiolabeling of mAbs is valuable during their preclinical and clinical evaluation. ^{89}Zr is the radionuclide of choice to label mAbs because of its good availability, its suitable physical half-life (78.4 h), which matches the biologic half-life of intact mAbs (6–8), and the availability of reliable radiolabeling procedures (9–14).

To date, more than 350 papers have been published describing preclinical and clinical studies with ^{89}Zr-mAbs (15,16). Nineteen clinical trials were published in which ^{89}Zr-mAbs have been used for PET imaging in oncology. Besides these, over 62 clinical trials are currently ongoing with ^{89}Zr-mAbs, of which 35 have been started since 2017 (17).

Generic methods for manual radiolabeling have been developed to support the continuously increasing number of clinical trials with ^{89}Zr-mAbs (6,8,18,19). Desferrioxamine B (DFO) is the only chelator applied for radiolabeling with ^{89}Zr. To this end, the mAbs are first conjugated with DFO and subsequently labeled with ^{89}Zr (described in the supplemental materials; available at http://jnm.snmjournals.org). To improve conjugate stability, a modified DFO has been introduced for octadentate instead of hexadentate chelation of ^{89}Zr while using identical labeling conditions (20,21).

To allow ^{89}Zr labeling, iron-protected tetrafluorophenol-N-succinyldesferal or p-isothiocyanatobenzyl-DFO is conjugated to a lysine residue of the mAb, resulting in an amide or thiourea linkage, respectively, followed by size-exclusion chromatography (SEC), such as by using a PD-10 column, to remove unconjugated chelator (8,19). DFO-mAbs might be stored as a labeling precursor for more than a year at −80°C, as demonstrated for DFO-N-suc-cetuximab. The desired ^{89}Zr-mAb is produced by ^{89}Zr complexation of the DFO-mAb at room temperature and pH 7. When ^{89}Zr complexation is completed, the product is purified by SEC using a buffer that allows administration of the product in vivo (the supplemental materials provide the reaction schemes) (8,19). Although other procedures have been described for conjugation of DFO, as well as novel chelators (20–25), these methods have not been applied for clinical use to date.

The increasing number of research sites that perform PET studies with ^{89}Zr-mAbs requires a suitable infrastructure and

enough trained researchers to produce the desired products. Furthermore, regulations should allow for the production and human application of the ^{89}Zr-mAbs. In general, for working with radioactivity, the radiation dose should be kept as low as reasonably achievable, better known as the ALARA principle. This means that exposure to the radioactive source, ^{89}Zr in this case, should be minimized or avoided (26). Furthermore, it is of the utmost importance that when a ^{89}Zr-mAb is prepared for preclinical or clinical application, the integrity and the sterility of the product are ensured. To ensure patient safety, the labeling procedure should be compliant with good manufacturing practices (GMPs) (27,28), be well documented and validated, give a reliable product yield, and comply with preset quality specifications. Quality control of the product consists of determination of product integrity (mAb integrity and binding affinity), radiochemical purity, and sterility, as well as the absence of endotoxins. Furthermore, product cross-contamination should be avoided. To address all these challenges, it would be ideal if a validated automated radiolabeling method were available to produce ^{89}Zr-mAbs in a GMP-compliant fashion. Automation should result in a reproducible labeling yield with minimal exposure of the production personnel to the radioactive source. Use of disposable cassettes should maximize the pharmaceutical product quality, minimize potential cross-contaminations, and avoid time-consuming cleaning procedures.

Automation in radiochemistry is commonly used for the production of ^{11}C- or ^{18}F-labeled small-molecule PET tracers (29). Publications describing the automated radiolabeling of biologicals are scarce. Only a few reports have been published describing the synthesis of pharmaceutical-grade ^{131}I-, ^{111}In-, or ^{177}Lu-labeled mAbs (30–32). To the best of our knowledge, the only automated system for ^{89}Zr-mAb production makes use of microfluidic chips and has been described by Wright et al. (33). Labeling yields reported by these authors were satisfactory; however, the product was purified manually.

Since there are no methods that describe a fully automated ^{89}Zr labeling and purification of mAbs, our goal was to develop such a procedure, focusing on production of a ^{89}Zr-mAb that can be formulated, filtered, and immediately injected into patients. In addition, this procedure should also be GMP-compliant, using reagents and disposables that meet the requirements for the production of ^{89}Zr-mAbs for clinical application. Reliable GMP-compliant manual ^{89}Zr-labeling protocols have been described by Vosjan et al. and Cohen et al. and are used herein as a basis to start the automated production of ^{89}Zr-mAbs (8,19). The challenges in automated production comprise accurate and complete reagent transfer, efficient mixing of all reagents, and the fact that the procedure should be at atmospheric pressure to allow PD-10 SEC purification of the ^{89}Zr-mAb. To develop this automated labeling of mAbs with ^{89}Zr, a Scintomics GRP 2V module was used.

MATERIALS AND METHODS

General

All reagents and solvents were purchased from Sigma Aldrich. Disposable tubing with Luer locks was obtained from Vygon. Disposable syringes were obtained from BD Plastipak. Cetuximab (5 mg/mL) directed against epidermal growth factor receptor and rituximab (10 mg/mL) directed against CD20 were obtained from the VU University Medical Center pharmacy. For this study, TFP-N-suc-Df-Fe was prepared in-house and stored in acetonitrile at −80°C (9). The chemical modification of mAbs to obtain DFO-N-suc-cetuximab and DFO-N-suc-rituximab for radiolabeling was performed according to literature procedures (6,8,19). General methods for quality control have been described previously and are also described in detail in the supplemental materials (6,8,19).

System Setup for Automated Radiolabeling

A double-cassette setup was used on a Scintomics GRP 2V module (Fig. 1). Both automated valve units were equipped with a stopcock-valve manifold with 5 3-way valves (ABX) and connected with fluidic transfer lines according to Figure 1. Details on the setup and connections of the tubing are described in the supplemental materials.

The software from Scintomics was used to program the automated reagent transfer to radiolabel DFO-N-suc-cetuximab or DFO-N-suc-rituximab with ^{89}Zr (supplemental materials). For the transfer of all reagents, a 20-mL syringe was used that allowed aspiration and squirting of the reagents into the desired vial (Fig. 1). The 3-way valves of the manifolds allowed for control of solvent and reagent transfer during the synthesis as required at each time point.

Procedure Description

At the start of the radiolabeling, 2 M Na_2CO_3 (0.09 mL) and 0.5 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7 (0.5 mL), were transferred from valve connection 3 to the reaction vial at connection 6 containing ^{89}Zr in 1 M oxalic acid (0.2 mL). After 3 min, DFO-N-suc-cetuximab or DFO-N-suc-rituximab (0.71 mL, 2–5 mg/mL) and 0.5 M HEPES, pH 7 (0.5 mL), from valve connection 2 were added to the reaction vial and mixed by drawing up the reaction mixture using the 20-mL syringe at valve 7 and then emptying it into the reaction vial again. The reaction mixture was kept at room temperature for 60 min, and then approximately 2 mL were transferred to the preconditioned PD-10 column, with immediate elution of the liquid through the column upon gravity into the 20-mL sterile vial, positioned at valve 10. After initial elution, the transfer line between valve 10 and the 20-mL product vial was vented through valve 9. Afterward, a 5 mg/mL solution of gentisic acid, pH 5.0 (3.0 mL for cetuximab and 2.5 mL for rituximab), was transferred from the syringe at position 4 to the PD-10 column and the liquid was eluted with gravity into the 20-mL product vial. In this automated procedure, the product vial contained approximately 5 mL for ^{89}Zr-DFO-N-suc-cetuximab (34) and 4.5 mL for ^{89}Zr-DFO-N-suc-rituximab.

Reliability and Reproducibility Study

The reliability and reproducibility of the automated ^{89}Zr-DFO-N-suc-cetuximab and ^{89}Zr-DFO-N-suc-rituximab preparation were examined by performing the described optimized procedure 2 times in
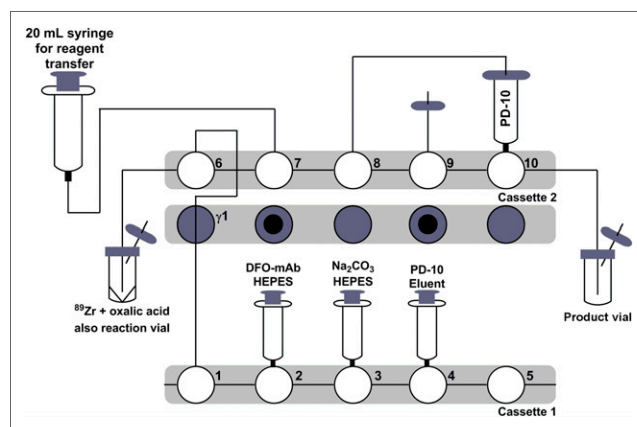

FIGURE 1. Schematic representation of complete and optimized automated ^{89}Zr labeling of DFO-mAbs on Scintomics GRP 2V module, with positions of reagent vials, ^{89}Zr-containing reaction vial, transfer lines, PD-10 desalting column, and product vial that will contain purified ^{89}Zr-labeled mAb indicated.
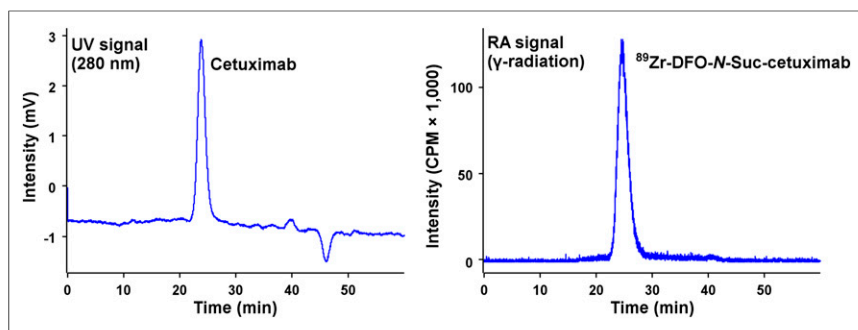
FIGURE 2. Analytic (radio)SEC-HPLC of ^{89}Zr-DFO-*N*-suc-cetuximab after automated radiolabeling and PD-10 purification. CPM = counts per minute; RA = radioactive; UV = ultraviolet.

triplicate for each product. For the first triplicate, approximately 20 MBq of ^{89}Zr were used at the start of synthesis, whereas approximately 60 MBq of ^{89}Zr were used for the second triplicate. The data for each individual labeling with either 20 or 60 MBq can be found in the supplemental materials. Calculation of yields was based on the amount of radioactivity at the start of synthesis and in the final product that was obtained after PD-10 purification. To make sure that the procedure was reliable and reproducible, no failing productions were tolerated during the consecutive runs.

Quality Control

The products obtained in the second series, starting with approximately 60 MBq of ^{89}Zr, were tested according to standards as applied for clinical use (*35–37*). The parameters and criteria investigated in these quality analyses were radiolabeling yield (>50%), radiochemical purity according to instant thin-layer chromatography (iTLC) and high-performance liquid chromatography (HPLC) (>90%), protein integrity (>90%), antigen binding (>70%) (*38*), and endotoxin content (<2.5 endotoxin units/mL). The results are summarized in Table 1. The test labelings that started with 20 MBq were analyzed for radiolabeling yield (>50%) and radiochemical purity according to iTLC and HPLC (>90%). Details of the quality tests are described in the supplemental materials.

Product Sterility

The sterility of the product was determined by performing a mockup labeling of DFO-*N*-suc-cetuximab with nonradioactive zirconium according to the same optimized automated procedure described above. After PD-10 purification of the product, it was filtered manually and tested for sterility.

RESULTS

Automated Process

For the automated ^{89}Zr labeling starting with 20 MBq using the optimized setup, ^{89}Zr-DFO-*N*-suc-cetuximab was obtained in 67.8%, 77.7%, and 75.8% radiochemical yield, and ^{89}Zr-DFO-*N*-suc-rituximab was obtained in 62.3%, 68.2%, and 58.0% radiochemical yield (supplemental materials). Radiochemical purity and protein integrity according to SEC-HPLC for both products was more than 95%, and antigen binding was 95.9% ± 0.6% and 87.1% ± 2.2% for ^{89}Zr-DFO-*N*-suc-cetuximab and ^{89}Zr-DFO-*N*-suc-rituximab, respectively.

Reliability and Reproducibility Study

To investigate the reliability and reproducibility of the procedure, radiolabeling reactions were conducted starting with 60 MBq of ^{89}Zr. The obtained radiochemical yields were 74.0%,

76.8%, and 72.9% for ^{89}Zr-DFO-*N*-suc-cetuximab and 59.2%, 65.0%, and 63.6% for ^{89}Zr-DFO-*N*-suc-rituximab.

Quality Control

When we started with 60 MBq of ^{89}Zr, radiochemical purity and protein integrity according to SEC-HPLC were 100%, and iTLC radiochemical purity was more than 98% for ^{89}Zr-DFO-*N*-suc-cetuximab and more than 99% for ^{89}Zr-DFO-*N*-suc-rituximab (Table 1). The antigen binding of ^{89}Zr-DFO-*N*-suc-cetuximab was more than 95%, whereas the antigen binding of ^{89}Zr-DFO-*N*-suc-rituximab was more than 85% (Table 1). The endotoxin content was below 0.5 endotoxin unit/mL.

Product Sterility

The nonradioactive zirconium–labeled DFO-N-suc-cetuximab was sterile.

DISCUSSION

Translation of a manual to an automated manufacturing process proved to be challenging. The automated ^{89}Zr labeling of DFO-*N*-suc-cetuximab or DFO-*N*-suc-rituximab was approached stepwise and appeared to contain multiple critical steps: efficient and complete reagent transfer, efficient reagent mixing, and sufficient and reliable product purity based on SEC. In addition, the automated labeling needs to be GMP-compliant and result in acceptable product quantities (~37 MBq/patient) with excellent quality.

Reagent Transfer

Efficient, complete, and accurate reagent transfer is crucial for successful automated ^{89}Zr-mAb labeling. The labeling is a well-defined and precise process in which relatively small volumes of reagents are used. The pH of the reaction mixture needs to be approximately 7 (optimally, 6.8–7.4); otherwise, the radiolabeling efficiency decreases (*8*). The current reagent amounts and volumes allow errors of 10% in the volumes being added to the reaction mixture. However, larger errors are not allowed because the optimal pH for radiolabeling will not be reached, resulting in suboptimal yields and quality. Therefore, for the automated ^{89}Zr-mAb labeling, where the total reaction volume is 2 mL, small volumes should be transferred and mixed accurately to obtain an optimal radiochemical yield.

Several setups for the automated ^{89}Zr labeling of mAbs were tested. Initially, capped and sealed vials were placed upside-down and punctured with plastic needles. Though this setup is often used for automation, accurate reagent transfer, especially of small volumes (<250 μL), was not possible. Therefore, this setup was not further investigated. The second setup made use of flat-bottomed glass vials containing stock solutions. With the syringe pump, and by programming the automation, the required reagent volumes were transferred. Because of inaccurate and delayed aspiration and dispensing, this approach never resulted in any successful radiolabeling (supplemental materials).

To come to a successful automated ^{89}Zr labeling, it was decided that all vials should contain the exact amount of reagent and solvent to be transferred and that V-shaped glass vials should be used, allowing complete aspiration via injection needles. These modifications allowed complete and accurate transfer of all

Reaction detail	^{89}Zr-DFO-N-suc-cetuximab	^{89}Zr-DFO-N-suc-rituximab
Start activity (MBq)	63.55 ± 2.13	63.01 ± 5.12
Radiochemical yield by iTLC (%)	92.0 ± 1.2	95.2 ± 0.2
Isolated product (MBq)	47.41 ± 2.88	39.56 ± 5.03
Labeling yield (non–decay-corrected) (%)	74.6 ± 2.0	62.6 ± 3.0
Quality control of isolated product		
Radiochemical purity by iTLC (%)	98.3 ± 0.4	99.1 ± 0.1
Radiochemical purity by SEC-HPLC (%)	99.5 ± 0.3	100 ± 0
Protein integrity by SEC-HPLC (%) at 280 nm	100 ± 0	100 ± 0
Immunoreactivity by Lindmo assay (%)	95.9 ± 0.6	87.1 ± 2.2
Sample value bacterial endotoxin content assay (<2.5 endotoxin units/mL)	0.226 ± 0.045	0.301 ± 0.175

Data on individual labeling are provided in supplemental materials.

solutions by the 20 mL syringe, although it was necessary that the 20 mL syringe be programmed for a larger volume than anticipated, to correct for the dead volume in the system. Using this setup, the first successful ^{89}Zr labeling runs were performed. Nevertheless, reliability was poor because of inaccurate transfer of the 90 μL 2 M Na_2CO_3 solution for neutralizing the ^{89}Zr solution. To circumvent this problem, we investigated whether dilution of the 2 M Na_2CO_3 stock solution with 0.5 M HEPES buffer (pH 7) would also result in successful production. When implemented on the automated system, this modification resulted in efficient and complete transfer of the 2 M Na_2CO_3 solution to the ^{89}Zr-containing vial, resulting in consistent radiochemical yields.

When the automated radiolabeling proved to be successful, with consistent radiochemical yields using 20 MBq of ^{89}Zr and V-shaped vials, we tried to make the process more cost-efficient. Because all materials are disposable to allow GMP-compliant radiolabeling, costs can be reduced using cheaper alternatives. The most expensive disposables were the V-shaped vials, and therefore these were replaced by syringes. The most suitable syringes for smaller and larger volumes were, respectively, 3 and 5 mL BD Plastipak syringes with a Luer lock tip (supplemental materials).

Yield Optimization

After optimizing the reagent transfer, we focused on optimal mixing of the solvents to improve and stabilize the yields. Initially, the reagents were transferred and left without shaking or stirring during radiolabeling, resulting in variable radiochemical yields. Use of an external shaking device resulted in stable radiochemical yields of more than 90% according to iTLC. However, a shaking device was required in addition to the synthesis unit, and this method was therefore not preferred. We then investigated whether aspirating and squirting back the reaction mixture into the reaction vial would result in proper mixing. This approach also resulted in stable high radiochemical yields of more than 90% according to iTLC and therefore became the method of choice.

Product Purification

Product purification appeared to be the greatest challenge because it is based on SEC with a PD-10. Initially, a copy of the manual elution setup was examined and tested in the automated system (8,19). This process consists of 3 steps: applying the mixture to the column (~2 mL); eluting a flow-through of 1 mL of a 5 mg/mL solution of gentisic acid, pH 5.0; and eluting product with a 5 mg/mL solution of gentisic acid, pH 5.0 (2.0 mL for cetuximab and 1.5 mL for rituximab). To automate this process, we tried mounting the PD-10 column on the cartridge and switching the 3-way valve to direct the elution to different vials. This approach, however, was unsuccessful since switching of the valves either blocked the column or increased the resistance in the tubing, hampering product elution. Alternatively, we investigated HiTrap desalting columns because these can handle pressure, making them more suitable for automated procedures, but we found their separation performance to be too low. Therefore, the PD-10 column was further investigated. The critical change for successful purification was collection of all fractions in a single product vial, without switching valves. The product vial thus contains 4.5–5 mL of purified and diluted ^{89}Zr-mAb: 2.0 mL from the reaction mixture, 1.0 mL of flow-through, and 1.5–2.0 mL of the product fraction. This purification differs from the manual synthesis as described by Vosjan et al. and Cohen et al., in which only the mAb-containing fractions are collected as product and the first 2 mL of eluent is discarded to avoid product dilution (8,19). Nevertheless, product purity is guaranteed since all small-molecule reagents were eluted from the PD-10 column after the product. The obtained purified ^{89}Zr-mAb can be formulated and sterile-filtered afterward, which is, to date, still a manual procedure. Despite the nearly quantitative production yields indicated by iTLC, the actual yields of isolated product were lower because only the radiochemically pure product fractions were collected, corresponding to the in-house GMP production protocols for manual ^{89}Zr-mAb labeling. By this means, the purity of the radioimmunoconjugates is ensured and GMP quality control requirements can be met. For successful automation of the PD-10 elution, it appeared to be crucial, after every addition of solvent, to ventilate the 3-way valve on which the PD-10 was mounted.

Higher-Quantity Productions

Important in the automated production are the quantities of ^{89}Zr-mAbs obtained after purification, which should be sufficient

for clinical application. For 1 scan, about 37 MBq of [89]Zr-mAb are injected into the patient; thus, for the clinical production 60 MBq of [89]Zr should be sufficient for a single patient batch. In summary, for the optimal automated [89]Zr labeling described here, the use of 60 MBq of [89]Zr was sufficient to reliably obtain the desired quantities of radiolabeled product.

CONCLUSION

We have demonstrated that the automated production of [89]Zr-mAbs is feasible on a Scintomics GRP 2V synthesis module. For both rituximab and cetuximab, this automated [89]Zr labeling was reliable and efficient and allowed the production of clinically relevant quantities of [89]Zr-mAbs. The entire procedure takes 77 min starting from reagent mixing to collection of the product after PD-10 purification. Furthermore, the quality of [89]Zr-DFO-N-suc-cetuximab and [89]Zr-DFO-N-suc-rituximab met the preset requirements for GMP production, allowing the clinical production of [89]Zr-mAbs. The herein described automated [89]Zr labeling of the TFP-N-suc-Df-Fe modified mAbs is also suitable for [89]Zr labeling of Df-Bz-NCS–modified mAbs, since the same radiolabeling procedure is used. Finally, with the now-available automated purification of the products with a PD-10 column, mAb radiolabeling with other diagnostic or therapeutic radiometals can also be developed.

DISCLOSURE

Hans-Jurgen Wester is a shareholder, and Saskia Kropf the CEO and a shareholder, of Scintomics GmbH. Maria Vosjan is employed by BV Cyclotron VU, responsible for [89]Zr production and distribution. No other potential conflict of interest relevant to this article was reported.

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