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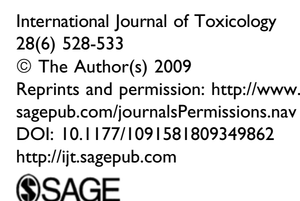

# 2,2-bis(4-Chlorophenyl)Acetic Acid (DDA), a Water-Soluble Urine Biomarker of DDT Metabolism in Humans

**Zhenshan Chen,[1] Francois Maartens,[2] Helen Vega,[1] Simon Kunene,[3] Jonathan Gumede,[4] and Robert I. Krieger[1]**

## Abstract

DDT metabolism in humans yields DDA as the principal urinary metabolite and potential exposure biomarker. A method for DDA analysis in human urine was developed using pentafluorobenzyl bromide and diisopropylethyl amine. Dried hexane extracts were reacted for 1 hour at room temperature. The stable DDA-pentafluorobenzyl-ester derivative was analyzed by gas chromatography–electron capture detector (GC-ECD) and confirmed by gas chromatography–mass spectrometry (GC-MS) in selective ion monitoring mode. The limit of detection for DDA was 0.1 μg/L urine by GC-ECD and 2 μg/L urine by GC-MS, with a relative standard deviation of 12%. Urine specimens from DDT applicators in Swaziland and South Africa were analyzed to evaluate the method. The mean DDA levels during the spray season and post season were 59 and 11 μg/L, respectively. These results must be interpreted cautiously because different groups of workers provided urine specimens in each case. The DDA urinalysis may be a feasible monitoring strategy for low-level occupational and residential DDT exposure assessment in antimalaria campaigns.

## Keywords

DDA, DDT, malaria, pentafluorobenzyl bromide, urine biomarker, human biomonitoring

When humans absorb DDT ([1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane]), some is rapidly transformed and excreted in urine as a water-soluble metabolite DDA (2,2-bis[4-chlorophenyl]acetic acid). In 1945, before the extensive use of DDT, persistence and environmental dispersal of DDT and its residues were documented; DDA was isolated from urine following oral administration of DDT to rabbits.[1,2] DDA was later characterized as a water-soluble DDT detoxification product in humans.[3] DDT and DDD (1,1-dichloro-2,2-bis[4-chlorophenyl]ethane], DDT's reductive dechlorinated derivative, are both insecticidal and potential sources of DDA (Figure 1). At about the same time, Telford and Guthrie[4] reported that administration of high levels of DDT to rats and goats produced milk lethal to animals that consumed it. The persistence of DDT and its lipophilic derivatives DDE (1,1-bis[4-chlorophenyl]-2,2-dichloroethene) and DDD as contaminants of the food supply and their occurrence in human adipose[5] and biota became centrally important public health and regulatory concerns.

DDA in urine correlated reasonably well with DDT storage in body fat.[6] Under medical supervision, pesticide applicators,[6] formulating plant workers,[7] and volunteers given daily dosages of 0, 3.5, and 35 mg per person for up to 25 months[8,9] excreted DDA in urine consistent with estimated levels of exposure. A survey of the status of the public's DDT exposures included DDA measured using the ion exchange methods of Cueto et al[10] (limit of quantification about 0.02 ppm DDA). Persons who ate an average diet and lacked known occupational exposure had urine levels that ranged from 0.02 or less to 0.35 ppm. Seventy-four percent of the 79 samples analyzed were below the limit of quantification.[6] Ortelee[7] estimated that background levels of DDA in the general population were 0.08 ppm at about the same time. These studies demonstrated the widespread occurrence of low levels of DDA in humans exposed to DDT in the general population.

Roan et al[11] studied the temporal relationship between DDT exposure and DDA excretion in volunteers receiving technical DDT, DDD, DDE, and DDA. These investigators clearly established that current DDT (or DDD) exposure, but not DDE, was linked with DDA excretion. This characteristic of DDT metabolism makes DDA excretion an especially valuable tool for

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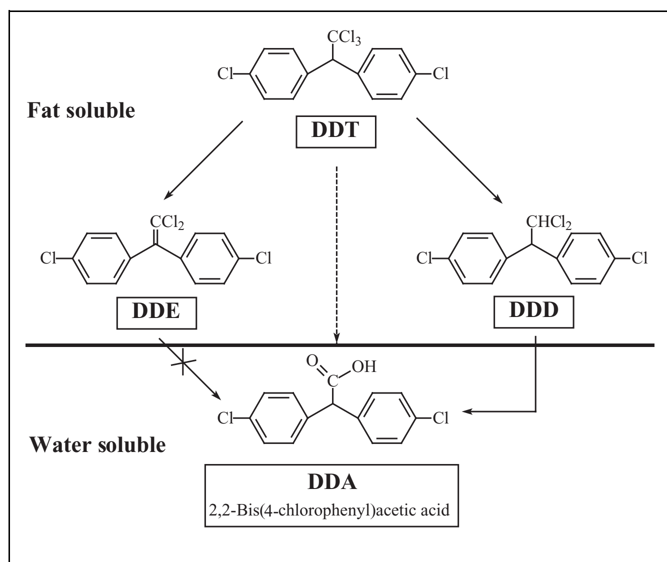


**Figure 1.** Sources of DDA formation after DDT exposure.

monitoring current DDT exposure and bioavailability. Rhesus monkeys (n = 3) were fed 100 ppm DDT diets for 224 days.[12,13] At the end of that period, blood levels ranged from 470 to 850 ppb DDT. During the same period, urinary DDA averaged 500 to 1000 µg/d. At the end of the feeding period, DDA excretion ranged from 800 to 1400 µg/d. Within 35 days, urine excretion of DDA dropped to 50 to 150 µg/d. Roan et al[11] observed that DDA returned to predose levels within 2 to 3 days of feeding DDA but returned to predose levels following DDT or DDD over 4 months following termination of DDT or DDD administration to individual volunteers. Because DDA derives only from DDT (or DDD) to the exclusion of DDE, urine biomonitoring is a potentially powerful means to assess ongoing DDT exposure.

Early methods for measuring DDA in biological samples have varied. The Schechter and Haller[14] colorimetric tests were used extensively in early DDT research that included DDA. An ion exchange procedure[10] and gas liquid chromatography with microcoulometric[11] or electron capture detection following formation of methyl[15] or chloroethyl[16] esters are also available analytical procedures. Early colorimetric and ion exchange methods lack the sensitivity and specificity of later gas chromatographic methods. An enzyme-linked immunosorbent assay method of limited application has also been published.[17]

Here we report a new method for the derivatization of low levels of DDA in human urine for use in DDT exposure assessment. After mild acid hydrolysis, DDA is derivatized with pentafluorobenzyl bromide and diisopropylethyl amine for gas chromatography–mass spectrometry (GC-MS) analysis. Additionally the initial human study of Neal et al[3] has been replicated. A sensitive new procedure has been developed and validated using authentic urine specimens from backpack DDT applicators in Swaziland and South Africa. The derivatization procedure is sensitive and specific and can be easily performed with readily available reagents and under simple laboratory conditions. DDA analysis provides a rapid means to assess DDT exposure and bioavailability. These analytical procedures can also be applied to forensic and ecologic applications where DDT exposure occurs.

## Materials and Methods

### *Human Subjects Research*

A human subject study protocol was reviewed and approved by the Institutional Review Board, University of California, Riverside, for the conduct of this research. Public health officials in Swaziland and South Africa obtained local permissions for subject participation and assurance of participant anonymity.

### *Chemicals*

Chemicals used in this study were 4,4-DDA, 98.0% (Sigma-Aldrich, St Louis, MO); 4,4-DDT, 98.6% (Supelco, Bellefonte, PA); 4,4-DDE, 99.2% (Supelco); 4,4-DDD, 97.9% (Supelco); pentafluorobenzyl bromide (PFBBr), 99% (Sigma-Aldrich); diisopropylethyl amine (DIPEA), 99% (Sigma-Aldrich); hexane, 99.9% (Fisher Scientific); ethyl acetate, 99.9% (Fisher Scientific, Hampton, NH); acetone, 99.9% (Fisher Scientific); and DDT technical used in Africa, DDT 75% WP (Avima, South Africa).

### *Pilot Oral DDT Metabolism Study*

The 1946 human oral DDT study by Neal et al[3] was repeated in a single adult male (170 kg) at 2 mg DDT/kg. After 6 preadministration complete 24-hour urine collections, DDT was ingested with a morning meal of fried potatoes and whole milk. Urine collection continued for 2 weeks. Specimens were stored frozen and were analyzed for DDT and DDA as described below. DDA (µg/d) results are presented in Figure 2.

### *Sample Collection and Handling*

Acid-washed 250-mL Nalgene bottles were used to collect urine specimens from applicators during DDT spray season and post season in Swaziland and South Africa. The maximum urine volume was about 230 mL to avoid overfilling for freezer storage. Urine samples were stored in coolers with blue ice for FedEx transport to the United States. The samples were ice-cold when received and were in good condition. Samples were weighed and their condition recorded. Sample weight was made up to 200 g with deionized water if the original weight was less than 200 g.

Each urine specimen was divided into eight 25-mL subsamples, and a 5-mL aliquot was taken for creatinine measurement. One subsample was analyzed for DDA on the day the urine specimens were received and processed. All the other subsamples were stored frozen until further analysis.

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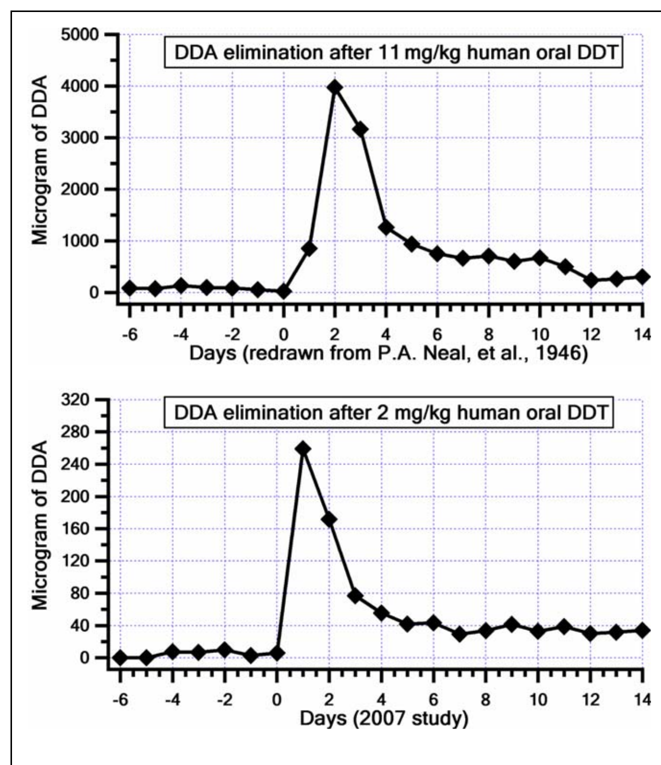


**Figure 2.** DDA excretion in urine following oral administration of DDT in original 1946 study and in present confirmatory research.

## DDT/DDD/DDE and DDA Extraction

*DDT/DDD/DDE.* The pH of an 8-mL aliquot of urine was adjusted to greater than pH 10 by addition of 5 N KOH. Sodium chloride was added to help minimize emulsification. The aqueous phase was extracted 3 times with 8 mL of hexane and the organic extract dried over anhydrous sodium sulfate. The organic extract was concentrated under nitrogen to less than 5 mL and transferred to an 8-mL vial. The organic extract was evaporated to dryness under nitrogen and redissolved in 0.4 mL of hexane for GC analysis.

*DDA.* The pH of the resulting aqueous phase from above was adjusted to less than pH 2 using 6 N HCl. The aqueous phase was extracted 3 times with 8 mL hexane and the organic extract dried over anhydrous sodium sulfate. The organic extract was concentrated under nitrogen to less than 5 mL and transferred to an 8-mL vial. The organic extract was evaporated to dryness under nitrogen and prepared for DDA derivatization.

## DDA Derivatization

Because of the thermal instability and the low volatility of organic acids, compounds like DDA must be derivatized for GC analysis.[18] In our method, 400 μL of PFBBr (2% in hexane vol/vol) and 200 μL of DIPEA (2% in hexane vol/vol) were added to the dried hexane extracts of acidified urine. After 1 hour at room temperature, the reactants were evaporated to dryness under nitrogen and redissolved in 0.4 mL of ethyl acetate for GC analysis.

## GC-ECD Analysis of DDT/DDD/DDE and DDA–Pentafluorobenzyl (PFB) Ester

Gas chromatography–electron capture detector (GC-ECD) analysis was done using an HP 5890 (Hewlett-Packard, Palo Alto, CA) gas chromatograph and a split/splitless injector operating in the splitless mode. The operating temperature of the injector was 250°C. Chromatographic separation was performed on an HP-5ms capillary column (30 m × 0.32 mm inner diameter × 0.25 μm film; Agilent Technologies, Santa Clara, CA). Helium was used as carrier gas and nitrogen as the makeup gas. The initial column temperature of 50°C was raised at a rate of 30°C/min to 180°C and then increased at a rate of 5°C/min to 220°C. Finally the temperature was raised by a rate of 1°C/min to 250°C. The detector temperature was 280°C. The injection volume was 1 μL.

## GC-MS Analysis for DDA-PFB Ester

GC-MS analysis was done using an HP 6890 (Hewlett-Packard) gas chromatograph with an HP MSD 5973 (Hewlett-Packard) mass spectrometer in electron impact ionization mode. Electron impact ionization mass spectra were obtained at an ionization energy of 70 eV. The MS transfer line temperature was maintained at 280°C. Injector temperature was 250°C. Injection (1 μL) was done in the pulsed splitless mode at a pressure of 45 psi. The pulse time was 1.5 minutes. Helium was used as carrier gas with constant flow of 1.0 mL/min. Chromatographic separation was performed on an HP-5ms capillary column (30 m × 0.25 mm inner diameter × 0.25 μm film; Agilent Technologies). The initial column temperature of 50°C was increased at 15°C/min to 300°C and held constant for 10 minutes. For quantification of DDA-PFB ester, the GC-MS was operated in a selective ion monitoring (SIM) mode. The characteristic ion m/z 460 $[M]^+$ was used as quantitative ion; m/z 235 and 237 were used as qualitative ions.

# Results

## Method Development

The DDA derivatization scheme is shown in Figure 3. This differs from a previously published procedure in that we used DIPEA instead of triethylamine as the catalyst and used a 1-hour reaction period at room temperature instead of 110°C for 1 hour.[19] Under the conditions used here, recovery studies were done by spiking 10, 50, and 100 ppb DDA in 8-mL control human urine specimens. The mean recovery ranged from 76% to 84% with a relative standard deviation (RSD) of 11% to 13% (Table 1).

The limit of detection for DDA was in the area of 0.1 μg/L urine by GC-ECD and 2 μg/L urine by GC-MS. For reproducibility of the GC-MS method, 5 aliquots from the same urine

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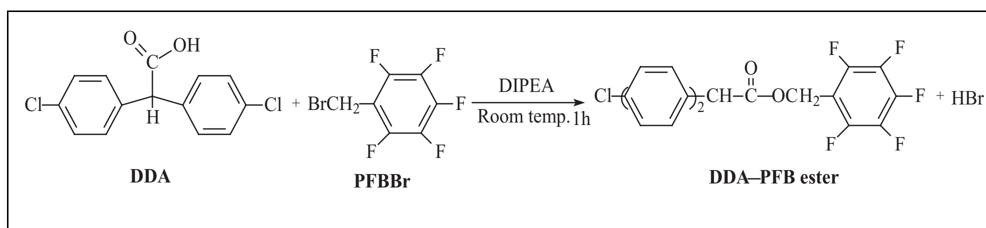


**Figure 3.** Reaction formula of DDA derivatization.

**Table 1.** Results of Recovery Experiments in Human Urine

| Spike Level, μg/L | Recovery, %, Mean ± SD | Relative Standard Deviation, % (n = 4) |
|---|---|---|
| 10 | 81.8 ± 9.3 | 11.5 |
| 50 | 83.8 ± 10.5 | 12.6 |
| 100 | 76.4 ± 8.5 | 11.2 |

sample were analyzed and yielded an RSD of 12%. The GC-MS procedure was adopted for routine analysis because it gave greater specificity and was not compromised by trace impurities.

### DDT Oral Repeat Study

In 1945, DDA was identified as the most important urine metabolite of DDT.[1,2] After developing the DDA PFBBr derivatization procedure, we confirmed the 1946 human oral study by Neal et al.[3] In the previous case, 11 mg/kg DDT was administered to a volunteer, and urine was collected and analyzed using the relatively nonspecific Schechter-Haller method for DDA.[14] Despite the extensive use of DDT and literally ubiquitous human exposure to measurable residues of DDT and its degradates, very limited contemporary studies of human DDT metabolism using specific determination of DDA have been published.[3,11] The volunteer in our present study was administered 2 mg of DDT per kilogram of body weight. The results are shown in Figure 2. Peak excretions appeared after 1 or 2 days.

Neal et al[3] used the colorimetric procedures for analysis of urine and demonstrated maximum excretion (4 mg of DDA) on day 2 following administration of DDT. Approximately 2% (mol%) of the dose was recovered as DDA. In the present study at a lower dosage, 0.4% of the 340 mg DDT dose was recovered as DDA and peak excretion occurred during the first 24 hours following administration. The specificity of the analysis probably contributed to the sharper excretion profile. Prolonged, low-level DDA excretion was measured during the 14-day postadministration period. No other analytes were detected in urine. In both cases, DDA excretion appeared within 24 hours of exposure, consistent with the reports of Roan et al,[11] who studied persons with continuing chlorinated hydrocarbon exposure. Rapid reduction of DDA excretion following termination of DDT exposure is a feature of DDT metabolism that is extremely important support for the use of DDA excretion in occupational and residential biomonitoring.

### DDT Applicator Urine Surveillance Study

The urine levels of DDA and DDT in occupationally exposed persons are summarized in Table 2. Four separate sets of urine specimens were collected. The set referred to as Swaziland-1 was collected from less experienced DDT applicators and Swaziland-2 from applicators with multiple years of experience. Both sets of urine specimens were obtained during the spray season. The mean DDA excretion of less experienced applicators was significantly higher than that of more experienced applicators ($P < .05$). This observation warrants further study and may contribute to the usefulness of biomonitoring during applicator training.

An additional set of urine specimens provided by active DDT applicators was designated KwaZulu-Natal-3 in Table 2. The mean DDA excretion of applicators during the spray season (collections 1, 2, and 3) and the mean excretion of a separate group post season (KwaZulu-Natal-4) were 59.1 μg/L (range, 3.6-407 μg/L; median 30 μg/L) and 10.6 μg/L (range, 0.5-44 μg/L; median 4.7 μg/L), respectively. A 2-sample $t$ test was applied, and the seasonal difference in DDA excretion was statistically significant ($P < .05$). The urine DDT excretion in these urine specimens was relatively stable at a very low level (range, 0.24-2.78 μg/L; median 0.52 μg/L). The urine levels must be compared very cautiously because they represent different groups of applicators in each case.

The DDA:DDT mole ratio can be used to represent the relationship between the water-soluble product and its lipophilic precursor. Mole ratios were calculated for each applicator and group means are listed in Table 2. When DDT metabolism occurs (Figure 1), the portion that results in DDA represents a product that is rapidly eliminated and is a putative biomarker of DDT exposure. During the spray season when DDT exposures occurred, the corresponding weighted average (n = 37; Table 2) DDA:DDT ratio was 143:1. About 1 month after the spray season (in other workers), the ratio was reduced to 31:1. The lower level of DDA excretion represents lower DDT availability after the spray period.

## Discussion

DDT metabolism in humans forms DDA, a stable, water-soluble metabolite that is a useful urine biomarker of active

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**Table 2.** Summary of DDA and DDT Urine Excretion in African Applicators

| Collection | No. of Specimens | DDA, μg/L, mean ± SD | DDT, μg/L, mean ± SD | DDA:DDT mole ratio |
|---|---|---|---|---|
| Swaziland-1[a] | 8 | 28 ± 9.1 | 1.3 ± 0.66 | 33:1 |
| Swaziland-2[a] | 9 | 14 ± 6.4 | 0.99 ± 0.30 | 19:1 |
| KwaZulu-Natal-3[b] | 20 | 92 ± 99 | 0.56 ± 0.48 | 243:1 |
| KwaZulu-Natal-4[b] | 19 | 11 ± 12 | 0.41 ± 0.15 | 31:1 |

[a] Swaziland-1 (less experienced applicators) and Swaziland-2 (more experienced applicators) specimens were obtained from the same time and area during the spray season.
[b] KwaZulu-Natal-3 specimens were obtained during the spray season and KwaZulu-Natal-4 specimens were obtained 1 month post season from different applicators.

DDT exposure. The characteristics of DDA as a biomarker seem to make it ideal for DDT exposure monitoring and surveillance. The determination of DDA in human urine has been demonstrated under laboratory conditions. The field tests of the procedures have provided useful evidence of their application in low-level DDT exposure scenarios as encountered among applicators in antimalaria programs. The procedures may be useful in assessing contemporary exposures as well.

The influence of various reaction temperatures such as –20°C, 4°C, room temperature, and 60°C on the DDA-PFB ester recovery was investigated. There was no significant difference among different temperatures ($P > .05$). This is an advantage of the method in that the derivatization can be done at room temperature without using special heating or cooling facilities. The procedures may be adopted for routine DDA analysis with gas–liquid chromatography in academic, public health, and commercial laboratories.

After a 1-hour derivatizing reaction, the DDA-PFB ester may still be formed during frozen storage because trace PFBBr and DIPEA residues are left in the final solution. We recommend that formation of DDA-PFB ester from both DDA standard and sample be done at the same time so that fresh DDA-PFB ester standard can be applied in sample analysis.

Using authentic urine specimens of DDT applicators, we found DDA to be present at higher levels during the spray season than the levels measured 1 month after spraying had concluded. These results must be interpreted cautiously because different groups of workers provided urine specimens in each case. The routine work practice includes 8 hours per day, 5 days per week for about half a year, therefore representing subchronic occupational exposure. From the greater than 2-order-of-magnitude person-to-person difference in daily DDA excretion, a large worker-to-worker exposure variability is inferred. When detailed work practice information becomes available, it is likely that exposure reduction measures can be developed. Existing data represent low exposures relative to no-adverse-effect levels determined in the United States during active DDT use in the 1960s.[6] The excretion of DDA in urine after active DDT exposure is therefore a promising biomarker to detect present DDT exposure in scenarios where DDT is used.

These pilot studies show the feasibility of sensitive DDA analysis of urinary DDA from people exposed to low-level DDT. The results may be useful for training and guidance for public health officials concerned about the extent and duration of DDT exposure in people under occupational exposure and in the general public. DDA analysis can be an important adjunct to future DDT exposure assessment studies.[20]

Indoor residual spraying is a primary intervention for malaria control in the World Health Organization (WHO) Global Malaria Program.[20] The Stockholm convention on persistent organic pollutants has given an exemption for the production and public health use of DDT for indoor applications to vector-borne diseases, mainly because of the absence of equally effective and efficient alternatives. It is expected that there will be a continued role for DDT in malaria control until equally cost-efficient alternatives are developed.[20] The precise temporal relationship of the DDA:DDT mole ratio will be evaluated as a rapid and readily available indicator of DDT availability and DDA metabolism in workers and residents in future studies.

A joint Food and Agriculture Organization/WHO Meeting on Pesticide Residues[21] undertook a reevaluation of DDT and its primary metabolites. The evaluation included storage of DDT and its lipophilic metabolites in human body fat, the presence of those residues in human milk and potential carcinogenicity, and biochemical and toxicological information, including hormone-modulating effects. The role of DDA as a hydrophilic excretion product for the purpose of biomonitoring DDT exposure was not included in this review.[21] Likewise, the current Concise International Chemical Assessment document undergoing peer review at this time[20] does not include the possibility that DDT exposure may be monitored using urinary DDA excretion.

The WHO program calls for DDT use to be closely monitored.[20] To avoid undue exposure of householders and spray operators, standard operating procedures must be in place and strictly followed. The methods and techniques for monitoring operator exposure outlined here provide a tool for monitoring DDT exposure that is relatively simple, sensitive, and proven to measure DDT exposure under conditions of use. The results reported here document the sensitivity and specificity of DDA analysis in authentic urine specimens of applicators.

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## Declaration of Conflicting Interests

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