Efficacy and Tolerability of a GD2-Directed Trifunctional Bispecific Antibody in a Preclinical Model: Subcutaneous Administration Is Superior to Intravenous Delivery

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Abstract

Trifunctional bispecific antibodies (trAb) are novel anticancer drugs that recruit and activate different types of immune effector cells at the targeted tumor. Thus, tumor cells are effectively eliminated and a long-lasting tumor-specific T-cell memory is induced. The trAb Ektomab is directed against human CD3 on T cells and the tumor-associated ganglioside GD2, which is an attractive target for immunotherapy of melanoma in humans. To optimize clinical applicability, we studied different application routes with respect to therapeutic efficacy and tolerability by using the surrogate trAb Surek (anti-GD2 × anti-murine CD3) and a murine melanoma engineered to express GD2. We show that subcutaneous injection of the trAb is superior to the intravenous delivery pathway, which is the standard application route for therapeutic antibodies. Despite lower plasma levels after subcutaneous administration, the same tumor-protective potential was observed in vivo compared with intravenous administration of Surek. However, subcutaneously delivered Surek showed better tolerability. This could be explained by a continuous release of the antibody leading to constant plasma levels and a delayed induction of proinflammatory cytokines. Importantly, the induction of counter-regulatory mechanisms was reduced after subcutaneous application. These findings are relevant for the clinical application of trifunctional bispecific antibodies and, possibly, also other immunoglobulin constructs. Mol Cancer Ther; 14(8): 1877–83. © 2015 AACR.

Introduction

Although treatment of cancer has substantially improved during the past years, the prognosis of many malignancies is still poor. Recent developments in the field of immunotherapy, however, may pave the way for new therapeutic approaches. Trifunctional bispecific antibodies (trAb) are promising reagents that harness the immune system to reject cancer (1). These novel therapeutic antibodies consist of two different binding arms, which are directed against a tumor-associated antigen and CD3 on T cells, respectively. In addition, they are endowed with an intact Fc region (comprising a rat and a mouse moiety) that simultaneously stimulates accessory cells via activating Fcγ receptors (2). Thereby, trAbs recruit and activate different types of immune effector cells at the targeted tumor where they mediate effective tumor cell destruction (3). The interplay between T cells, tumor cells, and accessory cells mainly leads to the induction of a Th1 response, which is a prerequisite for potent tumor rejection (4, 5). Furthermore, it has been shown that antigen-presenting cells recruited to the tumor site subsequently present the phagocytosed and processed tumor-associated antigens to T cells (2). This leads to the induction of tumor-specific T cells and a long-lasting immunologic memory, which was not achieved by using the corresponding bispecific F(ab’)_2 fragment (6, 7). Therefore, trAbs not only eliminate tumor cells directly, but also exert a long-term vaccination effect. This unique characteristic enables a long-lasting antitumor response and offers new perspectives for anticancer immunotherapy.

Catumaxomab, which has dual specificity for epithelial cell adhesion molecule (EpCAM) and CD3, is the first trAb routinely used in the clinic. It was approved in 2009 for the treatment of malignant ascites resulting from EpCAM-expressing tumors (8, 9). Other trAbs targeting different specificities are being developed and have already shown therapeutic benefits for cancer patients in clinical studies. A promising trAb, named Ektomab, is directed against human CD3 on T cells and the tumor-associated ganglioside GD2 (10). This cell-surface molecule is an attractive target for cancer immunotherapy in humans because it is strongly expressed on small-cell lung cancer and on malignancies of neuroectodermal origin such as neuroblastoma, glioma, and melanoma, while it shows a highly restricted pattern of expression in normal tissue.
of trAbs, the subcutaneous delivery may offer several bene-
tons for melanoma, we selected the GD2-targeting trAb Surek
A critical factor that may determine the antitumor efficacy as
possible adverse side effects exerted by trAbs is the appli-
ance and as possible adverse side effects exerted by trAbs is the appli-
ction route. Several clinical trials indicated that both intraperi-
toneal (8, 9, 16, 17) and intravenous (18–20) administration is feasible. However, we argued that for broader clinical applicability of trAbs, the subcutaneous delivery may offer several benefits, for example, a shortened application time (minutes vs. hours) and a further improved safety profile. Therefore, we directly compared intravenous administration, which has been mostly used in the clinic, and subcutaneous application. As this regimen will be particularly relevant for future trAb-mediated treatment of mela-
oma, we selected the GD2-targeting trAb Surek for preclinical evaluation. Here, we show that trAb-mediated therapy can indeed be optimized by choosing the appropriate application route.

Materials and Methods

Cell lines and tumor model

The GD2-positive B78-D14 mouse melanoma cell line (kindly provided by J.C. Becker, Julius-Maximilians-Universität, Würzburg, Germany) was derived from the B16F0 melanoma by transfection of

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(11, 12). Using the B16F0-derived murine melanoma cell line B78-
line (kindly provided by J.C. Becker, Julius-Maximilians-Universität, Würzburg,
GD2 and CD2 syntheses as described previ-
ously (13). B78-D14 cells were cultured in RPMI1640 medium supplemen-
ted with 8, 9% FCS, 2 mmol/L l-glutamine, 0.4 mg/mL 
GD18, 0.5 mg/mL hygromycin B, sodium pyruvate, and nonessen-
tial amino acids. Before in vivo application, cells were extensively washed in PBS. On a regular basis, the identity of the cell line was con-
firmed by morphology, in vivo growth behavior, and antigen ex-
pression.

TrAb construct

The trispecific bispecific antibody Surek is derived from the par-
ental antibodies 17A2 (anti-mouse CD3, rat IgG2b) and Me361 (anti-GD2, mouse IgG2a; ref. 10). Surek was generated by quadroma technology and purified by affinity and ion exchange chromatography (14).

Animals

Female C57BL/6 mice, purchased from Taconic and kept under specific pathogen-free conditions in our animal facility, were used at the age of 9–12 weeks in groups of 5 animals. Mice were injected intraperitoneally with 1 × 10^7 B78-D14 cells and 50 μg Surek intravenously or subcutaneously at the indicated time points. Control groups received only 1 × 10^7 B78-D14 cells or no treatment. All experiments were in accordance with relevant reg-
ulations and have been approved by Regierung von Oberbayern.

T-cell phenotyping by FACS

For analyses of T cells, mice were treated as mentioned before. Two and 3 days after treatment, splenocytes were analyzed by staining with directly labeled monoclonal antibodies against CD4
(RM4-5; BD Biosciences), CD8 (53-6-7; ebioscience), and CD69
(H1.2F3; BD Biosciences). Intracellular staining with anti-IFNγ
(XMG1.2; ebioscience) and anti-IL-10 (JESS-16E3; ebioscience) was performed after 4 hours of stimulation with PMA/ionomycin and Brefeldin A (ebioscience), whereas intracellular staining with

anti-Ki-67 (SolA15; ebioscience) and CTLA-4 (UC10-489; Bio-
Legend) was carried out without stimulation. Cells were fixed and permeabilized (ebioscience) according to the manufacturer’s instructions and subsequently analyzed by FACS using an LSRII flow cytometer (BD Biosciences).

Weight monitoring

After premonitoring, mice were treated as mentioned under the heading “animals.” Then, body weight was monitored on a daily basis over a period of 7 days by using the laboratory balance Quintix 5101-1S (Sartorius).

Histopathology and immunohistochemistry

After shaving mice, 50 μg Surek was injected subcutaneously. The skin was continuously monitored macroscopically. In addi-
tion, formalin-fixed skin biopsies (2 and 7 days after trAb injec-
tion) were processed and embedded in paraffin. For histologic analysis, 4 μm (for hematoyxlin and eosin stain) and 1 μm (for immunohistochemistry) sections were made from paraffin blocks. Slides were then stained with hematoyxlin/eosin or immunohistochemical evaluation was done by using antibodies against CD3 (Dianova), Mac3 (DCS), and B220 (BD Biosciences).

Measurement of plasma concentrations of antibodies

After injecting Surek (50 μg) intravenously and subcutaneous-
ly, respectively, blood samples were taken at different time points and sera were stored at −20°C. Surek plasma concentrations were measured by ELISA. Briefly, Surek was captured by an anti-rat IgG2b antibody (RC7/11.1; BD Biosciences) and detected via a biotin-labeled anti-mouse IgG2a antibody (R19-15; BD Bio-
sciences). Subsequently, streptavidin-β-galactosidase (Roche Diagnostics) and the substrate chlorophenol red-β-D-galactopy-
ranoside (Roche Diagnostics) were added. The colorimetric reac-
tion was measured at 570 nm. Surek concentration was calculated by interpolation on a standard curve.

To measure anti-antibody induction, blood samples were col-
clected at day 15, 30, and 45 after injection. The titers of mouse anti-
mouse antibodies (MAMA) as well as mouse anti-rat antibodies (MARA) were determined by ELISA. Briefly, anti-antibodies in serially diluted sera were captured by parental antibody 17A2 (21) for analyzing MARAs or Me361 (22) for analyzing MAMAs. Bound anti-antibodies were detected by a biotinylated goat-anti mouse IgG (H+L) antibody (MARAs) or a biotinylated goat-anti mouse IgG1 antibody (MAMAs), both obtained from SouthernBiotech. Then, streptavidin-β-galactosidase and substrate were added and optical density was measured at 570 nm. Titers were calculated as those reciprocal serum dilutions that yielded an extinction exceeding the mean background signal by 3 × SD.

Cytokine quantification

For analyzing cytokine production, mice were treated as men-
tioned before. One, 3, 24, and 48 hours after treatment, blood samples were taken. Cytokine levels in sera of mice were analyzed by using the Bio-Plex cytokine assay system (Bio-Rad).

Statistical analysis

GraphPad Prism software version 5.01 (GraphPad Software) was used for generation of survival curves, data plots, and statis-
tical calculations. Statistical analyses were done by using the unpaired, two-tailed Student’s t test or the log-rank test. P values < 0.05 were considered as statistically significant.
Results

Therapeutic efficacy of subcutaneously delivered Surek

As already shown, the in vivo efficacy of trAbs is reflected by T-cell activation in vivo and a predominant release of Th1 cytokines, particularly IFNγ, following trAb injection (6). To evaluate the efficacy of different application routes, we examined T-cell activation and proliferation after delivery of Surek intravenously and subcutaneously. Both CD8+ and CD4+ T cells showed a strong upregulation of CD69 and Ki-67 in mice treated with B78-D14 and Surek as compared with control mice (Fig. 1A and B), indicating trAb-induced T-cell activation and proliferation. Importantly, no differences were seen between intravenous and subcutaneous application. Likewise, after intravenous and subcutaneous treatment, CD8+ cells were equally capable of producing IFNγ 2 days after treatment (Fig. 1C).

The results encouraged us to compare the tumor-protective potential of Surek subcutaneously and Surek intravenously, respectively, in vivo (Fig. 2). Subcutaneous and intravenous treatment showed the same antitumor efficacy with a median survival of >100 days when Surek was injected twice (day 2 and 7 after B78-D14 challenge). As already published, a trAb with an unrelated tumor specificity (anti-HER2 × anti-CD3), which did not bind to B78-D14 cells, was completely ineffective (14). It is established that the potential of trAbs depends on the number of treatment cycles but increasing the number of intravenous injections is often limited by possible side effects. Therefore, we asked whether the subcutaneous route allows to extend the treatment cycles and to ameliorate the therapeutic effect. Indeed, the overall survival after subcutaneous administration was further increased up to 85% when Surek was administered three times (day 2, 7, and 11) after tumor challenge, while the intravenous group had to be euthanized immediately after the third antibody injection due to severe adverse effects, which were most likely caused by aggregation of Surek and anti-drug antibodies (see below). This suggests a better tolerability of Surek subcutaneously versus intravenously.

Local and systemic tolerability of Surek subcutaneously

For using subcutaneous injection of trAbs as an appropriate alternative to conventional intravenous application in a clinical setting, it is pivotal to provide data not only on efficacy, but also on safety. As subcutaneous trAb administration could be associated with inflammatory reactions at the injection site, we performed a close skin monitoring before and after Surek administration. One, 24, 48 hours, as well as 7 days after trAb administration, no notable skin reaction was observed, indicating that Surek subcutaneously was not associated with significant adverse events at the injection site. These observations were verified by histo- and immunopathologic analyses 2 and 7 days after subcutaneous administration of Surek. All skin biopsies showed a normal distribution of immune cells (data not shown). Taken together, no inflammatory reactions or other alterations were detected after subcutaneous administration of trAb Surek.

Systemic tolerability of subcutaneous trAb treatment is of special interest for clinical use. In mice, weight loss is used as a sensitive marker for health monitoring (23). Therefore, we performed a close weight monitoring following intraperitoneal injection of GD2-positive B78-D14 mouse melanoma cells and trAb (Fig. 3). After injection of Surek intravenously and subcutaneously, respectively, a massive weight loss in both treatment groups was observed by day 2. While weight loss continued in intravenously treated mice until day 3, subcutaneously treated mice started earlier to recover. At day 3, the relative weight loss of intravenously treated animals was about 40% higher than that of the subcutaneously treated group (Student t test, P = 0.0032).
Weight at day 0 was different between Surek intravenously (i.v.) and subcutaneously (s.c.), respectively, at day 0. Weight of control groups remained stable, while Surek-treated mice experienced a severe weight loss. At day 3, weight of intravenously treated animals was statistically different from subcutaneously treated mice. 

Bioavailability of trAb Surek and anti-drug antibody induction

The data show that subcutaneous administration of Surek results in equal efficacy and even better tolerability compared with intravenous injection of trAb. As this outcome might be related to the bioavailability of the trAb, we measured plasma concentrations over 10 days following injection and determined bioavailability by calculating the areas under the curves (Fig. 4A). Surek injected once intravenously was available immediately, reached its maximum concentration 10 minutes after injection, and was then eliminated constantly. In contrast, subcutaneously administered Surek showed a delayed absorption. A plateau concentration was observed between 8 and 24 hours, followed by trAb elimination. At day 5 after injection, Surek could not be detected any more in either setting. Taken together, bioavailability was only 50% after subcutaneous administration as compared with intravenous injection.

Previous studies showed that the antitumor efficacy of Surek was dose-dependent (14). To mimic the clinical situation more closely, we therefore measured the plasma levels of Surek in a treatment schedule involving three injections (day 0, 2, and 4; Fig. 4B). While each intravenous injection of Surek led to a concentration peak that subsequently declined rapidly, multiple subcutaneous administrations entailed a steadily continuing rise in plasma concentration. For clinical use, application of Surek subcutaneously looks promising because plasma peaks, which may cause systemic adverse effects, were absent.

The therapeutic potential of Surek may be limited by the development of neutralizing anti-drug antibodies. As high concentrations of trAb in the skin and the presence of numerous antigen-presenting cells may favor presentation of trAb epitopes to the immune system in the subcutaneous setting, we measured MAMAs and MARAs in mice sera. However, comparing Surek subcutaneously and intravenously did not reveal any difference in anti-antibody titers 15, 30, and 45 days after trAb injection. In both settings, anti-antibody titers were in the range of 10^5 to 10^6 (Supplementary Fig. S1).

Differential cytokine milieu induced by Surek injected intravenously versus subcutaneously

Adverse systemic effects associated with trAb treatment are induced by cytokines and closely correlate with the systemic cytokine profile after trAb administration. Therefore, we studied the cytokine pattern in sera of tumor-bearing animals after treatment with Surek intravenously and subcutaneously, respectively. As shown in Fig. 5A, we observed an increase of all Th1 cytokines tested after Surek administration. However, intravenous and subcutaneous treatment differed in the kinetics of cytokine response. Intravenously injected Surek led to an immediate increase of IL6, IFNγ, and TNF within 1 hour after injection, whereas trAb administered subcutaneously induced a delayed rise with the maximum concentration only 3 hours after injection and a more rapid decline at later time points compared with intravenous treatment. The moderate induction of cytokine release after subcutaneous administration in comparison with the rapid release after intravenous injection may have a favorable effect on the safety profile. Nonetheless, as shown above, T-cell activation (Fig. 1A) and especially IFNγ secretion of T cells (Fig. 1C) after subcutaneous treatment were obviously sufficient to secure a beneficial therapeutic outcome.

Given the lower bioavailability of Surek (50%) following subcutaneous application as compared with intravenous injection (Fig. 4A), the effective tumor killing by subcutaneously delivered Surek (Fig. 2) as well as the high expression of T-cell activation parameters (Fig. 1) were surprising. We hypothesized...
Optimization of trAb-Mediated Tumor Therapy

Antibody-mediated approaches of cancer immunotherapy have widely been used in the clinic (24, 25). However, such regimens are often associated with undesired adverse effects such as flulike symptoms, chills, and fever that are due to off-site T-cell activation and systemic cytokine release (18, 19, 26). trAbs, which recruit different types of immune effector cells to the tumor cells and thereby also induce a T-cell memory (6, 7), are one of the most potent immunologic antitumor reagents hitherto used in cancer patients. These Ig constructs, which are effective at nanogram/ml concentrations (18, 27), may raise particular safety concerns (18, 19). Although trAb-induced cytokine-related adverse events are manageable in the clinics by using low starting doses and subsequent dose escalation (18, 28), there must be constant efforts to further improve patients' safety thus increasing the treatment options for trAbs in the near future. In the current study, we directly compared conventional intravenous treatment and subcutaneous application of trAbs by using trAb Surek as a preclinical model, which targets GD2-expressing melanoma cells and CD3 on murine T cells (14). Many antibody-induced side effects as observed in humans (like chills, nausea, or headache; refs. 18) cannot be documented in mice. A reliable indicator of adverse health effects in mice, however, is the body weight (23). Both weight loss in animals and the clinical symptoms observed in humans are equally affected by cytokines released upon antibody delivery. Therefore, we assessed weight loss as a marker for systemic tolerability although specific effects that will be seen in humans after intravenous or subcutaneous injection of trAbs cannot be predicted. Our results suggest a better tolerability of the subcutaneous application.

Reduced CTLA-4 expression after Surek subcutaneous (s.c.) administration. Mice received 50 µg Surek intravenously (i.v.) and subcutaneously, respectively, together with 10^5 B78-D14 cells intraperitoneally or tumor cells alone or were left untreated. After 72 hours, T cells from spleens were analyzed. Intracellular staining of CTLA-4 in CD4+ (A) and CD8+ (B) cells. Left, representative experiments. Right, cumulative results with means and SEM (n = 5 per group). Statistical significance in comparison with intravenously treated group according to the Student t test: *, P < 0.05; **, P < 0.01, respectively.

Discussion

Antibody-mediated approaches of cancer immunotherapy have widely been used in the clinic (24, 25). However, such regimens are often associated with undesired adverse effects such as flulike symptoms, chills, and fever that are due to off-site T-cell activation and systemic cytokine release (18, 19, 26). trAbs, which recruit different types of immune effector cells to the tumor cells and thereby also induce a T-cell memory (6, 7), are one of the most potent immunologic antitumor reagents hitherto used in cancer patients. These Ig constructs, which are effective at nanogram/ml concentrations (18, 27), may raise particular safety concerns (18, 19). Although trAb-induced cytokine-related adverse events are manageable in the clinics by using low starting doses and subsequent dose escalation (18, 28), there must be constant efforts to further improve patients’ safety thus increasing the treatment options for trAbs in the near future. In the current study, we directly compared conventional intravenous treatment and subcutaneous application of trAbs by using trAb Surek as a preclinical model, which targets GD2-expressing melanoma cells and CD3 on murine T cells (14). Many antibody-induced side effects as observed in humans (like chills, nausea, or headache; refs. 18) cannot be documented in mice. A reliable indicator of adverse health effects in mice, however, is the body weight (23). Both weight loss in animals and the clinical symptoms observed in humans are equally affected by cytokines released upon antibody delivery. Therefore, we assessed weight loss as a marker for systemic tolerability although specific effects that will be seen in humans after intravenous or subcutaneous injection of trAbs cannot be predicted. Our results suggest a better tolerability of the subcutaneous application.

It should be noted that T-cell activation requires binding of Surek to CD3 but not simultaneous engagement of the tumor-specific binding arm. The latter is only necessary for redirecting the cytotoxic activity of activated T cells towards the target cells (15). Therefore, Surek also induced T-cell activation, weight loss, and
cytokine release in the absence of tumor cells (Supplementary Figs. S2–S4), which, of course, does not reflect the clinical situation.

While intravenous delivery of trAb Surek gave rise to sharp plasma peaks followed by a rapid decline after each injection, subcutaneous administration led to a continuous building up of plasma levels (Fig. 4B). Peaks observed after intravenous injection correlated with an immediate rise of all Th1 cytokines tested, whereas sera of subcutaneously treated animals exhibited the maximum concentration of these cytokines only 3 hours after administration (Fig. 5A). As shown earlier (6), T-cell activation and the release of Th1 cytokines, especially IFNγ, are crucial for the induction of an effective and long-lasting antitumor response, but proinflammatory cytokines also convey the undesired side effects. Although the bioavailability of Surek in the subcutaneous setting was considerably decreased (about 50% compared with intravenous injection; Fig. 4A), the IFNγ induction was apparently still sufficient to provide the same beneficial therapeutic effects as after intravenous delivery (Fig. 1C). Nonetheless, systemic side effects were greatly reduced (Fig. 3), which may be explained by the absence of trAb plasma peaks and a hereby conveyed delay of cytokine release, which is also reduced at most time points (Figs. 4B and 5A).

The development of anti-drug antibodies could pose a possible limitation to subcutaneous therapy with trAbs because the skin contains numerous antigen-presenting cells, which could engulf subcutaneously injected trAb and present trAb-derived epitopes to the immune system. However, no increased anti-antibody titers were observed following subcutaneous therapy as compared with intravenous application (data not shown). After several therapy cycles, anti-drug antibodies may hamper the clinical use not only by neutralizing the trAb-dependent therapeutic effect, but also by forming aggregates that could lead to severe adverse events in vivo. However, as trAbs are typically used in extremely low amounts, such effects have not been observed in clinical trials so far (8, 18, 19). Our preclinical studies demonstrate that application of much higher doses in humans would be feasible, provided that the appropriate application route is chosen. In contrast to intravenously treated animals, subcutaneously treated mice did never show any reaction related to immunogenicity of Surek despite identical amounts of anti-antibodies. This can be explained by the different maximum concentrations reached after trAb injection: After intravenous injection, high trAb doses are immediately available and can form aggregates with anti-antibodies that were induced before and are circulating in the bloodstream. After subcutaneous delivery of Surek, by contrast, the absence of plasma peaks and lower trAb concentrations lead to formation of less aggregates despite identical amounts of anti-antibodies. These data further support the assumption that subcutaneous application of Surek is better tolerable than intravenous delivery and open the possibility to apply high trAb concentrations in multiple treatment cycles. Indeed, a third subcutaneous injection of Surek was feasible and increased the overall survival (Fig. 2), while a third intravenous injection of trAb led to severe adverse events in mice.

The regulatory cytokine IL10 is known to mediate counter-regulatory signals and to suppress Th1-driven antitumor responses (29, 30). Interestingly, the plasma concentration of IL10 was higher in intravenously treated animals compared with subcutaneously injected mice (Fig. 5B). Intracellular FACS staining revealed that this difference was accounted for by CD8+IL10+ T cells, which were increased in intravenously treated animals compared with subcutaneously treated mice (Fig. 5C). Besides, CD4+ and CD8+ T cells of subcutaneously treated animals express significantly less CTLA-4 (Fig. 6), which is upregulated as a counter-regulatory mechanism during an immune response (31, 32). These findings indicate that the balance between T-cell activation and suppression is shifted after subcutaneous administration, thus enabling potent tumor rejection despite lower bioavailability.

Other monoclonal antibodies, which are already routinely used in the clinic, like trastuzumab or alemtuzumab, were also effective despite slower absorption rates when delivered subcutaneously (33, 34). To facilitate absorption of trastuzumab, a subcutaneous formulation containing recombinant hyaluronidase was developed (35). For rituximab, the subcutaneous application has also been approved (36). In our study, we provide for the first time, a systematic comparison of the application routes by using a trAb. Given the safety concerns emanating from the high tumoricidal efficacy of these constructs, our results are of particular relevance for refining therapeutic approaches using other monoclonal antibodies.

In summary, the efficacy of subcutaneously delivered Surek is comparable with intravenous administration (Fig. 2), and the safety profile may be even better than observed after intravenous injection (Fig. 3). The availability of a subcutaneous formulation dramatically simplifies trAb administration and thus opens completely new perspectives of redirecting immunity via trAbs in the future. As subcutaneous administration presents a well-tolerated treatment, trAb may additionally be applicable in a home setting after dose escalation (37, 38). This would increase patients’ quality of life and might also reduce costs in the health care sector.

Disclosure of Potential Conflicts of Interest

H. Lindhofer is a CEO/CSO at Lindis Biotech and has ownership interest (including patents) in a patent application. No potential conflicts of interest were disclosed by the other authors.

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