Arsenic trioxide inhibits viability and induces apoptosis through reactivating the Wnt inhibitor secreted frizzled related protein-1 in prostate cancer cells

Lei Zheng¹,²
Hui Jiang³
Zhi-Wei Zhang¹
Ke-Nan Wang¹
Qi-Fei Wang¹
Quan-Lin Li¹
Tao Jiang¹

¹Department of Urology, First Affiliated Hospital of Dalian Medical University, ²Department of Urology, The Fifth People’s Hospital of Dalian, ³Department of Urology, Third Affiliated Hospital of Beijing University, Beijing, People’s Republic of China

Background: Growing evidence suggests that arsenic trioxide (As₂O₃) induces apoptosis and inhibits tumor cell growth in prostate cancer (PCa), although details of the mechanism are still inconclusive. We investigated the antitumor effect of As₂O₃ in human PCa cell lines LNCaP and PC3 and the underlying mechanisms by focusing on the Wnt signaling pathway.

Methods: The effect of As₂O₃ on the viability and apoptosis of PCa cells was investigated by cholecystokinin-8 and flow cytometry. The expression of the related proteins in the Wnt signaling pathway and the downstream target genes of the Wnt signaling pathway was examined by Western blot and quantitative real-time PCR assay. The methylation status of the SFRP1 gene promoter was assessed by bisulfite sequencing.

Results: As₂O₃ inhibited the viability of PCa cells and induced apoptosis of PCa cells in a dose-dependent manner. The protein level of phospho-glycogen synthase kinase-3β was upregulated, whereas the protein level of β-catenin and the mRNA levels of c-MYC, MMP-7, and COX-2 were downregulated in a dose-dependent manner in PCa cells treated with As₂O₃. In addition, As₂O₃ upregulated the protein and mRNA levels of secreted frizzled related protein-1, and increased the demethylation of the SFRP1 gene promoter.

Conclusion: Our results suggest that As₂O₃ may inhibit cell viability and induce apoptosis through reactivating the Wnt inhibitor secreted frizzled related protein-1 in both androgen-dependent and -independent human PCa.

Keywords: arsenic trioxide, CpG island methylation, demethylation, prostate cancer, Wnt signaling pathway, SFRP1

Introduction

Prostate cancer (PCa), the most common malignant tumor of the urinary system, has become the second leading cause of cancer-related death in men in Western developed countries.¹ The incidence of PCa is gradually increasing due to increased awareness and screening. Clinically, one in six men are diagnosed with PCa over the age of 60.² Multiple risk factors may induce the occurrence of PCa, including androgens, dietary factors, ethnicity, family history, and old age.³ Although details of the pathogenesis of PCa are still inconclusive, aberrant activation of the Wnt signaling pathway may be involved. Currently, endocrine therapy, radical surgical resection, radiotherapy, and chemotherapy are used as treatments for PCa.⁴ However, after treatment with endocrine therapy for an average of 18 months, the disease is likely to be castration-resistant PCa with a poor prognosis.⁵ Radical surgical resection causes great surgical trauma...
and affects the sexual function of patients. Radiotherapy is accompanied by serious complications, such as acute or chronic gastrointestinal reaction, urinary incontinence, and erectile dysfunction. Therefore, it is important to identify new chemotherapeutic agents with lower toxicity and stronger antitumor effects and investigate their mechanisms of antitumor activity.

The Wnt signaling pathway is an evolutionarily conserved signaling cascade and plays important roles in multiple biological processes, including cell proliferation, differentiation, migration, apoptosis, and tumor development. Wnt proteins are secreted cysteine-rich proteins that serve as ligands for the Wnt signaling pathway. β-Catenin is the most important component of the Wnt signaling pathway, and its altered localization is a marker of pathway activation. Once the Wnt ligand binds to a specific member of the frizzled (FZD) family of receptors, a stable receptor complex consisting of Wnt protein, coreceptor lipoprotein receptor-related protein (LRP5/6), and FZD is formed. The Wnt–LRP–FZD complex increases the stability of β-catenin through inhibiting the β-catenin destruction complex (casein kinase 1α, AXIN, adenomatous polyposis coli, glycogen synthase kinase-3β [GSK-3β]) that targets β-catenin for degradation, resulting in the accumulation of β-catenin in cytoplasm and its translocation to the nucleus. In the nucleus, β-catenin interacts with T-cell factor/lymphoid enhancer factor transcription factor and then stimulates the expression of downstream target genes, including several oncogenes, such as Cyclin D1, JUN, and c-MYC. Deregulation of the Wnt signaling pathway is closely associated with many human diseases including cancer.

Secreted frizzled related protein-1 (SFRP1) is a well-known antagonist of the Wnt signaling pathway and belongs to the secreted glycoprotein SFRP family. SFRP1 can bind to Wnt proteins via its cysteine-rich domain that is homologous to the FZD receptors, and inhibits the interaction between the Wnt ligand and FZD receptor competitively, leading to the inhibition of signal transduction. Silencing of SFRP1 gene expression by aberrant cytosine-phosphate-guanine (CpG) methylation has been reported in many malignant tumors, including PCa, suggesting the possibility of its tumor-specific inactivation. SFRP1 is a candidate mediator of stromal-to-epithelial signaling in PCa. Moreover, SFRP1 inhibits the transcriptional activity of androgen receptor and the proliferation of androgen-dependent LNCaP cells. However, the effect of SFRP1 gene methylation in PCa cells is still inconclusive.

Arsenic trioxide (As$_2$O$_3$) is a well-studied chemotherapeutic agent that has been widely used to treat acute promyelocytic leukemia and multiple myeloma with good results. Preclinical studies have demonstrated that As$_2$O$_3$ can induce apoptosis and inhibit tumor cell growth in a wide range of malignant solid cancers including PCa. As$_2$O$_3$ may exert its antitumor function in PCa through p38 and/or Akt/mTOR signaling pathways. As$_2$O$_3$ also decreases the methylation level of CDKN2B/CDKN2A genes in human hematologic malignant cells. Aberrant activation of the Wnt signaling pathway is closely associated with the pathogenesis of PCa. However, the relationship between the Wnt signaling pathway and As$_2$O$_3$ has not been clarified. In this study, we examined whether the Wnt signaling pathway is involved in the mechanisms by which As$_2$O$_3$ inhibits the growth of PCa cells. In this study, androgen-dependent human PCa cell line LNCaP and androgen-independent human PCa cell line PC3 were used to investigate the anticancer effect of As$_2$O$_3$. We demonstrated that As$_2$O$_3$ inhibited viability and induced apoptosis in LNCaP and PC3 cells. In addition, As$_2$O$_3$ suppressed the Wnt signaling pathway and downregulated the expression of its target genes. We also showed that As$_2$O$_3$ increased the protein and mRNA levels of SFRP1, and induced the demethylation of the SFRP1 gene promoter. Our data suggest that As$_2$O$_3$ may inhibit the viability of PCa cells through the Wnt signaling pathway in addition to the p38 and Akt/mTOR signaling pathways.

**Materials and methods**

**Cell culture and reagents**

This study was approved by the Ethics Committee of Dalian Medical University, and conformed with the provisions of the Declaration of Helsinki. Human PCa cell lines LNCaP and PC3 were obtained from Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, People’s Republic of China). LNCaP cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 µg mL$^{-1}$ penicillin, and 100 µg mL$^{-1}$ streptomycin. PC3 cells were cultured in fresh Ham’s F12 medium (HyClone, Logan, UT, USA) plus 10% fetal bovine serum (Gibco), 100 µg mL$^{-1}$ penicillin, and 100 µg mL$^{-1}$ streptomycin. All cells were incubated at 37°C in a humidified incubator with 5% CO$_2$.

Injectable As$_2$O$_3$ (SL Pharmaceutical Co., Ltd, Beijing, People’s Republic of China) was dissolved in 0.9% sodium chloride injection solution to prepare a 1.0 mmol L$^{-1}$ stock solution, and stored at 4°C. The As$_2$O$_3$ solution was diluted in culture medium just before use. DAPI, sodium hydrogen sulfite, and hydroquinone were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Annexin V and propidium
iodine (PI) were purchased from KeyGen Biotech (Nanjing, People’s Republic of China). Rabbit monoclonal anti-SFRP1 (ab126613) and mouse monoclonal anti-β-catenin (ab22656) were obtained from Abcam (Cambridge, UK). Mouse monoclonal anti-actin, goat antimouse IgG, and goat antirabbit IgG were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Protease inhibitor mixture was obtained from Roche Applied Science (Basel, Switzerland).

**Cell viability assay**

Cell viability was measured with cholecystokinin-8 (CCK8) kit (Dojindo, Kumamoto, Japan). Cells in a 96-well plate were treated with various concentrations of As$_2$O$_3$ (0, 1, 2.5, 5, 10, and 20 μmol L$^{-1}$). After incubation for 24 hours, CCK-8 stock solution (20 μL) was added to each well and incubated for 3 hours at 37°C. The absorbance of each well was measured by a microplate reader (Spectramax190, Molecular Devices LLC, Sunnyvale, CA, USA) at 450 nm. The inhibition rates of cell proliferation were calculated according to the following formula: inhibition rate (%) = (1 – (mean absorbance of experimental group – mean absorbance of blank group))/(mean absorbance of control group – mean absorbance of blank group)×100%. All experiments were repeated three times independently.

**Cell apoptosis assay**

Cell apoptosis was examined by flow cytometry. Cells were seeded into a six-well plate. When the cell density reached 8×10$^4$ mL$^{-1}$, the medium was replaced with new medium containing various concentrations of As$_2$O$_3$ (0, 2.5, 5, and 10 μmol L$^{-1}$) for 24 hours. Cells were harvested and fixed in 70% ethanol at −20°C overnight. The ethanol was removed and the samples were stained with Annexin V binding buffer (500 μL), Annexin V–FITC (5 μL), and PI (5 μL), and were kept in the dark at room temperature for 10 minutes. Cells in early apoptosis were assayed by using a flow cytometer (FACS Calibur, BD Bioscience, Franklin Lakes, NJ, USA). All experiments were repeated three times independently.

**Western blot assay**

Proteins from cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%–12%). After electrophoresis, the separated protein bands in the gel were transferred to a polyvinylidene fluoride membrane (Merck Millipore, Billerica, MA, USA). The PVDF membrane was blocked in 5% nonfat milk at room temperature for 1 hour, and then incubated with the specific primary antibodies, followed by the appropriate secondary antibodies. The results were analyzed by chemiluminescence detection. Immunoblot data were quantified by scanning the appropriate bands of interest and the relative density was plotted in gray scale. All experiments were repeated three times independently.

**RNA extract and quantitative real-time PCR assay**

Total RNA from cells was extracted by using RNAiso Plus reagent (Takara Biotechnology [Dalian] Co., Ltd, Dalian, People’s Republic of China). Reverse transcription was carried out by using a reverse transcription kit (Takara). Real-time polymerase chain reaction (PCR) was conducted with a real-time PCR System (LightCycler, Roche Diagnostics, Basel, Switzerland) by using the following primers: 5′-CCCGAGATGCTTAAGTGTGACAA-3′ (sense) and 5′-ACTCGTGCCACAGAGATGTTC-3′ (antisense) for SFRP1; 5′-CACCACCAACGAGCACTCT-3′ (sense) and 5′-CAGACTCTGACTTTTGCCAGG-3′ (antisense) for c-MYC; 5′-GTATGGGACATCTCCTCTGATCC-3′ (antisense) and 5′-CCAATGAAATGATGATGAGT-3′ (sense) for MMP-7; 5′-AGCTATCTGTTAACCAGATGGTGA-3′ (sense) and 5′-TGTTCTATAGGAACACTATGTT-3′ (antisense) for COX-2; and 5′-CATTGGCCAGAAGATGAC-3′ (sense) and 5′-CATCTGCTGAAGGTGACACG-3′ (antisense) for GAPDH. The samples were subjected to the following reaction: initial denaturation step of 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 55°C for 20 seconds, and a final step at 72°C for 90 seconds. The 2$^\Delta$ΔCt method was used for data analysis. The mRNA levels of SFRP1, c-MYC, MMP-7, and COX-2 were normalized to GAPDH, which was used as an endogenous control.

**Bisulfite sequencing PCR assay**

Total DNA from cells was extracted using a DNA purification kit (Tiangen Biotech, Beijing, People’s Republic of China), and then subjected to bisulfite conversion by using the CpGenome Fast DNA Modification Kit (Millipore). The bisulfite-treated DNA was amplified by a PCR system (GeneAmp 9600, Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences were as follows: CpG island 1 of SFRP1 promoter: 5′-GGTTACTTACTATGAGTTAGTATTGAGGT-3′ (sense) and 5′-AACCTAAATCATACTTAACACAAACTTCC-3′ (antisense); and CpG island 2 of SFRP1 promoter: 5′-TTGTTTTTTAAGGGTGTTGAGTGT-3′ (sense) and 5′-TACCACAAACTTCCAAAACCTTCC-3′ (antisense). The following reaction conditions were used: ten cycles of 95°C for 5 minutes, 95°C for 30 seconds, then 60°C–50°C for 45 seconds beginning at 60°C for the first
cycle, and then decreasing by 1°C for each subsequent cycle, and 72°C for 45 seconds; 33 cycles of 95°C for 30 seconds, 50°C for 45 seconds, and 72°C for 45 seconds; and finally 60°C for 30 minutes. The PCR products were inserted into the pMD-19T vector (Takara). After transformation, ten clones were selected and sequenced from each sample.

Statistical analysis
Data were analyzed by using ANOVA followed by multiple comparison Student–Neuman–Keuls tests and bi-variant relationship analyses. Data are presented as mean ± standard deviation. P<0.05 was considered statistically significant.

Results
As$_2$O$_3$ inhibits viability and induces apoptosis in PCa cells
As$_2$O$_3$ has a cytotoxic effect on a wide range of cancer cells derived from solid tumors and hematopoietic malignancies.19–23 To investigate the inhibitory effect of As$_2$O$_3$ on the viability of PCa cells, LNCaP and PC3 cells were incubated with increasing concentrations of As$_2$O$_3$ (0, 1, 2.5, 5, 10, and 20 μmol L$^{-1}$) for 24 hours. The inhibition rates were evaluated by CCK8 assay. As$_2$O$_3$ reduced the viability of LNCaP and PC3 cells in a dose-dependent manner (Figure 1).

We also assessed the effect of As$_2$O$_3$ on the apoptosis of PCa cells by a flow cytometry assay. LNCaP and PC3 cells were treated with As$_2$O$_3$ (0, 2.5, 5, and 10 μmol L$^{-1}$) for 24 hours, and then stained with Annexin V/PI. As$_2$O$_3$ increased the protein and mRNA expression of SFRP1 in a dose-dependent manner (Figure 2). These results demonstrated that As$_2$O$_3$ inhibited PCa cell viability and induced apoptosis in a dose-dependent manner.

As$_2$O$_3$ inhibits the Wnt signaling pathway in PCa cells
Deregulation of the Wnt signaling pathway plays important roles in the initiation and development of PCa. To examine whether the Wnt signaling pathway is involved in the treatment of PCa by As$_2$O$_3$, LNCaP and PC3 cells were treated with different concentrations of As$_2$O$_3$ (0, 2.5, 5, and 10 μmol L$^{-1}$) for 24 hours. Western blot assay showed that the expression of phospho-GSK-3β increased, whereas the expression of β-catenin decreased in LNCaP and PC3 cells treated with As$_2$O$_3$ in a dose-dependent manner, and the expression of GSK-3β was not affected by As$_2$O$_3$ (Figure 3A and B). Moreover, we examined the effect of As$_2$O$_3$ on the expression of downstream target genes on the Wnt signaling pathway. Real-time PCR assay showed that the mRNA level of e-MYC, MMP-7, and COX-2 decreased in LNCaP (Figure 3C) and PC3 (Figure 3D) cells treated with As$_2$O$_3$ in a dose-dependent manner, respectively. These results showed that As$_2$O$_3$ inhibited the Wnt signaling pathway and downregulated the expression of its target genes.

As$_2$O$_3$ increases the protein and mRNA levels of SFRP1 in PCa cells
SFRP1 is a well-studied competitive inhibitor of the FZD receptor, which binds Wnt ligands and inhibits Wnt signal transduction. To investigate whether SFRP1 is involved in the inhibition of As$_2$O$_3$ on the Wnt signaling pathway, we examined the expression of SFRP1 in LNCaP and PC3 cells treated with different concentrations of As$_2$O$_3$. Figure 4A and B shows that the protein and mRNA levels of SFRP1 in LNCaP cells were increased by As$_2$O$_3$ in a dose-dependent manner in the experimental group compared with the
control group. Similar results were obtained in PC3 cells (Figure 4C and D). These results showed that the expression of SFRP1 was upregulated substantially by As$_2$O$_3$, indicating the involvement of SFRP1 in the inhibition of the Wnt signaling pathway by As$_2$O$_3$.

As$_2$O$_3$ promotes the demethylation of the SFRP1 gene promoter

The methylation of the SFRP1 gene downregulates SFRP1 expression in a wide range of malignant tumors. To determine whether As$_2$O$_3$ downregulated the expression of SFRP1 through promoting demethylation of the SFRP1 gene, we examined the methylation level of the SFRP1 gene promoter in LNCaP and PC3 cells. Methylation usually occurs in CpG-rich promoter regions, known as CpG islands. Sequence analysis revealed that two CpG islands might be methylated in the SFRP1 gene promoter (Figure 5A, blue regions). A bisulfite sequencing PCR assay showed that the two CpG islands were methylated in LNCaP and PC3 cells. The methylation rates of CpG islands 1 and 2 in LNCaP cells not treated with As$_2$O$_3$ were 43.3% and 3.6%, respectively, whereas the methylation rates dropped to 12.5%...
and 1.3%, respectively, after As$_2$O$_3$ treatment (Figure 5B).

In PC3 cells, the methylation rates of CpG islands 1 and 2 decreased from 86.7% and 71.4% to 48.3% and 34.1%, respectively, after As$_2$O$_3$ treatment (Figure 5C). These results were consistent with the data from Western blot and RT-PCR assays (Figure 4A–D), indicating that the As$_2$O$_3$-induced upregulation of SFRP1 is probably caused by inducing demethylation of the SFRP1 gene.

Discussion

PCa is the most commonly diagnosed nonskin cancer in men in industrialized countries. Despite continuous biomedical research, PCa is still a major public health problem. Currently, common treatment strategies for PCa include endocrine therapy, radical surgical resection, radiotherapy, and chemotherapy. However, the side effects of these therapies are severe. Therefore, much research is focusing on identifying new chemotherapeutic agents with lower toxicity and stronger antitumor effects. In this study, we showed that As$_2$O$_3$ could inhibit cell viability and induce apoptosis in both androgen-dependent and -independent human PCa cells. As$_2$O$_3$ exerted its antitumor effect, at least partly, through downregulating the Wnt signaling pathway by promoting demethylation of the SFRP1 promoter.
As$_2$O$_3$ is a safe, effective treatment for patients with acute promyelocytic leukemia, and it has also shown promising results in patients with relapsed multiple myeloma.\textsuperscript{30} In addition, a wide range of malignancies, including hematologic cancer and solid tumors derived from several tissue types, are susceptible to As$_2$O$_3$ treatment.\textsuperscript{31} Here, we found that As$_2$O$_3$ inhibited the viability (Figure 1) and induced apoptosis (Figure 2) of LNCaP and PC3 cells in a dose-dependent manner. These results were consistent with previous studies,\textsuperscript{24,25,29} suggesting that As$_2$O$_3$ possessed excellent antitumor effects in both androgen-dependent and -independent PCa cells.

The mechanism of As$_2$O$_3$ in treating acute promyelocytic leukemia is mediated by targeting disease-specific PML–RAR fusion proteins.\textsuperscript{32,33} However, the detailed mechanism of As$_2$O$_3$ in treating PCa is still inconclusive. In our previous studies, As$_2$O$_3$ inhibited the growth of PC3 cells and induced apoptosis through the p38 signaling pathway.\textsuperscript{23,24} As$_2$O$_3$ combined with ionizing radiation increases reactive oxygen species generation, and induces autophagy and apoptosis of LNCaP and PC3 cells. As$_2$O$_3$ also can induce the cell death through inhibition of the Akt/mTOR signaling pathways in LNCaP and PC3 cells.\textsuperscript{25} Increasing evidence suggests that aberrant activation of the Wnt signaling pathway contributes...
to tumorigenesis by upregulation of downstream target genes, such as Livin, cyclin D1, and c-MYC. However, it has not been reported whether As\textsubscript{2}O\textsubscript{3} exerts its antitumor function through regulating the Wnt signaling pathway in PCa. In this study, we demonstrated that As\textsubscript{2}O\textsubscript{3} inhibits the Wnt signaling pathway with a corresponding reduction in the mRNA levels of its downstream target genes (Figure 3A–D), indicating that the inhibition of Wnt signaling by As\textsubscript{2}O\textsubscript{3} might be a molecular mechanism by which As\textsubscript{2}O\textsubscript{3} exerts its antitumor effects in PCa. These results support the use of As\textsubscript{2}O\textsubscript{3} as an anticancer drug for treating PCa.

SFRP1 is recognized as a tumor suppressor in several human cancers. Silencing of SFRP1 expression has been observed in a wide range of malignancies, which prevents SFRP1 from performing its physiological role of inhibiting the Wnt signaling pathway. In our previous study, the expression of SFRP1 decreased significantly with increasing PCa malignancy. Here, we found that As\textsubscript{2}O\textsubscript{3} could induce the re-expression of the SFRP1 gene, and upregulate the protein and mRNA levels of SFRP1 in a dose-dependent manner in PCa cells (Figure 4A–D), indicating that As\textsubscript{2}O\textsubscript{3} probably inhibits the Wnt signaling pathway through reactivating the Wnt inhibitor, SFRP1. In addition, SFRP1 is a negative regulator of androgen receptor activity in PCa. Therefore, the role of SFRP1 in prostate carcinogenesis may not be limited to inhibiting the Wnt signaling pathway. As\textsubscript{2}O\textsubscript{3} may also inhibit the transcriptional activity of androgen receptor via SFRP1.

DNA methylation is one major epigenetic modification that plays crucial roles in the control of gene activity and nuclear architecture. Aberrant hypermethylation of gene promoter regions induces the inactivation of tumor suppressor genes, leading to the occurrence of human malignancies. Methylation is the main mechanism of SFRP1 gene silencing, which has been observed in multiple malignant tumors including PCa. Moreover, SFRP1 is a good predictive and prognostic biomarker of PCa. Therefore, detecting the methylation status of the SFRP1 gene could provide a basis for monitoring and early diagnosis of cancers. As\textsubscript{2}O\textsubscript{3} decreases the methylation level of CDKN2B/CDKN2A genes in human hematologic malignant cells, indicating its role in demethylation. In this study, we found that the methylation level of the SFRP1 gene promoter was also downregulated by As\textsubscript{2}O\textsubscript{3} in PCa cells (Figure 5B and C). This is consistent with its role in the demethylation of CDKN2B/CDKN2A genes. These data suggest that As\textsubscript{2}O\textsubscript{3} increased the protein and mRNA levels of SFRP1 by promoting demethylation of
the SFRP1 gene promoter. However, the detailed mechanism through which As$_2$O$_3$ promotes demethylation of the SFRP1 gene is still unclear.

In summary, we showed that As$_2$O$_3$ inhibited viability and induced apoptosis in both the androgen-dependent human PCa cell line LNCaP and the androgen-independent human PCa cell line PC3. The mechanism of the antimutant function of As$_2$O$_3$ in PCa cells at least partly involved its regulation of the Wnt signaling pathway. As$_2$O$_3$ increased SFRP1 expression through inducing demethylation of the SFRP1 gene promoter, thereby inhibiting Wnt signal transduction. Moreover, As$_2$O$_3$ has emerged as a potential treatment for PCa patients owing to its lower toxicity and stronger antitumor effects. Further research is required to address the mechanism through which As$_2$O$_3$ promotes the demethylation of the SFRP1 gene and evaluate the safety of As$_2$O$_3$ in clinical treatment of PCa.

Acknowledgment
This study was supported by the National Natural Science Foundation of China (no 21272032).

Disclosure
Tao Jiang and Hui Jiang are full brothers. The authors report no other conflicts of interest in this work.

References


