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Overcoming physical barriers in cancer therapy

Ines Beyer,[1,2,†] Ruan van Rensburg[1,3,†] and André Lieber[1,4,*]

[1]University of Washington; Department of Medicine; Division of Medical Genetics; Seattle, WA USA; [2]University of Duesseldorf; Department of OB/GYN; Dusseldorf, Germany; [3]Leibnitz Institute for Age Research; Jena, Germany; [4]University of Washington; Department of Pathology; Seattle, WA USA

[†]These authors contributed equally to this work.

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*Correspondence to: André Lieber; Email: lieber00@u.washington.edu

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Most solid tumors are of epithelial origin and, although malignant cells are de-differentiated, they maintain intercellular junctions, a key feature of epithelial cells, both in the primary tumor as well as in metastatic lesions. These intercellular junctions represent a protective mechanism against attacks by the host's immune system and pose as physical barriers that prevent intratumoral penetration and dissemination of cancer therapeutics. A key protein of epithelial junctions is desmoglein 2 (DSG2). DSG2 is consistently upregulated in all cancers analyzed. Recently, we demonstrated that a group of human adenoviruses (Ad serotypes 3, 7, 11 and 14) use DSG2 as a primary attachment receptor for the infection of cells. We subsequently created a small recombinant protein derived from Ad serotype 3, which binds to DSG2 and triggers transient opening of epithelial intercellular junctions. We named the protein "JO-1" ("junction opener-1"). JO-1 is a small protein that can easily be produced in *E. coli*. JO-1 binding to and clustering of DSG2 triggers an epithelial-to-mesenchymal-transition that results in transient opening of epithelial junctions. We have shown in over 25 xenograft tumor models that the intravenous injection of JO-1 increased the efficacy of monoclonal and chemotherapy, subsequently reducing the required treatment dose and concomitantly reducing the toxic side effect of these treatments. The application of JO-1 has not been associated with toxicities in safety studies performed in human DSG2-transgenic mice and monkeys.

Epithelial Features and Junction Proteins in Cancer

Greater than 80% of all cancer cases are carcinomas, formed by the transformation of epithelial cells. For most carcinomas, progression to malignancy is accompanied by a loss of epithelial differentiation and a shift toward a mesenchymal phenotype, i.e., epithelial to mesenchymal transition (EMT).[1] EMT increases migration and invasiveness of many cell types and is often seen as one of the conditions for tumor infiltration and metastasis. However, following invasion or metastasis, cells that have undergone the process of epithelial to malignant transition can also revert to a well-differentiated epithelial phenotype.[2] In support, there exists numerous examples of advanced carcinomas showing that mesenchymal cells can regain characteristics of epithelial cells or undergo mesenchymal to epithelial transition (MET).[2] This ability to change back to an epithelial phenotype is especially of note as it allows for the formation of physical barriers, which have been hypothesized to represent a mechanism that decreases access of immune cells, drugs or antibodies to the sites of tumors.[3-5] Overall, two key features, which characterize epithelial cells, are (1) polarized membranes (apical and basolateral) that have distinct density of surface molecules and differ in the composition of proteins and lipids and (2) adherens and intercellular tight junctions that are connected with the underlying apical actin-myosin ring. A third component of the epithelial junctional complex are desmosomes, which are built out of desmogleins, e.g., DSG2. The tight junctions (TJ) are situated just

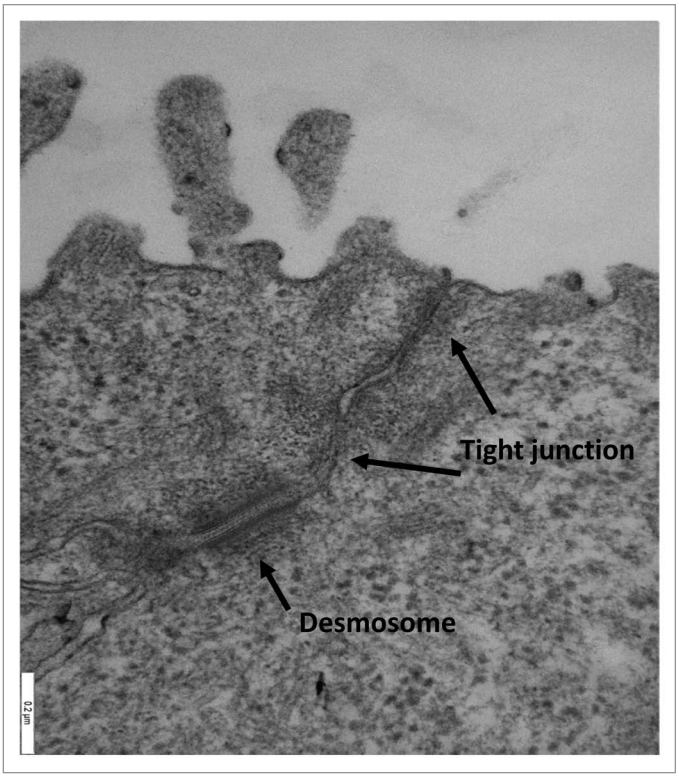

Figure 1. Epithelial junctions. Transmission electron microscopy of junctional areas of colon cancer T84 cells. Magnification is 100,000×. The scale bar is 0.2 μm.

above the adherens junctions (AJ) at the apical side of the lateral membrane in epithelial cells. The electron-microscopy image of polarized cancer epithelial cells in **Figure 1** depicts the localization of tight and desmosomal junctions at the lateral membrane. A network of interconnected strands is formed by TJ proteins, i.e., several claudins and a single occludin protein, that form the paracellular barrier, which prohibit the flow of molecules across the epithelial layer.[6-8] A current concept of the paracellular barrier implies the existence of two permeability pathways: the major "pore" pathway, which is permeable for small molecules with molecular radius below four angstroms; and an alternative "leaky" pathway, which can be permeable for larger molecules.[9,10] However, it is the pore pathway, which plays the major role in the permeability of drugs through the paracellular barrier. The TJ and AJ proteins interact with a number of intracellular signaling proteins that trigger morphological changes and changes in gene expression. Desmosomes probably do not directly regulate paracellular permeability, but they seem to do this indirectly by altering the structure and the stability of tight junctions.[11]

As mentioned above, the epithelial junctions of a tumor can significantly restrict the penetration of therapeutic agents within the tumor, thus severely decreasing the efficacy of such therapeutic modalities.[8] Although the basolateral surface of polarized epithelial cells would be readily accessible to the underlying blood vessels, antigens on the apical membrane would be less accessible to intravenously (i.v.) administered therapeutics due to this barrier function of the tight junctions.[12] Malignant cells with intact tight junctions would be relatively resistant to targeted therapies directed against apical antigens, such as monoclonal antibodies targeted against the Her2/*neu*, epidermal growth factor receptor or prostate-specific membrane antigen. Several studies have also shown that an upregulation of epithelial junction proteins occur in tumors, which is correlated with an increased resistance to therapy, including monoclonal antibodies and chemotherapeutics.[13-15] One of these overexpressed junction proteins in malignant cells is desmoglein 2 (DSG2).[16,17] mRNA profiling of more than 60 ovarian cancer biopsies revealed the overexpression of DSG2 mRNA, as well as consistently higher levels of DSG2 proteins in the ovarian cancer cells when compared with the surrounding normal tissue or tumor stroma cells.[5] **Figure 2** illustrates DSG2 expression in ovarian and breast cancer biopsies.

Working on the hypothesis that the epithelial phenotype of tumors enables the formation of physical barriers that protect the tumor cells from attacks by the host-immune system or elimination by cancer therapeutics, it has been shown by Beyer et al. 2011 and 2012 that the opening of tight junctions increases monoclonal antibody or chemotherapy penetration of tumor masses, leading to greater efficacy of immuno- and chemotherapies.[4,5]

Tight Junction Openers

Various pathogens must first breach the epithelial barrier before gaining access to the body in order to initiate infection. Several mechanisms to disrupt junctional integrity have been developed by these pathogens, e.g., Clostridium perfringens enterotoxin removes claudins-3 and -4 from the TJ to facilitate bacterial invasion.[18] Also, Zona Occludens toxin (Zot), produced by Vibrio cholerae strains, possesses the ability to reversibly modify intestinal epithelial TJs, granting the passage of macromolecules through mucosal barriers.[19] Notably Cox et al. have shown that Zot increases the transport of drugs with low bioavailability (e.g., paclitaxel, doxorubicin, aciclovir and cyclosporin A) up to 30-fold.[20] Additionally, oncoproteins encoded by human papillomavirus (HPV), human adenovirus and human T-lymphotropic virus 1 (HTLV-1) can transiently open TJs by the mislocalization of the TJ protein ZO-1, thereby enhancing the paracellular permeability in epithelial cells.[21] To date, however, there are no epithelial junction openers used clinically for cancer therapy. A number of chemical detergents, surfactants, calcium-chelating agents and phospholipids have been used to increase drug absorption through the gastrointestinal (GI) tract epithelium.[22] Recently, Kytogenics Pharmaceuticals, Inc. has developed a tight junction opener

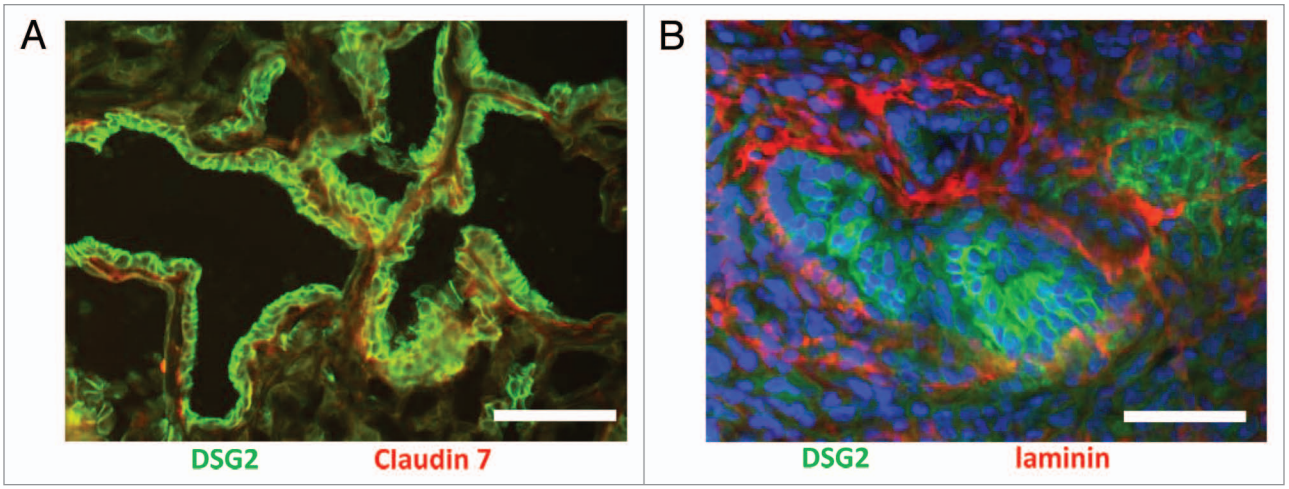

Figure 2. DSG2 expression in human cancers. Cryosections of ovarian cancer (**A**) and breast cancer (**B**) were stained for DSG2 (green), the epithelial junction marker Claudin 7 (red) or the extracellular matrix protein laminin (red). The scale bar is 20 μm.

based on chitosan derivatives. It is thought to act by electronegative forces applied to tight junction proteins (www.kytogenics.com). However, all of these agents act indiscriminately to mechanically disrupt junctions and cannot be applied systemically without major toxic side effects.

Junctions Opener JO-1

We recently developed a recombinant protein (JO-1) for the transient opening of the intercellular junctions in epithelial tumors. This work is based on our finding that DSG2 is a high-affinity receptor for a number of human adenovirus (Ad) serotypes, including, most notably, Ad serotype 3.[23,24] JO-1 is a self-dimerizing recombinant protein derived from the Ad3 fiber, which utilizes DSG2 as binding protein.[25] JO-1 has a molecular weight of approximately 60 kiloDaltons (kDa). It can be easily produced in *E. coli* and purified by affinity chromatography. It has been shown that JO-1 triggers the transient opening of TJ in vitro, in polarized epithelial cancer cells.[4,23]

Mechanism of JO-1

As stated above, desmosomes, of which the DSG2 protein is a part, probably do not directly regulate paracellular permeability. These proteins do, however, seem to regulate paracellular permeability indirectly by altering the structure and the stability of tight junctions.[11] Studies utilizing ultraviolet (UV)-inactivated Ad3, as well as Ad3 fiber-derived dodecahedral particles (PtDd)—the predecessor of JO-1 and JO-1 itself have indicated that binding to DSG2 transiently triggers EMT. EMT is characterized by decreased expression of epithelial markers, activation of kinases and the altered location of transcription factors.[1] Incubation of epithelial cancer cells with UV-inactivated Ad3 or PtDd caused remodelling of junctions as reflected by the decrease in membrane/junction-localized E-cadherin and Claudin 7 signals and an increase in mesenchymal markers such as Vimentin and Lipocalin 2.[23] mRNA expression profiles of PtDd treated cells indicated a marked activation of a number of signaling pathways involved in EMT, including mitogen activated protein kinase (MAPK a.k.a. ERK), phosphatidylinositol, focal adhesion, adherens junctions, Wnt and regulation of actin cytoskeleton signaling pathways.[23] In addition, Western blot analysis of JO-1 treated xenograft tumors showed an upregulation of proteins of the ERK pathway and a decrease in E-cadherin.[4] We have also shown in mouse xenograft tumor models that the i.v. administration of JO-1 mediates the cleavage of DSG2 dimers found in the TJs between epithelial tumor cells.[5] The changes triggered by JO-1 were detectable within one hour after its i.v. injection. This, subsequently, enabled the increased intratumoral penetration of the anti-Her2/*neu* mAb trastuzumab.[4] These biological effects of JO-1 translated into an increased therapeutic efficacy of several mAbs, including trastuzumab and cetuximab, in xenograft tumor models, e.g., models of colon, breast, gastric, lung and ovarian cancer.[4] JO-1 co-administration also enhanced the therapeutic efficacy of several chemotherapy drugs, including pegylated liposomal doxorubicin (PLD or Doxil) (**Fig. 3**), paclitaxel (Taxol), nanoparticle albumin bound paclitaxel (Abraxane) and irinotecan (Camptosar) in tumor xenograft models of breast, lung and prostate cancer.[5] Furthermore, chemotherapy doses could be decreased without compromising the antitumor effects due to JO-1 co-therapy, and this also provided protective effects to normal tissues.[5] For example, we demonstrated that it was possible to decrease the effective dose of PLD with JO-1 cotherapy in xenograft models, i.e., with orthotopic ovarian cancer cells (ovc316)—a primary tumor cell line that was established from an ovarian cancer biopsy.[3] The combination of JO-1 and PLD was significantly more effective than PLD alone (**Fig. 3**). JO-1 also relieved adverse side effects from PLD treatment, e.g., liver enzymes (AST, ALT and alkaline phophatase) were significanly decreased in animals treated with JO-1 and PLD compared with mice treated with PLD alone.[5] Mice that received JO-1 injections also had less severe tissue damage in the bone marrow and intestine caused by PLD treatment. We speculate that the ability of JO-1 to open the

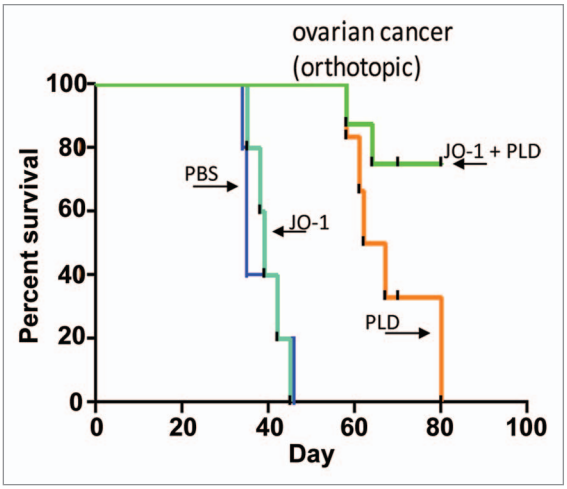

Figure 3. JO-1 enhances PEGylated liposomal doxorubicin (PLD) therapy in an ovarian cancer model. CB17-SCID/beige mice with intraperitoneal tumors derived from primary human ovarian cancer cells ovc316. Treatment was started at day 35 after tumor cell implantation and repeated weekly. Mice were injected intravenously with 2 mg/kg JO-1 or PBS, followed by an intravenous injection of liposomal doxorubicin (3 mg/kg) or PBS 1 h later. Onset of ascites was taken as an endpoint in therapy studies. Shown is the survival of animals in a Kaplan Meier graph. n = 10. $p < 0.001$ for PLD vs JO-1 + PLD.

intercellular junctions in tumors increases the uptake and amount of chemotherapeutics in the tumor environment. This then results in the reduced drug levels in normal tissues, thereby providing a larger therapeutic window. Using an ELISA to measure PEGylated compounds in tissues,[26] we found support for our hypothesis by documenting significantly more PLD in tumors and less in normal tissues in mice that received JO-1 prior to i.v. PLD injection. Immunofluorescence analysis of tissue sections also revealed higher levels of PLD in tumors of JO-1+PLD treated mice compared with mice treated with PLD alone. In these animals, PLD is found to be more broadly distributed over a greater distance from blood vessels, suggesting better intratumoral penetration and absorption by tumor tissue.

In addition to epithelial junctions, extracellular matrix in solid tumors affects the effectiveness of therapeutics through blocking of intratumoral diffusion.[27] We have recently shown that transient degradation of tumor stroma proteins by intratumoral expression of the peptide hormone relaxin significantly enhanced trastuzumab therapy.[28] In a more recent study, we demonstrated in a breast cancer model, that intratumoral relaxin expression in combination with JO-1 treatment had an additive effect and stopped tumor growth.[4]

Tumor-Specificity of JO-1 Action

The necessity arose to generate human DSG2 (hDSG2) transgenic mice with tumors that overexpressed human DSG2 for biodistribution and safety studies, due to the failure of JO-1 to bind to mouse DSG2.[29] Using the hDSG2-transgenic mouse model with syngeneic $hDSG2^{high}$ tumors, we demonstrated that JO-1 predominantly accumulates in tumors.[5] This could be explained by either one of the following factors: (1) the overexpression of DSG2 by tumor cells, (2) better accessibility of DSG2 on tumor cells due to a lack of strict cell polarization compared with DSG2-expressing normal epithelial cells or (3) a high degree of vascularization and vascular permeabilty in tumors. JO-1 appears to function as a magnet to draw therapeutic drugs into tumors, which decreases the levels and exposure of these drugs in normal tissues, due to its binding to and action on epithelial junctions of tumors.

Toxic Side Effects and Immunogenicity

The i.v. injection of JO-1 at a dose of 2 mg/kg into hDSG2 transgenic mice had no observed adverse side effects, except for mild, transient diarrhea. There were also no abnormalities found in laboratory parameters as well as histopathological studies of tissues. We speculate that this is due to the fact that DSG2 in tissues, other than the tumor and a subset of epithelial cells in the intestine/colon, is not accessible to i.v. injected JO-1. The hDSG2 transgenic mouse model was also used to obtain biodistribution and pharmacokinetics data for JO-1.[5] Although, JO-1 is a protein derived from adenovirus 3 and therefore potentially immunogenic. This might not be a critical issue if JO-1 is used in combination with chemotherapy, which suppresses immune responses, this prediction is supported by studies with oncolytic adenovirus vectors in which immunosuppression allowed for the repeated application of the vector.[30-32] In addition, we have shown that JO-1 remains active in vitro and in vivo, even in the presence of anti-JO-1 antibodies generated by the JO-1 vaccination of mice.[5] This may be due to the fact that JO-1 binds to DSG2 with a very high avidity, thus potentially disrupting the complexes between JO-1 and antibodies against JO-1. Notably, JO-1 is a dimer of a trimeric fiber knob, which contributes to the picomolar avidity to DSG2.[25] We performed repeated injections of JO-1 in an immunocompetent hDSG2 mouse tumor model to test the effect of anti-JO-1 antibodies on the therapeutic efficacy of JO-1. Importantly JO-1 had an enhancing effect on PLD therapy after repeated JO-1 pre-treatment, demonstrating that JO-1 continues to be effective after multiple treatment cycles, even in the presence of detectable antibodies.

JO-1 Safety Studies in Non-Human Primates

Our preliminary data indicate that human DSG2 can interact with mouse proteins and thereby allowing for the use of hDSG2 transgenic mice in JO-1 toxicity and efficacy studies. The hDSG2 transgenic mouse model also provides for the possibility to efficiently study variables in a large number of animals. Nonetheless, it is unknown whether the hDSG2-mouse system accurately models a homologous human system. A better model seems to be non-human

primates. We have shown that DSG2 biodistribution in *Macaca fascicularis* is similar to humans and that JO-1 binds to monkey DSG2.[29] This led us to perform studies on two animals, which have been injected with JO-1 (0.6 mg/kg) followed by a full necropsy after 72 h. Thus far, no histological or laboratory abnormalities have been observed. JO-1 serum clearance and JO-1 biodistribution was similar to that observed in DSG2 transgenic mice, i.e., JO-1 was found in the GI-tract as well as macrophages in the liver and lymphnodes.

Risk of Metastasis

JO-1 binding to DSG2 on tumor cells triggers pathways involved in EMT, a process which, as mentioned above, has been associated with tumor metastasis. However, over 20 in vivo studies conducted with JO-1 combined with a range of cancer therapeutics in various different cancers with long-term follow-up, have not provided any evidence of metastases.[5] Transient activation of the EMT pathway is only one of many steps required for tumor metastasis. Detachment of tumor cells from epithelial cancers and their subsequent migration is only possible after long-term crosstalk between malignant cells and the tumor microenvironment, resulting in changes in the tumor stroma and phenotypic reprogramming of epithelial cells into mesenchymal cells.[33]

Transitions between epithelial and mesenchymal cell stages, namely EMT and its reverse mesenchymal-epithelial transition (MET) have been recently accredited important roles in cancer progression, specifically in the induction and maintenance of cancer stem cells.[34] We are therefore currently studying the effect of JO-1 on cancer stem cells in xenograft models. To monitor cancer stem cells by non-invasive in vivo imaging, we are using primary ovarian cancer cells that express luciferase under Nanog or CD133 promoters.

Affinity Enhanced Junction Openers

Affinity enhancement of biologics is used to (1) decrease their effective dose, (2) increase their half-lives, (3) potentially increase their therapeutic effects and (4) circumvent the adverse effects of antibodies generated by patients against the biological (e.g., neutralization). To make JO-1 analogs with increased affinity, we screened an *E. coli* expression library with random mutations within JO-1 for variants for increased affinity. We selected three mutants (JO-2, JO-3, JO-4) with dissociation constants (K_ds) that were 3.5-, 11.4- and 27.8-fold lower than JO-1. We also generated a JO-1 mutant, which was unable to bind to DSG2 (JO-neg). The efficacies of these mutants in junction opening were measured in vitro by adding these JO-1 variants to the apical side of polarized epithelial colon cancer cells and measuring the transepithelial electrical resistance (TEER). The addition of JO-neg had no effect on the TEER, while JO-2, -3 and -4 decreased the TEER significantly more than JO-1. This indicates that JO-2, JO-3 and JO-4 were more potent in the opening of epithelial junctions compared with JO-1. These observations were confirmed by an in vivo study in a xenograft tumor model, where the affinity-enhanced variants increased therapy with the chemotherapy drug irinotecan (MW 590 Da) significantly more than JO-1. We are currently analyzing the safety and pharmacokinetic profiles of JO-2, -3 and -4. Based on these data, we will select the best candidate for future studies.

Clinical Development

We are currenlty focused on the clinical translation of JO-1 in combination with PEGylated liposomal doxorubicin (PLD)/Doxil) for ovarian cancer therapy. PLD is used to treat patients with ovarian cancer that has progressed or recurred after platinum-based chemotherapy. Although a drug commonly used, response rates to PLD are low, the response duration is short, and the toxicity is significant, implying a need for improvement.

In conclusion, we have demonstrated that the protective epithelial barrier created by tumors can be overcome, allowing for the better penetration of cancer therapeutics. A minority of research has, to date, concentrated on this aspect of treatment, the majority focusing rather on increasing targeting specificity or stronger inhibition of tumor growth. However, the fact that the opening of the tight junctions allows not only for greater access to the tumor cells but also greater access to the binding receptors on said cells, provides the potential for greater therapeutic effects of targeted therapies and the possibility of cost reduction for the already stressed health systems.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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