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REPORT



Individualized doxorubicin sensitivity testing of undifferentiated soft tissue sarcoma (USTS) in a patient-derived orthotopic xenograft (PDOX) model demonstrates large differences between patients

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ABSTRACT

Doxorubicin (DOX) is often first-line treatment of undifferentiated/unclassified soft tissue sarcoma (USTS). However, the DOX response rate for USTS patients is low. Individualized precision-medicine technology that could identify DOX responders as well as non-responders would be of high value to cancer patients. In the present study, we established 5 patient-derived orthotopic xenograft (PDOX) nude mouse models from 5 USTS patients and evaluated the efficacy of DOX in each PDOX model. USTS's were grown orthotopically in the right thigh of nude mice to establish the PDOX models. Two weeks after implantation, the mouse models were randomized into two groups of 8 mice each: untreated control; and DOX (3 mg/kg, i.p., once a week for 2 weeks). DOX showed significant growth inhibition in only 2 USTS PDOX models out of 5 ($p = 0.0054$, $p = 0.0055$, respectively) on day 14 after initiation. DOX was ineffective in the other 3 PDOX models. However, even in the DOX-sensitive cases, DOX could not regress the PDOX tumors responding to treatment. The present study has important implications since this is the first *in vivo* study to compare the DOX sensitivity for USTS on multiple patient tumors. We showed that only two of five USTS were responsive to DOX, despite DOX being first line chemotherapy for USTS. The 3 resistant cases should not be treated with DOX clinically, in order to spare the patients' unnecessary toxicity. This PDOX model is useful for precise individualized drug sensitivity testing, especially for rare heterogeneous recalcitrant sarcomas such as USTS.

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Introduction

Soft tissue sarcoma (STS) is a heterogeneous group of neoplasms comprising more than 50 subtypes. Undifferentiated/unclassified soft-tissue sarcoma (USTS) is described in the fourth edition of the World Health Organization (WHO) Classification of Tumors of Soft Tissue and Bone in 2013 [1]. USTS is the most common type of STS observed in adults [2–4].

Surgical resection remains the only curative option for USTS which often metastasizes and then is not a candidate for surgery [5–7]. Anthracycline-based chemotherapy, including doxorubicin (DOX), is first-line treatment of USTS, but the DOX response rate for USTS patients is low [8,9]. The use of DOX for USTS is controversial since the disease is heterogeneous. Therefore, novel more effective individualized and precision treatment is necessary for USTS.

Toward this goal, our laboratory pioneered the patient-derived orthotopic xenograft (PDOX) nude mouse model with

the technique of surgical orthotopic implantation (SOI), including breast [10], ovarian [11], lung [12], cervical [13,14], colon [15–17], stomach [18], pancreatic [19–23], melanoma [24–28], and sarcoma [29–38]. The PDOX model, developed by our laboratory over the past 30 years, has many advantages over subcutaneous-transplant models which are growing ectopically under the skin [39].

In the present study, we established PDOX nude-mouse models with USTS from 5 patients and evaluated DOX efficacy for each patient.

Results and discussion

USTS PDOX nude mouse models were established from 5 patients. Patient characteristics from 5 patients are shown in Table 1. The median age was 60 years (range 56–65). Three male and 2 female patients were included. Tumor presentation was 3 primary and 2 recurrent cases.

Table 1. Patients characteristics.

| No. | Age | Sex | Tumor origin site | Primary or recurrence |
|-----|-----|-----|----------------------|-----------------------|
| 1 | 65 | M | Right thigh | Primary |
| 2 | 62 | F | Left upper extremity | Recurrence |
| 3 | 56 | M | Right shoulder | Primary |
| 4 | 59 | F | Left thigh | Recurrence |
| 5 | 60 | M | Right thigh | Primary |

DOX showed significant growth inhibition in only 2 PDOX models ($p = 0.0054$, $p = 0.0055$, respectively) and resistance in 3 on day 14 after treatment initiation (Figures 1 and 2).

The body weight on day 14, compared with day 0, did not significantly differ between control and treatment group in each PDOX model (Figure 3). There were no animal deaths in any group.

Histologically, untreated control tumors comprised mostly viable cells. In DOX-sensitive tumor No. 1 described in Table 1,

some necrosis and fibrosis were observed after DOX treatment (Figure 4A). In contrast, in DOX-resistant tumor No. 4, there were no notable changes after DOX treatment compared to the control (Figure 4B).

In the present study, DOX could inhibit tumor growth in only 2 of 5 PDOX models. The other 3 PDOX models were resistant. In addition, even in the sensitive cases, there was no tumor regression. In such cases, a second drug to effectively combine with DOX could possibly be identified. Future experiments will focus on this goal. The present results demonstrate the urgent need for individualized, precision treatment of USTS. The DOX-resistant PDOX cases should not receive DOX therapy in the clinic. The probable result would be toxicity without efficacy. In the current study, recurrent tumors were both DOX-sensitive and- resistant. Therefore, the PDOX model can be used for precise individualized

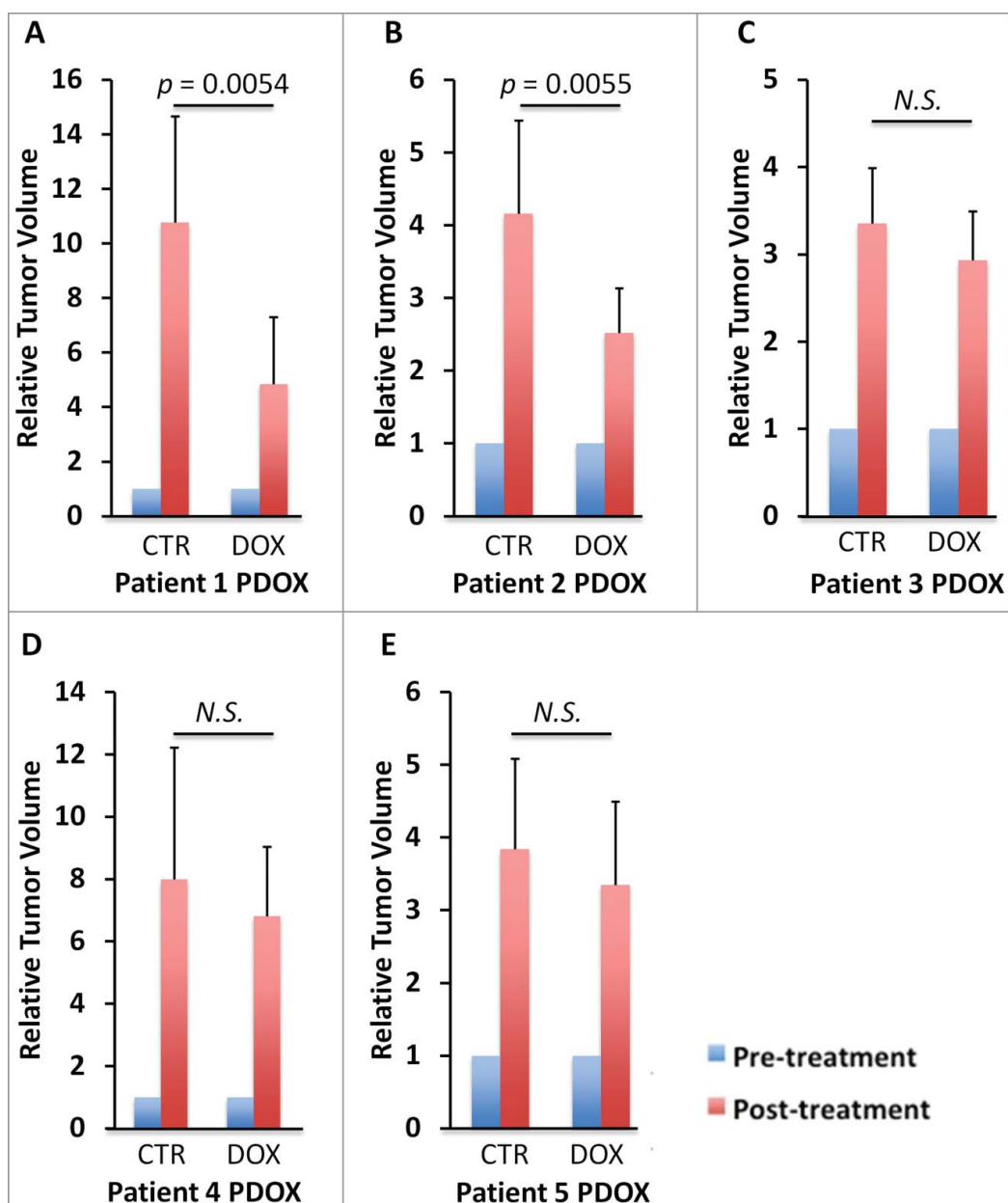


Figure 1. DOX sensitivity of 5 different USTS PDOX models. Bar graphs show relative tumor volume at post-treatment relative to pre-treatment tumor volume. Error bars: \pm SD.

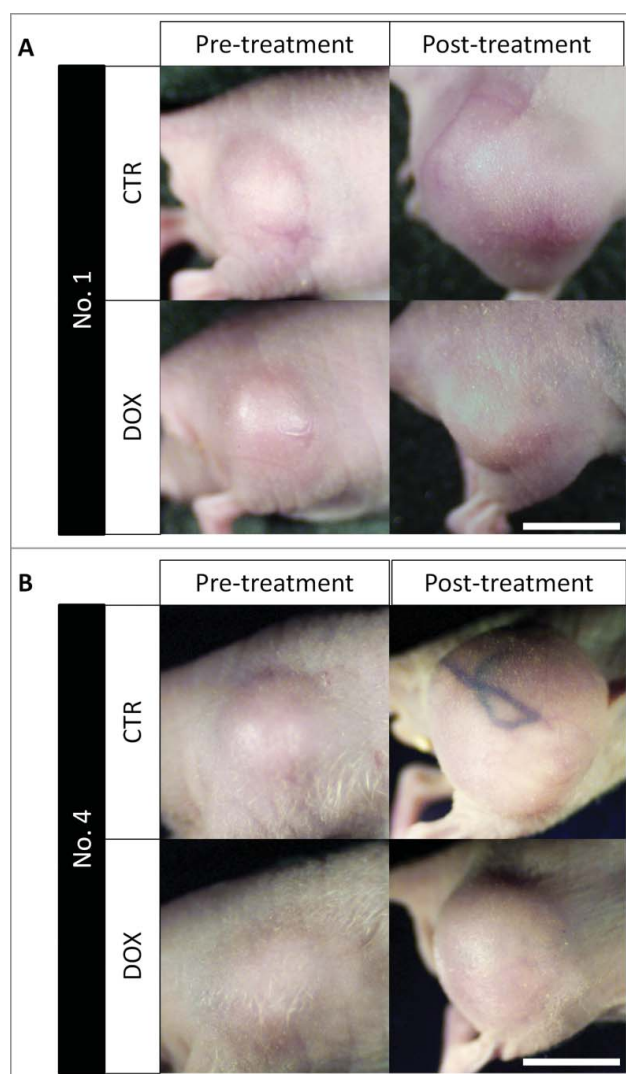


Figure 2. Treated and untreated PDOX tumors. Tumors from USTS PDOX models of patients 1 and 4 are shown before and after treatment with DOX. Scale bar: 5 mm.

drug-sensitivity testing, especially for rare recalcitrant heterogeneous sarcomas [29–38].

DOX resistance has been reportedly due to a number of different cellular changes including: overexpression of efflux transporters, reduction of α topoisomerase II (TOP2A) [40,41], p53 mutation [42], activation of NF- κ B [43], elevated FOXO3 [44], activation of the PI3K/Akt pathway [45], activation of the MEK/ERK pathway [46], as well as other possible mechanisms, including DNA repair.

Previously-developed concepts and strategies of highly-selective tumor targeting can take advantage of molecular targeting of tumors, including tissue-selective therapy which focuses on unique differences between normal and tumor tissues [47–52].

Conclusion

The present study has demonstrated significant differences of DOX sensitivity of 5 different USTS PDOX models. These results demonstrate the powerful potential of the PDOX model for precision oncology and suggest that patients with failed

DOX therapy could possibly benefit from drugs shown to be active against PDOX models of their tumor.

Materials and methods

Mice

Athymic *nu/nu* nude mice (AntiCancer Inc., San Diego, CA), 4–6 weeks old, were used in this study. Animals were housed in a barrier facility on a high efficacy particulate arrestance (HEPA)-filtered rack under standard conditions of 12-hour light/dark cycles. The animals were fed an autoclaved laboratory rodent diet. All mouse surgical procedures and imaging were performed with the animals anesthetized by subcutaneous injection of a ketamine mixture (0.02 ml solution of 20 mg/kg ketamine, 15.2 mg/kg xylazine, and 0.48 mg/kg acepromazine maleate). The response of animals during surgery was monitored to ensure adequate depth of anesthesia. The animals were observed on a daily basis and humanely sacrificed by CO₂ inhalation if they met the following humane endpoint criteria: severe tumor burden (more than 20 mm in diameter), prostration, significant body weight loss, difficulty breathing, rotational motion and body temperature drop. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

Patient-derived tumors

The tumors diagnosed with USTS were resected in the Department of Surgery, University of California, Los Angeles (UCLA). Undifferentiated soft tissue sarcomas include those that show no specific identifiable line of differentiation by currently-available methods including immunohistochemistry, special stains, and cytogenetic or molecular-pathology techniques. Written informed consent was provided by each patient. The Institutional Review Board (IRB) of UCLA approved this experiment [25,26]. Of the 2 relapsed patients, one failed adjuvant chemotherapy and the other was untreated. Patient no. 2, who relapsed, previously failed pazopanib, then received gemcitabine and docetaxel, where the patient's disease was stabilized, then underwent tumor resection which was used to construct the PDOX model. Patient no. 4 received DOX, ifosfamide and MESNA, followed by radiation. However, the tumor continued to grow and the patient underwent tumor resection from which her PDOX was constructed. The patient subsequently succumbed to her disease.

Establishment of PDOX models of USTS by surgical orthotopic implantation (SOI)

Fresh samples of the USTS of the patients were obtained and transported immediately to the laboratory at AntiCancer, Inc., on wet ice. The samples were cut into 5-mm fragments and implanted subcutaneously in nude mice. After the subcutaneously-implanted tumors grew to more than 10 mm in diameter, the tumors were then harvested and cut into small fragments (3 mm³). After nude mice were anesthetized with the ketamine solution described above, a 5-mm skin incision was made on

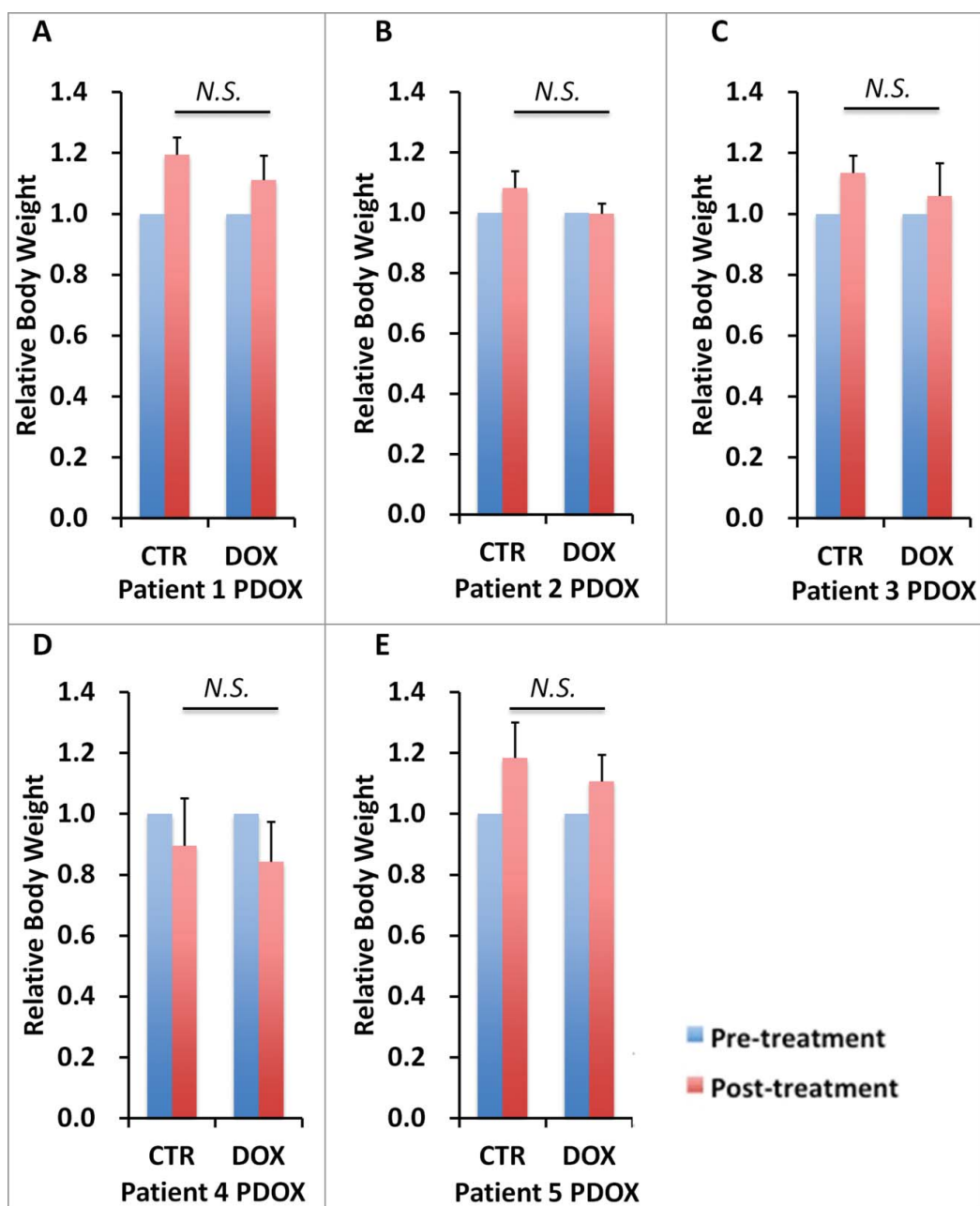


Figure 3. Effect of each treatment on mouse body weight. Bar graphs shows mouse body weight in each mouse at pre- and post-treatment.

the right high thigh, then the biceps femoris was split to make space. A single tumor fragment was implanted orthotopically into the space to establish the PDOX model. The wound was closed with a 6-0 nylon suture (Ethilon, Ethicon, Inc., NJ, USA).

Treatment study design in the USTS PDOX model

PDOX mouse models were randomized into two groups of 8 mice each: untreated control ($n = 8$); and DOX (3 mg/kg, i.p., once a week for 2 weeks, $n = 8$). Tumor length and width were measured both pre- and post-treatment. Tumor volume was calculated with the following formula: Tumor volume (mm^3) = length (mm) \times width (mm) \times width (mm) \times 1/2. Data are presented as mean \pm SD. The tumor volume ratio is

defined as the tumor volume at post-treatment point relative to pre-treatment tumor volume.

Histological examination

Fresh tumor samples were fixed in 10% formalin and embedded in paraffin before sectioning and staining. Tissue sections ($5 \mu\text{m}$) were deparaffinized in xylene and rehydrated in an ethanol series. Hematoxylin and eosin (H&E) staining was performed according to standard protocols. Histological examination was performed with a BHS System Microscope (Olympus Corporation, Tokyo, Japan). Images were acquired with INFINITY ANALYZE software (Lumenera Corporation, Ottawa, Canada) [25,26].

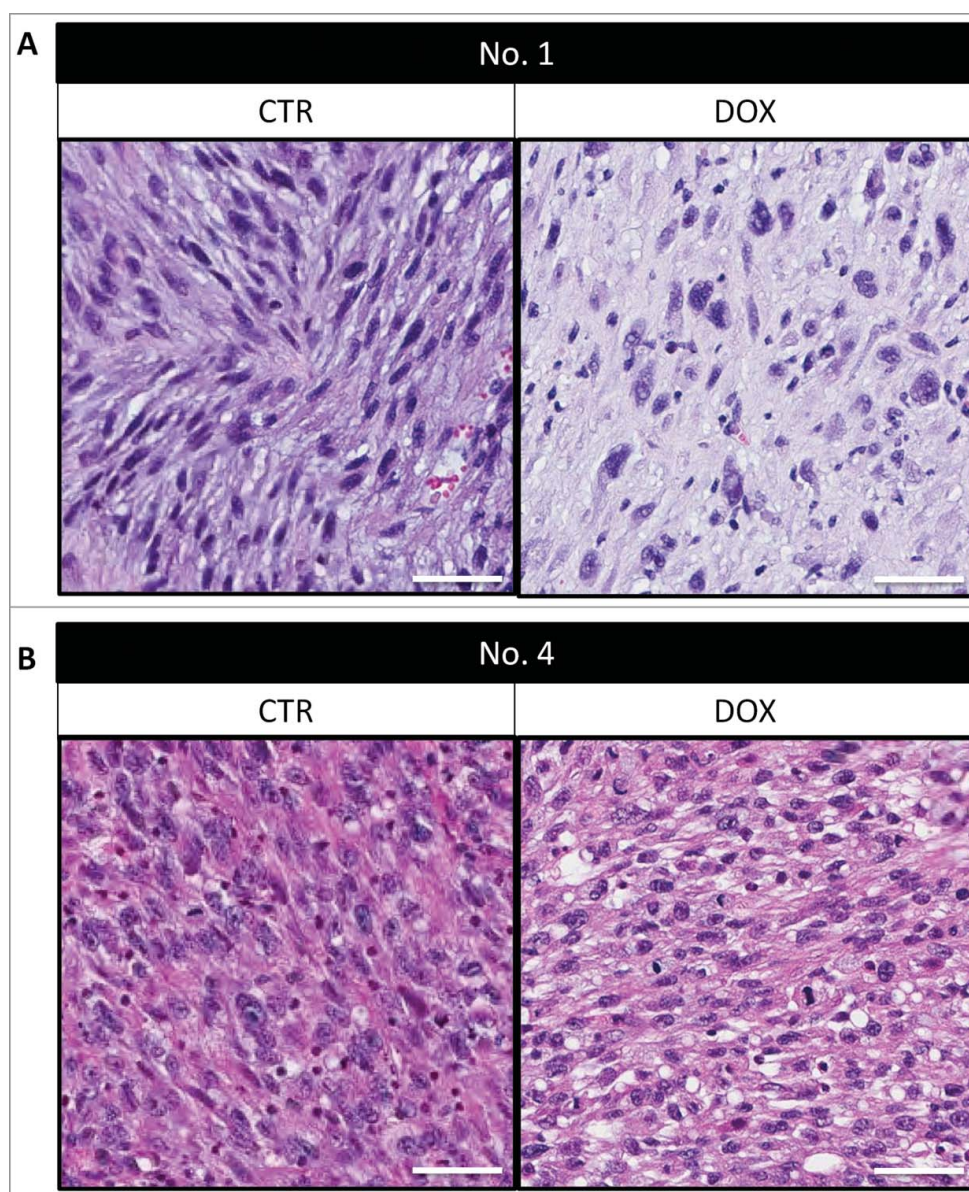


Figure 4. Tumor histology. Tumors from PDOX models 1 and 4 are shown both untreated and after treatment. Scale bars: 50 μ m.

Statistical analysis

JMP version 11.0 was used for all statistical analyses. Significant differences for continuous variables were determined using the Mann-Whitney U test. Bar graphs expressed average values and error bar showed SD. A probability value of $P \leq 0.05$ was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.




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