



Genetically Modified DR5-Specific TRAIL Variant DR5-B Revealed Dual Antitumor and Protumoral Effect in Colon Cancer Xenografts and an Improved Pharmacokinetic Profile

Anne V. Yagolovich^{a,b}, Artem A. Artykov^{a,b}, Tatiana A. Karmakova^c, Maria S. Vorontsova^c, Andrey A. Pankratov^c, Alexander A. Andreev-Andrievsky^b, Dmitry A. Dolgikh^{a,b}, Mikhail P. Kirpichnikov^{a,b}, Marine E. Gasparian^{a,*}

^a Department of Bioengineering, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, Russia

^b Faculty of Biology, Lomonosov Moscow State University, Moscow, Russia

^c Department of modifiers and protectors of antitumor therapy, National Medical Research Radiological Center of the Ministry of Health of the Russian Federation, Moscow, Russia

ARTICLE INFO

Article history:

Received 20 January 2020

Received in revised form 11 March 2020

Accepted 14 March 2020

Available online xxx

ABSTRACT

Despite the weak clinical efficacy of TRAIL death receptor agonists, a search is under way for new agents that more efficiently activate apoptotic signaling. We previously created a TRAIL DR5-selective variant DR5-B without affinity for the DR4, DcR1, DcR2, and OPG receptors and increased proapoptotic activity in tumor cells. Here we showed that DR5-B significantly inhibited tumor growth in HCT116 and Caco-2 but not in HT-29 xenografts. The antitumor activity of DR5-B was 2.5 times higher in HCT116 xenografts compared to TRAIL. DR5-B at a dose of 2 or 10 mg/kg/d for 10 days inhibited tumor growth in HCT116 xenografts by 26% or 50% respectively, and increased animal survival. Unexpectedly, DR5-B at a higher dose (25 mg/kg/d) inhibited tumor growth only during the first 8 days of drug exposure, while at the end of the monitoring, no effect or even slight stimulation of tumor growth was observed. The pharmacokinetic parameters of DR5-B were comparable to those of TRAIL, except that the half-life was 3.5 times higher. Thus, enhancing TRAIL selectivity to DR5 may increase both antitumor and proliferative activities depending on the concentration and administration regimens.

Introduction

Cytokine TRAIL induces apoptosis in transformed cell lines without affecting normal cells, being a potentially valuable candidate for treatment of malignant neoplasms [1]. At least five TRAIL receptors have been identified, two of which, DR4 and DR5, are capable of transmitting an apoptosis signal, while so-called decoy receptors DcR1, DcR2, and soluble OPG block TRAIL-mediated apoptosis [2,3]. Moreover, it was shown that receptors DcR1 and DcR2 not only act in a cell-autonomous or cis-regulatory manner but also exert transcellular regulation [4].

The possible range of application of antitumor therapy using TRAIL death receptor agonists is wide since TRAIL death receptors are expressed in almost all types of tumors. TRAIL death receptor agonists have shown promising antitumor activity in a number of preclinical studies [5,6]. Clinical trials suggest that TRAIL or agonistic antibodies to death receptors are well tolerated and exhibit some antitumor efficacy [7–9]. However, the therapeutic effect of recombinant wild-type TRAIL (Dulanermin Genetech, USA) was limited to partial responses or disease stabilization [10–13].

According to recent phase III clinical trials, Dulanermin treatment resulted in increased tumor progression-free survival and an objective response rate only in combined action with cisplatin [14]. Novel apoptosis-inducing agents with higher potency for activation of death receptors, mainly to DR5, are in ongoing clinical trials for the treatment of cancer [15].

To date, several TRAIL mutant variants were obtained with altered affinity to death receptors [16–18]. All these mutant variants have improved *in vitro* cytotoxic activity, and some of them demonstrated slightly improved or comparable to TRAIL antitumor activity *in vivo* [18,19]. Previously, we have generated TRAIL mutant variant DR5-B, which selectively binds DR5 with no affinity to DR4, DcR1, DcR2, and OPG [20]. The recombinant mutant variant DR5-B showed increased proapoptotic activity compared to wild-type TRAIL *in vitro* in tumor cell lines of various origins, either alone or in combination with chemotherapeutic drugs [21,22].

In the present study, we tested DR5-B in human colon cancer cell lines with different sensibility to TRAIL *in vitro* and *in vivo*. Antitumor activity of DR5-B was highly pronounced in HCT116 and Caco-2 but not in HT-29 xenografts. DR5-B inhibited HCT116 tumor xenograft growth more efficiently compared with wild-type TRAIL. However, at a higher dose (25 mg/kg/d), DR5-B inhibited tumor growth only during the first 8 days of exposure to the drug, while at later times in the monitoring period, no effect or even slight stimulation of tumor growth was observed. The

* Address all correspondence to: Marine E. Gasparian, Department of Bioengineering, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, Russia.

E-mail address: marine_gasparian@yahoo.com. (M.E. Gasparian).

pharmacokinetic parameters of DR5-B studied in mice and rabbits were comparable to those of TRAIL, except that the half-life was 3.5 times higher.

Materials and Methods

Cell Lines and Cell Culture Reagents

Human cancer cell lines of colorectal carcinoma HCT116 and Caco-2 were from ATCC (Washington, USA); cell lines of colorectal carcinoma HT-29 and T-lymphoblastic leukemia Jurkat were from the Institute of Cytology, Russian Academy of Sciences (St. Petersburg, Russia). Cell culture media (DMEM, RPMI1640), 0.05% trypsin-EDTA solution, and phosphate-buffered saline tablets were from PanEco (Russia). Fetal bovine serum was from HyClone (USA). WST1 was from Sigma-Aldrich (USA).

Antibodies

Monoclonal antibodies to the receptors DR4 (clone DR-4-02), DR5 (clone DR5-01-1), and mouse IgG1 isotype control (clone 15H6) antibodies were from GeneTex (USA). Secondary antibody Alexa Fluor 488 was from Thermo Scientific (USA). Monoclonal antibodies to TRAIL and goat anti-mouse peroxidase-conjugated IgG were from R&D Systems Inc. (USA). Recombinant extracellular domain of DR5 receptor was from R&D Systems (USA).

Animals

BALB/c nu/nu, female, 8-week-old mice were obtained from the Puschino Animal Breeding Facility, a branch of the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry Russian Academy of Sciences, accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International). MD, F1 hybrid (C57BL/6j × CBA) female 8- to 9-week-old mice were obtained from the Andreevka branch of Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency (Russia). Female 8- to 10-week-old Soviet chinchilla breed rabbits were from Krolinfo Ltd. (Russia). All animals were admitted with a veterinary passport and certificate of quality. All procedures for routine animal care were performed in accordance with standard operational procedures and the sanitary rules for the design, equipment, and maintenance of experimental biological clinics and the Laboratory Animals manual. Animal experiments were carried out in accordance with the EU Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 «On the protection of animals used for scientific purposes».

Expression and Purification of Recombinant Proteins TRAIL and DR5-B

Highly purified TRAIL and DR5-B recombinant proteins were obtained from *E. coli* SHuffle B strain as described previously [23]. Briefly, the cells were transformed by plasmid pET32a/sdr5-b or pET32a/strail. Cell cultures were grown at 28°C for 20 hours. Cells were disrupted by French Press (Spectronic Instruments Inc., USA) under a pressure of 2000 psi. TRAIL and DR5-B were purified from the soluble fraction of cytoplasmic proteins by immobilized metal-affinity chromatography on Ni-NTA agarose (Qiagen, USA), followed by ion exchange chromatography on SP Sepharose (GE Healthcare, Sweden). DR5-B and TRAIL were further purified on Pierce High Capacity Endotoxin Removal Resin (Thermo Fisher Scientific, USA). The total content of endotoxins in the purified preparations did not exceed 0.48 U/mg. Protein preparations were dialyzed against 150 mM NaCl for 24 hours at 4°C, sterilized by filtration, lyophilized, and stored at -70°C.

Cell Viability Assay

HCT116, Caco-2, and Jurkat cells were maintained in DMEM supplemented with 10% fetal bovine serum; HT-29 cell line was maintained in RPMI1640 supplemented with 10% fetal bovine serum at 37°C and 10%

CO₂. The cells were seeded in 96-well plates (1 × 10⁴ cells per well) and incubated for 24 hours with TRAIL or DR5-B. Colorimetric WST-1 assay was used for quantification of cell viability. WST-1 solution (Sigma Aldrich, USA) was added to each well, and after 2-hour incubation at 37°C, the optical density was measured at a wavelength of 450 nm subtracting the background at 655 nm using an iMark Microplate Absorbance Reader (Bio-Rad, USA).

Detection of Death Receptors Surface Expression by Flow Cytometry

For each sample, 2 × 10⁵ cells were maintained in culture medium in six-well plates for 24 hours. Cells were rinsed with PBS, detached from culture plates by 1 ml of 0.05% Trypsin-EDTA solution, and centrifuged at 1100 rpm for 5 minutes. After washing by ice-cold PBS with 1% BSA, cells were resuspended in 50 µl of PBS-BSA containing primary antibodies (10 µg/ml) to death receptors or a mouse IgG1 as an isotype control and incubated for 1 hour at 4°C with gentle agitation. Stained cells were washed twice and incubated with secondary antibody Alexa Fluor 488 (10 µg/ml) for 1 hour at 4°C in dark. After washing twice by PBS-BSA solution, cells were analyzed on a Cytoflex flow cytometer (Beckman Coulter, USA).

Xenograft Studies

Assessment of DR5-B and TRAIL effect *in vivo* was performed on a colon carcinoma xenograft model in BALB/c nu/nu nude mice. HCT116 (4 × 10⁶ cells per mouse) or Caco-2 (5 × 10⁶ cells per mouse) cells in Matrigel (BD Biosciences) or HT-29 cells (3 × 10⁶ cells per mouse) without Matrigel were inoculated subcutaneously in the right dorsal flank of 8-week-old mice. Tumors were developed in the absence of treatment until they were well established. DR5-B and vehicle were administered intravenously in the tail vein. Once tumors reached a volume of 0.26 ± 0.01 cm³, 0.23 ± 0.02 cm³, and 0.15 ± 0.02 cm³ in HCT116, HT-29, and Caco-2 xenografts, respectively, mice were randomly divided into groups. To determine the effective doses, DR5-B was administered to HCT116 xenografts in single doses of 2, 10, and 25 mg/kg/d in two exposure regimens. In regimen 1, the exposure was carried out daily for 10 days with an interval of 2 days after the fifth injection. In regimen 2, the exposure was carried out seven times every 3 days. In both regimens, control animals were injected with physiological saline in a volume, equivalent to the volume of administered DR5-B. During the observation time, general condition and animal weight were monitored. Tumor volume was measured once (for Caco-2 xenografts) or twice a week (for HCT116 and HT-29 xenografts) by percutaneous measurement of tumor formations using a caliper in three mutually perpendicular projections and calculated by formula $V = \pi/6 \times (\text{width})^2 \times (\text{length})$. The tumor growth inhibition rate was calculated by formula $[(V_c - V_{ex})/V_c] \times 100\%$, where V_c is the median tumor volume in animals of experimental group and V_{ex} is the median tumor volume in animals of control group. In animals with tumors, a decision concerning euthanasia was based on the assessment of the general condition, measuring the tumor size, critical loss in body weight, and the severity of tumor necrosis.

Histology

Dissected tumors were fixed in 10% neutral-buffered formalin, routinely processed, and embedded in paraffin. Paraffin 4-µm sections were stained with hematoxylin and eosin for histopathological examination. The sections were analyzed on Olympus BX51 microscope (Olympus Corporation, Japan).

Mouse Pharmacokinetics

Female F1 hybrid C57BL/6j × CBA mice ($n = 16$, body weight 20 ± 1 g, laboratory animal nursery Puschino, Russia) were used for pharmacokinetic studies. DR5-B was administered intravenously *via* the tail as a single bolus at doses of 5, 10, or 20 mg/kg/d (100, 200, or 400 µl of 1 mg/ml solution, respectively, $n = 5$ per each dose). Blood samples

(300–400 µl) were collected between 5 minutes and 4 hours after the DR5-B injection ($n = 5$ per time point) from the inferior vena cava at sacrifice. Blood was allowed to clot at room temperature, and the serum was harvested and stored at -70°C for further analysis of DR5-B concentrations by enzyme-linked immunosorbent assay (ELISA).

Rabbit Pharmacokinetics

DR5-B was administered to female Soviet chinchilla breed rabbits ($n = 3$, body weight 2.9 ± 0.2 kg) at an equitherapeutic dose of 1.8 mg/kg/d intravenously via the lateral ear vein (5.4 ml of 1 mg/ml solution). Serial blood samples (500 µl) were collected between 5 minutes and 4 hours postdose from the lateral ear vein. Blood was processed to serum and stored at -70°C until analyzed for DR5-B concentration.

DR5-B ELISA

Recombinant extracellular domain of the DR5 receptor (R&D Systems Inc., USA) was immobilized on ELISA plates overnight at 4°C at concentration of 1 µg/ml in sodium phosphate buffer (PBS). The plates were washed three times with PBST (PBS + 0.05% Tween), and wells were blocked by 2% BSA in PBST for 1 hour at room temperature. After blocking, serum samples (in four replicates) or recombinant DR5-B standard at concentration rate of 5 to 500 ng/ml was added, and the plates were incubated for 1 hour at 37°C . Captured DR5-B was detected by subsequent incubation with monoclonal antibodies to TRAIL (MAB375, R&D systems, USA) and mouse IgG (HAF007, R&D Systems, USA) conjugated with horseradish peroxidase. Unbound antibodies were washed three times with PBST buffer, and color was developed by OPD colorimetric substrate. After 15-minute incubation with substrate, reaction was stopped by 1 N H_2SO_4 solution. The optical density was determined at 490 nm by iMark spectrophotometer (Bio-Rad, USA). DR5-B concentrations in the samples were extrapolated from a four-parameter fit of the DR5-B standard curve.

Noncompartmental Pharmacokinetics Analysis

Pharmacokinetic parameters of DR5-B, including area under the plasma concentration-time curve (AUC), apparent total volume of distribution, clearance, and serum half-life, were estimated by standard noncompartmental methods. The area under the first moment curve (AUMC) was calculated by the trapezoidal rule. The mean residence time (MRT) was calculated as $\text{AUMC}_{0-\infty}/\text{AUC}_{0-\infty}$. In mice, the averaged data were used, and in rabbits, individual data were analyzed. Calculation of pharmacokinetic parameters was performed by Microsoft Excel (version 16.3) and GraphPad Prism (version 8.0).

Statistical Analysis

Data are presented as mean \pm standard deviation. Significances of differences were determined using the Student's *t* test or Mann-Whitney *U* test using Statistica (version 8.0) and Microsoft Office Excel 2013 (USA) software. Differences between groups were analyzed by one- or two-way analysis of variance (ANOVA). Survival curves were drawn according to Kaplan-Meier analysis. *P* values $< .05$ were considered statistically significant.

Results

In Vitro Activity of TRAIL and DR5-B on Colorectal Carcinoma Cells

Cytotoxic activity of recombinant TRAIL and DR5 selective variant DR5-B was tested in human colorectal carcinoma cell lines HCT116, Caco-2, and HT-29. All these lines expressed death receptors DR4 and DR5 on the cell surface (Figure 1, A and B). HCT116 cells were sensitive to both TRAIL and DR5-B (65% and 70% of maximum viability reduction for TRAIL and DR5-B, respectively). However, EC_{50} value for DR5-B was

0.279 ± 0.02 ng/ml, which is 102-fold lower compared to TRAIL ($\text{EC}_{50} = 28.6 \pm 0.5$ ng/ml). Caco-2 cell lines were practically resistant to wild-type TRAIL, while DR5-B inhibited the growth of Caco-2 cells by 60% at concentrations above 100 ng/ml. HT-29 cells were practically resistant to both TRAIL variants (Figure 1C).

Suppression of the Growth of Various Colon Cancer Xenografts by DR5-B

Before testing the antitumor activity of DR5-B, histological studies of established tumors in BALB/c nu/nu mice were done. Histological sections of HCT116, HT-29 and Caco-2 subcutaneous xenograft tumors showed significant difference in tumor formation by different cell lines (Figure 2A). Seventeen days after the introduction of the cells, the tumor size was 3.4 ± 0.17 cm³ and 1.72 ± 0.11 cm³ for xenografts HCT116 and HT-29, respectively. Caco-2 xenografts grew quite slowly forming tumors 2.0 ± 0.16 cm³ in size after 60 days of injection. While HT-29 formed tumors without Matrigel, HCT116 and Caco-2 needed Matrigel for tumor formation. HCT116 formed low-grade tumors with homogeneous structure. HT-29 formed moderately differentiated tumors containing cricoid cells with intracellular accumulation of mucus during the tumor growth, which may indicate changes in differentiation. Caco-2 cells formed slow-growing heterogeneous tumors with prevailing tubular-glandular structures and high or moderate degree of differentiation, which are the likely cause of its retarded/impaired growth.

Antitumor activity of DR5-B was analyzed *in vivo* in HCT116, Caco-2, and HT-29 xenografts (Figure 2, B and C). Treatment was started on the 6th, 10th, and 14th day after the inoculation of HCT116, HT-29, and Caco-2 cells, respectively. DR5-B was administered intravenously at a dose of 10 mg/kg/d for 10 days with a break of 2 days after the fifth injection. The animals with HCT116 and HT-29 xenografts were monitored for 30 days (twice a week) and those with Caco-2 xenografts were monitored for 65 days (once in 7–8 days) from the start of exposure. DR5-B effectively reduced tumor volume in HCT116 xenografts ($P < .0001$). Inhibition of tumor growth was 37% to 47%, and this effect was stably maintained from 8 to 30 days after the start of exposure. In Caco-2 xenografts, DR5-B inhibited tumor growth by 28%–34% ($P < .0001$), and this trend continued after exposure was completed until the end of the observation. An insignificant and short-term delay in tumor growth (14%–22%) was observed in 8–10 days after the start of DR5-B exposure in HT-29 xenografts ($P = .6787$). However, later, this effect decreased and was almost imperceptible at the end of the experiment. Thus, DR5-B antitumor activity on xenograft models *in vivo* is in good agreement with its cytotoxic activity *in vitro* (Figure 1).

Comparative Analysis of TRAIL and DR5-B Antitumor Activity

Recombinant human TRAIL and DR5-B were expressed and purified as described previously [23]. The antitumor activity of TRAIL and DR5-B was compared on HCT116 xenografts in BALB/c nu/nu mice at a dose of 10 mg/kg/d administered six times every 3 days. Mice were inoculated with 4×10^6 cells, and once the average size of the tumors reached 0.26 ± 0.1 cm³ (on day 6), vehicle or TRAIL and DR5-B were injected intravenously six times every 3 days. All animals ($n = 5$ per group) were alive during monitoring period (29 days).

The inhibition of tumor growth by DR5-B and TRAIL ranged from 39% to 60% and from 12% to 23%, respectively, for the entire observation period. *P* values for treated groups with DR5-B and TRAIL versus vehicle control were $P < .0001$ and $P = .0012$, respectively (Figure 3). On day 29, while the average tumor size in the vehicle-treated group reached 755.2 mm³, in DR5-B-treated mice, the average tumor size was only 443.2 mm³, and in mice treated with TRAIL, it was 645.1 mm³. Thus, the average inhibition of tumor growth using DR5-B was 2.5 times higher compared to TRAIL.

Dose-Dependent Activity of DR5-B In Vivo

Dose-dependent antitumor efficacy of DR5-B was analyzed in HCT116 xenograft model. Subcutaneous HCT116 xenografts in BALB/c nu/nu

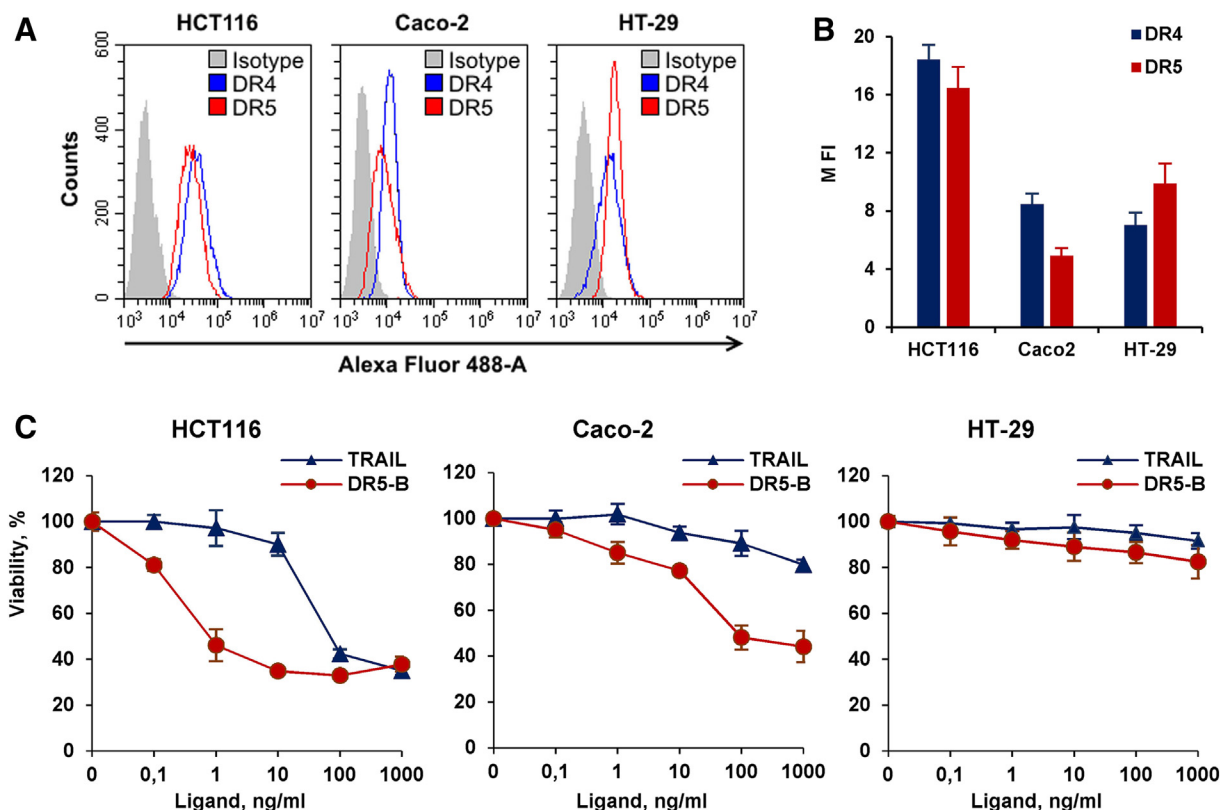


Figure 1. Cytotoxic activity of TRAIL and DR5-B in human colorectal carcinoma cells. (A) Death receptor surface expression in HCT116, HT29, and Caco-2 cell lines was analyzed by flow cytometry. (B) Median fluorescent intensity of the surface death receptors. (C) HCT116, Caco-2, and HT-29 cells were incubated with TRAIL or DR5-B at the indicated concentrations for 24 hours, and cell survival was determined by WST-1 colorimetric assay.

mice were obtained by inoculation of 4×10^6 cells, and DR5-B administration was started when the average tumor volume reached $0.26 \pm 0.1 \text{ cm}^3$. The tumor-bearing mice were randomly divided into four groups ($n = 8$), and DR5-B was administered daily in three dosages (2, 10, and 25 mg/kg). Dose-dependent activity of DR5-B was studied in two regimens. In regimen 1, DR5-B was administered daily for 10 days with an interval of 2 days after the fifth injection, while in regimen 2, DR5-B was administered seven times every 3 days.

At a dose of 2 mg/kg/d, the inhibition of tumor growth by DR5-B was 33%-34% in the first week after the start of exposure in both regimens. However, after 8 days, inhibition decreased to 22%-26% in the regimen 1 ($P < .0001$) and reached statistically insignificant values in regimen 2 ($P = .0632$) (Figure 4, A and B). In both regimens, statistically significant inhibition of tumor growth ($P < .0001$) by DR5-B was observed at a dose of 10 mg/kg/d. The inhibition values ranged from 41% to 48% from the 8th to the 27th day after the start of drug administration in regimen 1. In regimen 2, a tendency toward deeper inhibition of tumor growth was observed from the 6th to 15th day (54%-65%). However, this effect decreased to 29% in the end of monitoring period.

Inhibition values were 41%-48% from the 8th to the 27th day after the start of taking the drug in regimen 1. In regimen 2, there was a tendency to a deeper inhibition of tumor growth from 6 to 15 days (54%-65%). However, this effect decreased to 29% at the end of the monitoring period.

Surprisingly, DR5-B was less effective at a higher dose (25 mg/kg/d) in both regimens. Inhibition of tumor growth was observed only during first 8 days after drug exposure. Moreover, in regimen 1, stimulation of tumor growth (13%-15%) was observed in HCT116 xenografts ($P = .004$) (Figure 4B). The weight of the animals receiving any dose of DR5-B did not differ from the weight of the animals of the control group (data not shown), which indicates that the effect of the high dose was not associated with significant general toxicity. Besides growing evidences that TRAIL can trigger alternative prosurvival pathways [24], the induction of tumor

growth upon exposure to TRAIL variants or other death receptor agonists to our knowledge was not observed earlier in xenograft models. Recently, it has been shown that death receptor agonists, in addition to activating apoptotic signaling pathways in HCT116 cells, also activate noncanonical signaling pathways such as JNK, p38, and ERK1/ERK2. [25]. We performed additional experiments and demonstrated that the cytotoxic activity of DR5-B in HCT116 and Jurkat cells was significantly reduced at concentrations above 1000 ng/ml (Figure 5, A and B). This effect was not observed for TRAIL, although both recombinant proteins were prepared under identical conditions. A short exposure (1 hour) of DR5-B in high concentrations was not sufficient to achieve the reduction of the cytotoxic activity, indicating that constant ligand exposure is required for this process (Figure 5, C and D). Obviously, additional research is needed to find out the nature of this phenomenon.

Kaplan-Meier analysis of mice survival demonstrated an increase in survival at a dose of 10 mg/kg/d DR5-B ($P = .0021$ and $P = .0028$ in regimen 1 and regimen 2, respectively) (Figure 4C). The administration of DR5-B at a lower dose (2 mg/kg/d) increased animal survival only in regimen 1 ($P = .0016$), while the data in regimen 2 were statistically insignificant ($P = .0769$). Finally, in both regimens, there was no improvement in animal survival at a higher dose of 25 mg/kg/d ($P = .7561$ and $P = .1327$ in regimen 1 and regimen 2, respectively). Median survival was 34 days with the vehicle control and reached 42 days at doses of 2 mg/kg/d or 10 mg/kg/d of DR5-B in regimen 1. In regimen 2, median survival was 43 days at dose of 10 mg/kg/d of DR5-B and 35 days with the vehicle control.

Pharmacokinetics Study of DR5-B

As shown in Figure 6, DR5-B was detected in murine and rabbit serum for up to 4 hours after a single intravenous administration. The pharmacokinetic curves followed a distinct biexponential pattern. In mice, the AUC

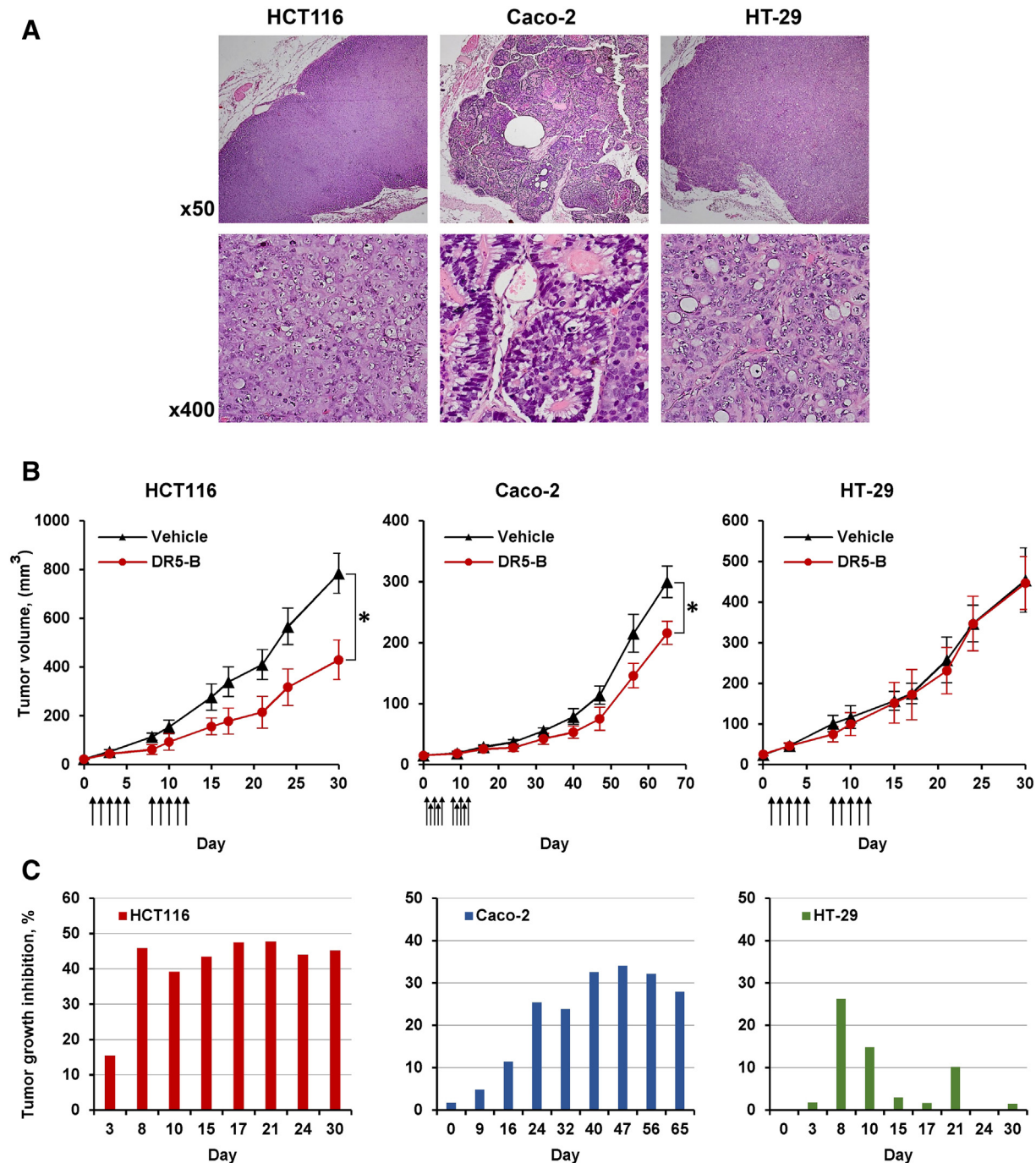


Figure 2. Antiproliferative effect of DR5-B in BALB/c-nu/nu mice xenograft models. (A) HCT116 (4×10^6 cells per mouse) and Caco-2 (5×10^6 cells per mouse) cells in Matrigel (BD Biosciences) or HT-29 cells (3×10^6 cells per mouse) without Matrigel were inoculated subcutaneously in the right dorsal flank of 8-week-old mice. Paraffin 4- μ m sections were stained with hematoxylin and eosin on 17 days (for HCT116 and HT-29) and 60 days (for Caco-2) of cell inoculation. (B) Effect of DR5-B on the kinetics of tumor growth in HCT116, Caco-2, and HT-29 xenografts. After cell inoculation (6, 10, and 14 days for HCT116, HT-29, and Caco-2, respectively), animals ($n = 5$ /group) were treated with DR5-B (intravenous administration in a dose of 10 mg/kg/d in cycles of 5 days with 2-day interval) or vehicle. Arrows show the scheme of drug administration. Significance between groups was tested using one- or two-way ANOVA; $*P < .0001$. (C) Values of tumor growth inhibition were calculated by the formula $[(V_c - V_{ex})/V_c] \times 100\%$, where V_{ex} and V_c are the average tumor volumes in the experimental and control groups.

was proportional to the administered dose (Table 1), while the DR5-B clearance was similar in the dose range of 5–20 mg/kg/d (the slope of clearance dependence on the dose did not differ from zero, $F_{1,1} = 25.22$, $P = .1252$). Mean residence time varied within tens of minutes in range in mice, while the distribution volume in both mice and rabbits was numerically close to blood volume (Table 1). DR5-B rapid elimination is probably governed by renal excretion, as can be inferred from the pharmacokinetic curves (Figure 6) and the rapid clearance, as well as the lower clearance in rabbits

as compared to mice. In pharmacokinetic study of TRAIL, performed in several rodent and primate species, renal clearance was suggested as the mean route of protein elimination, explaining for the good interspecies scaling found over three orders of magnitude [26]. The half-life of DR5-B in mice is 12.54 minutes, which is 3.44 fold higher compared with TRAIL (3.64 minutes). We suggest that a higher receptor specificity of DR5-B and, therefore, lower tissue binding may be one of the factors contributing to a longer half-life of this protein.

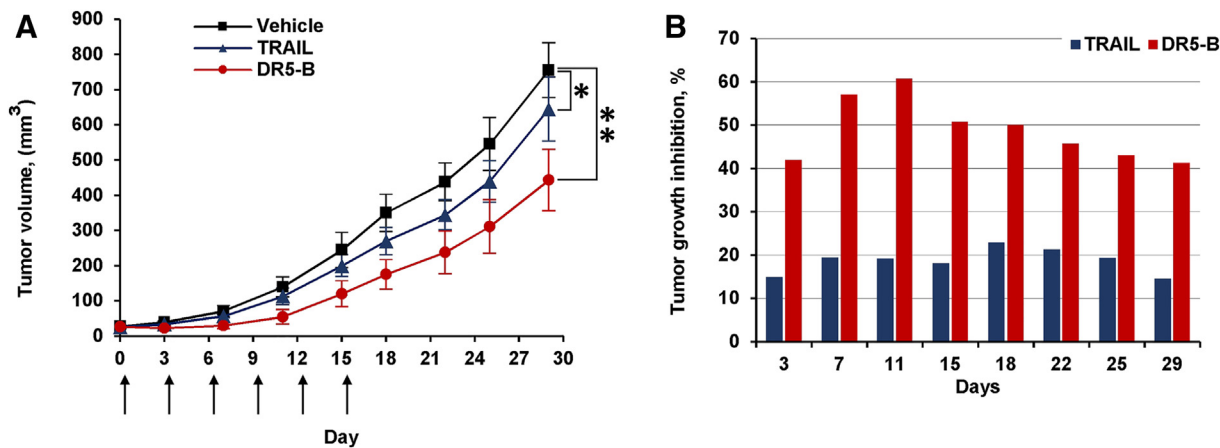


Figure 3. Comparison of TRAIL and DR5-B antitumor activity. HCT116 xenograft tumors were established in Balb/c nu/nu mice by subcutaneous injection of 4×10^6 cells. When tumor volume reached $0.26 \pm 0.01 \text{ cm}^3$, mice were treated by intravenous injection of 10 mg/kg/d TRAIL or DR5-B (six times every 3 days). Arrows show the scheme of drug administration. (A) Tumor volumes were determined every 3 days by percutaneous measurement using a caliper. Significance between groups was tested using one- or two-way ANOVA, $**P < .0001$, $*P = .0012$. (B) Values of tumor growth inhibition were calculated by the formula $[(V_c - V_{ex})/V_c] \times 100\%$, where V_{ex} and V_c are the average tumor volumes in experimental and control groups.

Discussion

During the last 15 years, a variety of TRAIL death receptor agonists, including recombinant TRAIL variants and agonistic death receptor-targeted antibodies, have been developed and tested in preclinical and clinical studies [9]. However, most of them showed weak therapeutic efficacy in clinics. Although the low effectiveness of the TRAIL cytokine can be explained by the short half-life, agonistic antibodies exhibiting a significantly longer half-life also showed limited antitumor efficacy [27]. In addition, monoclonal antibody target distinct receptor in tumor cells, whereas soluble TRAIL interacts with full range of its receptors. Therefore, soluble TRAIL has a wider target range but unpredictable level of apoptosis-inducing activity compared to highly specific antibodies. There is still no certainty about what may be preferable in a clinic: TRAIL variants or agonistic antibodies to DRs. Anyway, death receptor-induced apoptosis is still attractive for antitumor therapy due to its tumor specificity and safety compared with most chemotherapeutic drugs. As a result, new death receptor-targeting agents including TRAIL-conjugated magnetic, PLGA and HSA nanoparticles, PEG liposomes are being developed to make TRAIL-based therapy more effective [28,29]. Interestingly, the nanoparticles that carry the TRAIL and E-selectin proteins are able to form “TRAIL coated leukocytes” in the bloodstream and effectively target metastatic cancer cells [30].

Preclinical studies demonstrated that antitumor efficiency of TRAIL monotherapy is limited in many type of tumors. Tumor resistance can be overcome by combining TRAIL with other chemotherapeutic agents or by modification to increase apoptotic activity [31–33]. To enhance the cytotoxicity of TRAIL, we previously generated recombinant DR5-specific TRAIL mutant variant DR5-B with six substitutions of amino acid residues Y189N/R191K/Q193R/H264R/I266L/D269H, which selectively binds only to DR5 without showing affinity for other TRAIL receptors (DR4, DcR1, DcR2, and OPG) [20]. DR5-B demonstrated enhanced apoptotic activity over wild-type TRAIL in cancer cell lines of various origins, either alone or in combination with chemotherapeutic drugs [21,22].

In the present work, we studied the antitumor activity of DR5-B in colon cancer xenograft models. DR5-B demonstrated antitumor activity in HCT116 and Caco-2 but not in HT-29 xenografts. Average tumor growth inhibition was 37%–47% and 28%–34% in HCT116 and Caco-2 xenografts, respectively, and did not decrease within at least 20 days after discontinuation of the drug. The results are in good agreement with the sensitivity of these cell lines to DR5-B in experiments *in vitro*. We also showed that DR5-B inhibits HCT116 xenograft tumor growth approximately 2.5 times more effectively compared with TRAIL, considering that both proteins were

expressed and purified under identical conditions. The obvious reason is that DR5-B does not bind to decoy receptors that inhibit TRAIL-induced tumor regression in nude mice [33].

Notably, we obtained unusual results on the dose-dependent antitumor activity of DR5-B on HCT116 xenografts. When DR5-B was administered at doses of 2 mg/kg/d and 10 mg/kg/d, inhibition of tumor growth was observed during the entire monitoring period (27 days), whereas at a higher dose of 25 mg/kg/d, DR5-B inhibited tumor growth only during the first 8 days of exposure in two tested regimens. Moreover, in regimen 1 (two 5-day cycles with an interval of 2 days), the higher dose of DR5-B caused an average 15% acceleration of tumor growth ($P = .004$) within at least 15 days after discontinuation of the drug. Such effect was not observed earlier with recombinant TRAIL administered at up to 60 mg/kg/d in xenografts of human non-Hodgkin lymphoma and lung and colorectal carcinoma [33–36]. Acceleration of tumor growth under the influence of TRAIL was described only in the murine orthotopic model of pancreatic cancer during therapy with murine recombinant TRAIL [37]. Mice possess only one death receptor (mDR5), more homologous to human DR5 than to DR4, which is capable to transduce TRAIL apoptotic signal. Although the two TRAIL death receptors can transmit apoptotic signal independently, their cooperation cannot be excluded, although there are not enough data for a final conclusion [38].

Additional experiments on HCT116 cell line *in vitro* revealed a decrease in the cytotoxic activity of DR5-B but not TRAIL with the higher concentration of ligand ($>100 \text{ ng/ml}$). At higher concentrations, it appears that DR5-B signaling shifts from proapoptotic to prosurvival. Recently, it was demonstrated that DR5 is able to assemble proapoptotic/prosurvival platforms that propagate TRAIL signaling to both death and survival pathways in clonal populations of cancer cells [24,39]. It has been shown that, in addition to its role as a proapoptotic molecule, TRAIL can activate an NF- κ B-dependent proinflammatory program resulting in the expression and secretion of multiple cytokines and chemokines in diverse cell types [39]. Shifting the TRAIL response from apoptosis to prosurvival is likely a strategy of cancer cells to survive and proliferate *in vivo*. Moreover, evidence has recently been presented that when TRAIL binds to DR at the plasma membrane, the receptors can translocate to the nucleus [40], and nuclear DR5 can counteract apoptosis and promote cell proliferation [41]. Based on our data, it can be assumed that in the absence of DR4 and decoy receptors signaling, prolonged exposure to DR5-B leads to activation of both apoptotic and proliferative or prosurvival properties of DR5. In addition, many studies have shown that TRAIL regulated immune system homeostasis [42,43]. In mouse models, TRAIL modulated the tumor microenvironment toward a more immunosuppressive type and thereby

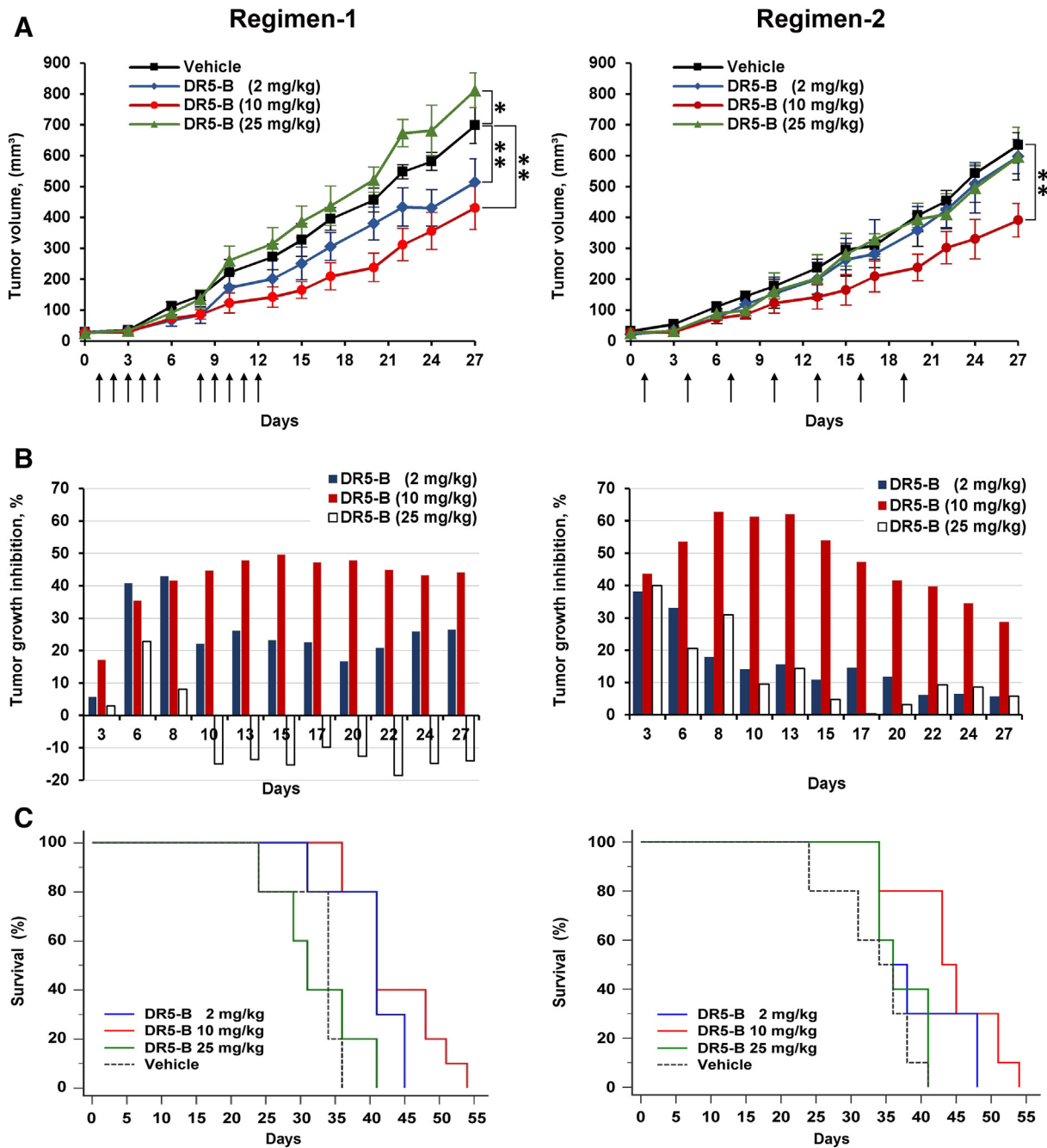


Figure 4. Dose-dependent effect of DR5-B on growth of established HCT116 tumors. (A) HCT116 cells were inoculated into BALB/c nu/nu mice and after 6 days (tumor volume $0.26 \pm 0.01 \text{ cm}^3$) were divided to groups ($n = 8$). DR5-B was administrated intravenously in two regimens at dosages of 2, 10, and 25 mg/kg/d (at 7, 8, 9, 10, 11 and 14, 15, 16, 17, 18 days in regimen 1, and 7 injections with an interval of 3 days in regimen 2). Arrows show the scheme of drug administration. Tumor volumes were determined twice a week. Significance between groups was tested using one- or two-way ANOVA, $**P < .0001$, $*P = .004$. (B) Values of tumor growth inhibition were calculated by the formula $[(V_c - V_{ex})/V_c] \times 100\%$, where V_{ex} and V_c are the average tumor volumes in the experimental and control groups. (C) Survival curves of mice treated with DR5-B at indicated doses were drawn up by Kaplan-Meier analysis using Medcalc version 19.1.3 software. Significance between vehicles and DR5-B-treated groups was: at a dose of 10 mg/kg/d $P = .0021$ and $P = .0028$, at a dose 2 mg/kg/d $P = .0016$, and $P = .0769$, at a dose of 25 mg/kg/d $P = .7561$ and $P = .1327$ in regimen 1 and regimen 2, respectively.

contributed to tumor growth. Tumor cells can respond to TRAIL by expressing or secreting factors that inhibit apoptosis induced by immune effector cells, or even destroy immune cells using TRAIL/TRAIL-R signaling pathway. BALB/c nu/nu mice lack thymus and therefore are not able to produce T cells. However, they still produce macrophages and natural killer cells. Thus, it can also be assumed that exposure to DR5-B can lead to immunosuppression in the microenvironment of the tumor and promote tumor growth. Additional experiments are needed to understand the mechanisms for simultaneously inducing death and survival signaling via DR 5-B.

The pharmacokinetic parameters of DR5-B studied in mice and rabbits were comparable to those of TRAIL [26]; however, the half-life of DR5-B in mice (12.54 minutes) was approximately 3.5-fold higher compared with TRAIL (3.64 minutes). In rabbits, half-life of DR5-B was 90.6 minutes, which is also 3.7-fold higher if comparing with TRAIL in primates (23-24 minutes) [26].

We speculate that higher receptor specificity of DR5-B compared with Apo2L/TRAIL and thus the lower DR5-B tissue binding can be one of the factors contributing to the longer half-life of this protein.

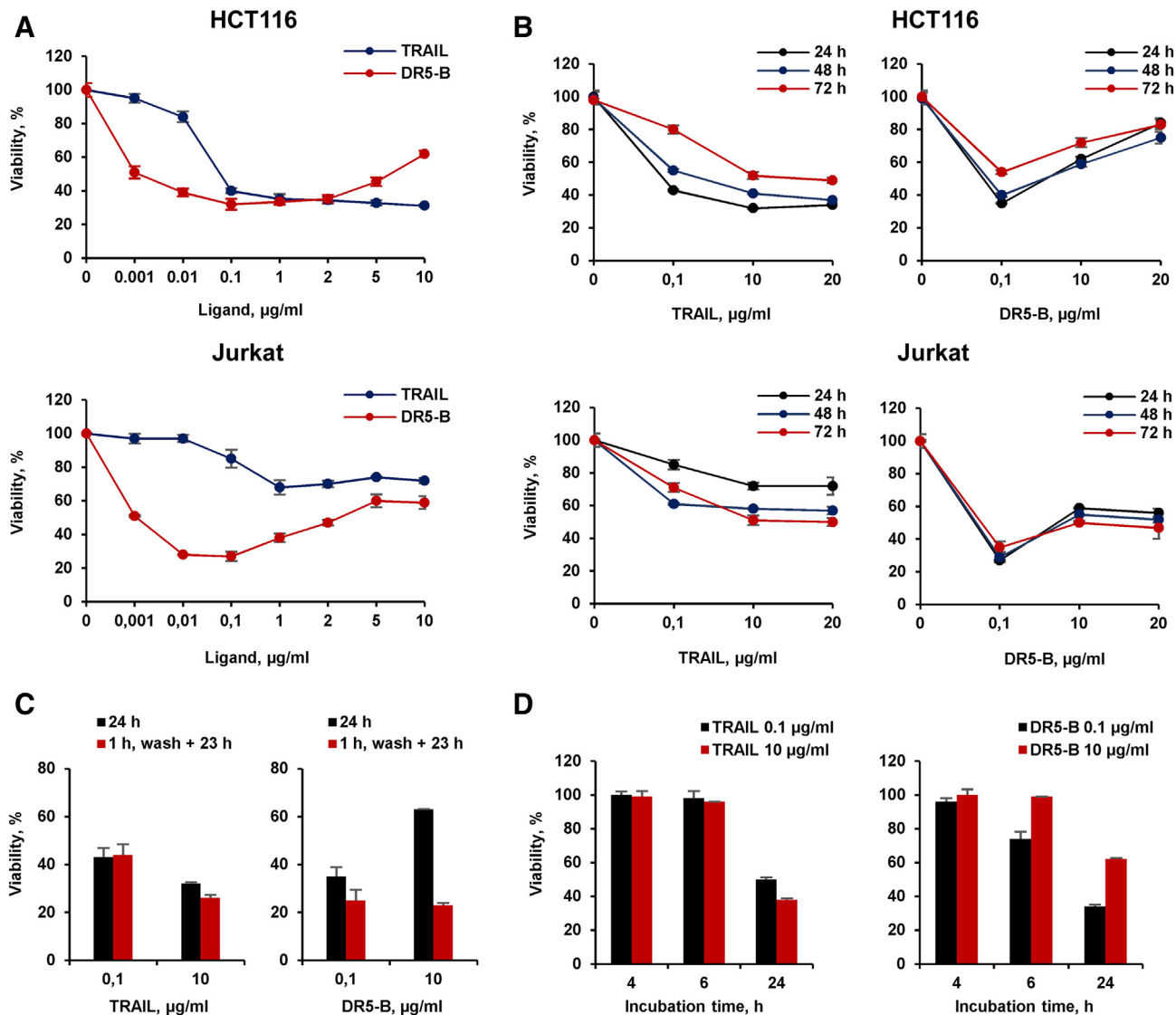


Figure 5. Influence of high concentrations of TRAIL and DR5-B on HCT116 and Jurkat cells viability. Cells (1×10^4 and 5×10^4 cells per well for HCT116 and Jurkat, respectively) were incubated with indicated concentrations of ligands for 24 hours (A) or for 24, 48, and 72 hours (B). (C) HCT116 cells were stimulated with either TRAIL or DR5-B at concentrations of 0.1 µg/ml or 10 µg/ml for 1 h, washed with ligand-free medium, and incubated for another 23 hours. (D) HCT116 cells survival over time when exposed to TRAIL or DR5-B. In all experiments, cells were maintained in serum-free medium at ligands addition. Cell viability was determined by WST-1 colorimetric assay.

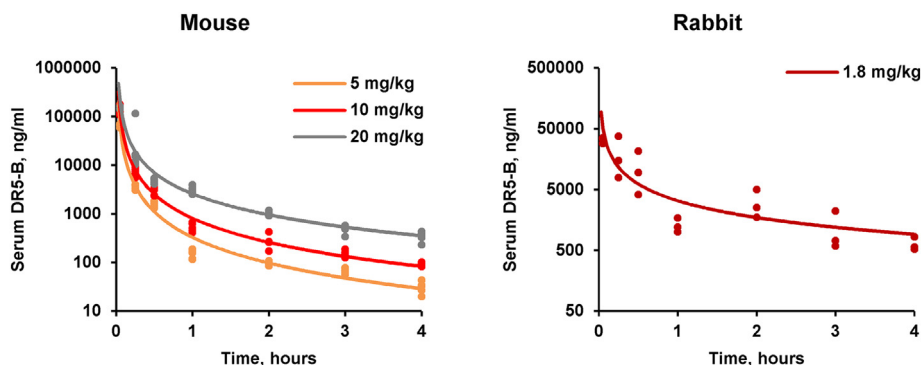


Figure 6. Pharmacokinetic curves of DR5-B upon intravenous administration in mice ($n = 5$ per time point per dose) and rabbits ($n = 3$). Serum DR5-B level was measured by ELISA.

Table 1
Noncompartmental Pharmacokinetic Parameters of DR5-B in Mice and Rabbits

Dose	Animal	C_{max}	$AUC_{0-\infty}$	$t_{1/2}$	CL	V_{ss}
mg/kg	no.	$\mu\text{g/ml}$	$\mu\text{g/ml/h}$	min	ml/kg/h	ml/kg
Mouse						
5	5	65.5 ± 2.0	9.6	10.68	523	134
10	5	125.2 ± 12.9	18.6	12.54	537	162
20	5	148.7 ± 3.7	32.4	22.62	618	336
Rabbit						
1.8	3	58.0 ± 2.5	22.1 ± 2.2	90.6 ± 15.2	83.2 ± 7.6	176 ± 11

C_{max} , maximum concentration; $AUC_{0-\infty}$, area under the concentration time curve; $t_{1/2}$, terminal half-life; CL, clearance; V_{ss} , volume of distribution.

In conclusion, it should be noted that targeting the TRAIL death receptor in cancer therapy obviously needs to be refined taking into account the still-growing list of their cell death-independent activities. Apparently, by selection of the drug dose and administration regimens, the predominance of survival signaling transmitted by death receptors can be avoided. Thus, DR5-B can be considered a promising candidate for the treatment of colon cancer at certain doses and the regimens.

Funding

This work was supported by federal program “Development of the pharmaceutical and medical industry of the Russian federation” of the Ministry of Education and Science of the Russian federation (contract 14.N08.11.0155), by the grant of the Presidium of the Russian Academy of Sciences “Molecular and Cell Biology,” and by the Russian Foundation for Basic Research (project #18-34-00812).

Declaration of Competing Interest

None.

References

- [1] A Ashkenazi, RC Pai, S Fong, S Leung, DA Lawrence, SA Marsters, C Blackie, L Chang, AE McMurtrey, A Hebert, et al., Safety and antitumor activity of recombinant soluble Apo2 ligand, *J. Clin. Invest.* 104 (1999) 155–162, <https://doi.org/10.1172/JCI6926>.
- [2] D Mérimo, N Lalaoui, A Morizot, P Schneider, E Solary, O Micheau, Differential inhibition of TRAIL-mediated DR5-DISC formation by decoy receptors 1 and 2, *Mol. Cell. Biol.* 26 (2006) 7046–7055, <https://doi.org/10.1128/MCB.00520-06>.
- [3] MED Chamuleau, GJ Ossenkoppele, A van Rhenen, L van Dreunen, SMG Jirka, A Zevenbergen, GJ Schuurhuis, AA van de Loosdrecht, High TRAIL-R3 expression on leukemic blasts is associated with poor outcome and induces apoptosis-resistance which can be overcome by targeting TRAIL-R2, *Leuk. Res.* 35 (2011) 741–749, <https://doi.org/10.1016/j.leukres.2010.12.032>.
- [4] L O’Leary, AM van der Sloot, CR Reis, S Deegan, AE Ryan, SPS Dhami, LS Murillo, RH Cool, P Correa de Sampaio, K Thompson, et al., Decoy receptors block TRAIL sensitivity at a supracellular level: the role of stromal cells in controlling tumour TRAIL sensitivity, *Oncogene* 35 (2016) 1261–1270, <https://doi.org/10.1038/ncr.2015.180>.
- [5] R. Trivedi, D.P. Mishra, Trailing TRAIL resistance: novel targets for TRAIL sensitization in cancer cells, *Front. Oncol.* 5 (2015). <https://doi.org/10.3389/fonc.2015.00069>.
- [6] A Ashkenazi, P Holland, SG Eckhardt, Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL), *J. Clin. Oncol.* 26 (2008) 3621–3630, <https://doi.org/10.1200/JCO.2007.15.7198>.
- [7] CY Cheah, D Belada, MA Fanale, A Janikova, MS Czucman, IW Flinn, AV Kapp, A Ashkenazi, S Kelley, GL Bray, et al., Dulanermin with rituximab in patients with relapsed indolent B-cell lymphoma: an open-label phase 1b/2 randomised study, *The Lancet Haematology.* 2 (2015) e166–e174, [https://doi.org/10.1016/S2352-3026\(15\)00026-5](https://doi.org/10.1016/S2352-3026(15)00026-5).
- [8] RS Herbst, SG Eckhardt, R Kurzrock, S Ebbinghaus, PJ O’Dwyer, MS Gordon, W Novotny, MA Goldwasser, TM Tohny, BL Lum, et al., Phase I dose-escalation study of recombinant human apo2L/trail, a dual proapoptotic receptor agonist, in patients with advanced cancer, *J. Clin. Oncol.* 28 (2010) 2839–2846, <https://doi.org/10.1200/JCO.2009.25.1991>.
- [9] PM Holland, Death receptor agonist therapies for cancer, which is the right TRAIL? *Cytokine Growth Factor Rev.* 25 (2014) 185–193, <https://doi.org/10.1016/j.cytogfr.2013.12.009>.
- [10] J-C Soria, E Smit, D Khayat, B Besse, X Yang, C-P Hsu, D Reese, J Wizezorek, F Blackhall, Phase 1b study of dulanermin (recombinant human Apo2L/TRAIL) in combination with paclitaxel, carboplatin, and bevacizumab in patients with advanced non-squamous non-small-cell lung cancer, *J. Clin. Oncol.* 28 (2010) 1527–1533, <https://doi.org/10.1200/JCO.2009.25.4847>.

- [11] J-C Soria, Z Márk, P Zatloukal, B Szima, I Albert, E Juhász, J-L Pujol, J Kozielski, N Baker, D Smethurst, et al., Randomized phase II study of dulanermin in combination with paclitaxel, carboplatin, and bevacizumab in advanced non-small-cell lung cancer, *J. Clin. Oncol.* 29 (2011) 4442–4451, <https://doi.org/10.1200/JCO.2011.37.2623>.
- [12] ZA Wainberg, WA Messersmith, PF Peddi, AV Kapp, A Ashkenazi, S Royer-Joo, CC Portera, MF Kozloff, A phase 1B study of dulanermin in combination with modified FOLFOX6 plus bevacizumab in patients with metastatic colorectal cancer, *Clin. Colorectal Cancer* 12 (2013) 248–254, <https://doi.org/10.1016/j.clcc.2013.06.002>.
- [13] B Lim, A Scicchitano, C Beachler, N Gusani, N Sarwani, Z Yang, K Staveley-O’Carroll, A Ashkenazi, C Portera, WS El-Deiry, FOLFIRI plus dulanermin (rhApo2L/TRAIL) in a patient with BRAF-mutant metastatic colon cancer, *Cancer Biol. Ther.* 14 (2013) 711–719, <https://doi.org/10.4161/cbt.25310>.
- [14] X Ouyang, M Shi, F Jie, Y Bai, P Shen, Z Yu, X Wang, C Huang, M Tao, Z Wang, et al., Phase III study of dulanermin (recombinant human tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand) combined with vinorelbine and cisplatin in patients with advanced non-small-cell lung cancer, *Invest. New Drugs* 36 (2018) 315–322, <https://doi.org/10.1007/s10637-017-0536-y>.
- [15] Greer Lim, Lipkowitz, Takebe, Novel apoptosis-inducing agents for the treatment of cancer, a new arsenal in the toolbox, *Cancers.* 11 (2019) 1087, <https://doi.org/10.3390/cancers11081087>.
- [16] RF Kelley, K Totpal, SH Lindstrom, M Mathieu, K Billeci, L DeForge, R Pai, SG Hymowitz, A Ashkenazi, Receptor-selective mutants of apoptosis-inducing ligand 2/ tumor necrosis factor-related apoptosis-inducing ligand reveal a greater contribution of death receptor (DR) 5 than DR4 to apoptosis signaling, *J. Biol. Chem.* 280 (2005) 2205–2212, <https://doi.org/10.1074/jbc.M410660200>.
- [17] AM van der Sloot, V Tur, E Szegezdi, MM Mullally, RH Cool, A Samali, L Serrano, WJ Quax, Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 8634–8639, <https://doi.org/10.1073/pnas.0510187103>.
- [18] CR Reis, AM van der Sloot, E Szegezdi, A Natoni, V Tur, RH Cool, A Samali, L Serrano, WJ Quax, Enhancement of antitumor properties of rhTRAIL by affinity increase toward its death receptors, *Biochemistry* 48 (2009) 2180–2191, <https://doi.org/10.1021/bi801927x>.
- [19] R Yu, SM Albarenque, RH Cool, WJ Quax, A Mohr, RM Zwacka, DR4 specific TRAIL variants are more efficacious than wild-type TRAIL in pancreatic cancer, *Cancer Biol. Ther.* 15 (2014) 1658–1666, <https://doi.org/10.4161/15384047.2014.972183>.
- [20] ME Gasparian, BV Chernyak, DA Dolgikh, AV Yagolovich, EN Popova, AM Sycheva, SA Moshkovskii, MP Kirpichnikov, Generation of new TRAIL mutants DR5-A and DR5-B with improved selectivity to death receptor 5, *Apoptosis* 14 (2009) 778–787, <https://doi.org/10.1007/s10495-009-0349-3>.
- [21] ME Gasparian, ML Bychkov, AV Yagolovich, DA Dolgikh, MP Kirpichnikov, Mutations enhancing selectivity of antitumor cytokine TRAIL to DR5 receptor increase its cytotoxicity against tumor cells, *Biochemistry (Mosc)* 80 (2015) 1080–1091, <https://doi.org/10.1134/S0006297915080143>.
- [22] ME Gasparian, ML Bychkov, AV Yagolovich, MP Kirpichnikov, DA Dolgikh, The effect of cisplatin on cytotoxicity of anticancer cytokine TRAIL and its receptor-selective mutant variant DR5-B1, *Dokl. Biochem. Biophys.* 477 (2017) 385–388, <https://doi.org/10.1134/S1607672917060114>.
- [23] AV Yagolovich, AA Artykov, DA Dolgikh, MP Kirpichnikov, ME Gasparian, A New efficient method for production of recombinant antitumor cytokine trail and its receptor-selective variant DR5-B, *Biochemistry (Mosc)* 84 (2019) 627–636, <https://doi.org/10.1134/S0006297919060051>.
- [24] D Siegmund, I Lang, H Wajant, Cell death-independent activities of the death receptors CD 95, TRAILR 1, and TRAILR 2, *FEBS J.* 284 (2017) 1131–1159, <https://doi.org/10.1111/febs.13968>.
- [25] A Willms, H Schitteck, S Rahn, J Sosna, U Mert, D Adam, A Trauzold, Impact of p53 status on TRAIL-mediated apoptotic and non-apoptotic signaling in cancer cells, *PLoS One* 14 (2019), e0214847, <https://doi.org/10.1371/journal.pone.0214847>.
- [26] SK Kelley, LA Harris, D Xie, L DeForge, K Totpal, J Bussiere, JA Fox, Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety, *J. Pharmacol. Exp. Ther.* 299 (2001) 31–38.
- [27] GP Amarante-Mendes, TS Griffith, Therapeutic applications of TRAIL receptor agonists in cancer and beyond, *Pharmacol. Ther.* 155 (2015) 117–131, <https://doi.org/10.1016/j.pharmthera.2015.09.001>.
- [28] D de Miguel, J Lemke, A Anel, H Walczak, L Martinez-Lostao, Onto better TRAILs for cancer treatment, *Cell Death Differ.* 23 (2016) 733–747, <https://doi.org/10.1038/cdd.2015.174>.
- [29] H Wajant, G Gerspach, K Pfizenmaier, Engineering death receptor ligands for cancer therapy, *Cancer Lett.* 332 (2013) 163–174, <https://doi.org/10.1016/j.canlet.2010.12.019>.
- [30] N. Jyotsana, Z. Zhang, L.E. Himmel, F. Yu, M.R. King, Minimal dosing of leukocyte targeting TRAIL decreases triple-negative breast cancer metastasis following tumor resection, *Sci Adv.* (2019) 5 eaaw4197, <https://doi.org/10.1126/sciadv.aaw4197>
- [31] E Brin, K Wu, E Dagostino, MM-C Kuo, Y He, W-J Shia, L-C Chen, M Stempniak, R Hickey, R Almassy, et al., TRAIL stabilization and cancer cell sensitization to its proapoptotic activity achieved through genetic fusion with arginine deiminase, *Oncotarget* 9 (2018) 36914–36928, <https://doi.org/10.18632/oncotarget.26398>.
- [32] W Ding, T Cai, H Zhu, R Wu, C Tu, L Yang, W Lu, Q He, B Yang, Synergistic antitumor effect of TRAIL in combination with sunitinib in vitro and in vivo, *Cancer Lett.* 293 (2010) 158–166, <https://doi.org/10.1016/j.canlet.2010.01.005>.
- [33] G Saturno, M Valenti, ADH Brandon, GV Thomas, S Eccles, PA Clarke, P Workman, Combining TRAIL with PI3 kinase or HSP90 inhibitors enhances apoptosis in colorectal cancer cells via suppression of survival signaling, *Oncotarget* 4 (2013) 1185–1198, <https://doi.org/10.18632/oncotarget.1162>.
- [34] R Ravi, AJ Jain, RD Schulick, V Pham, TS Prouser, H Allen, EG Mayer, H Yu, DM Pardoll, A Ashkenazi, et al., Elimination of hepatic metastases of colon cancer cells

- via p53-independent cross-talk between irinotecan and Apo2 ligand/TRAIL, *Cancer Res.* 64 (2004) 9105–9114, <https://doi.org/10.1158/0008-5472.CAN-04-2488>.
- [35] D Daniel, B Yang, DA Lawrence, K Totpal, I Balter, WP Lee, A Gogineni, MJ Cole, SF Yee, S Ross, et al., Cooperation of the proapoptotic receptor agonist rhApo2L/TRAIL with the CD20 antibody rituximab against non-Hodgkin lymphoma xenografts, *Blood* 110 (2007) 4037–4046, <https://doi.org/10.1182/blood-2007-02-076075>.
- [36] H Jin, R Yang, S Fong, K Totpal, D Lawrence, Z Zheng, J Ross, H Koeppen, R Schwall, A Ashkenazi, Apo2 ligand/tumor necrosis factor–related apoptosis-inducing ligand cooperates with chemotherapy to inhibit orthotopic lung tumor growth and improve survival, *Cancer Res.* 64 (2004) 4900–4905, <https://doi.org/10.1158/0008-5472.CAN-04-0408>.
- [37] K Beyer, L Normann, M Sendler, A Käding, C-D Heidecke, LI Partecke, W von Bernstorff, TRAIL promotes tumor growth in a syngeneic murine orthotopic pancreatic cancer model and affects the host immune response, *Pancreas* 45 (2016) 401–408, <https://doi.org/10.1097/MPA.0000000000000469>.
- [38] IAM van Roosmalen, WJ Quax, FAE Kruyt, Two death-inducing human TRAIL receptors to target in cancer: Similar or distinct regulation and function? *Biochem. Pharmacol.* 91 (2014) 447–456, <https://doi.org/10.1016/j.bcp.2014.08.010>.
- [39] Y Shlyakhtina, V Pavet, H Gronemeyer, Dual role of DR5 in death and survival signaling leads to TRAIL resistance in cancer cells, *Cell Death Dis.* 8 (2017) e3025, <https://doi.org/10.1038/cddis.2017.423>.
- [40] U Mert, A Adawy, E Scharff, P Teichmann, A Willms, V Haselmann, C Colmorgen, J Lemke, S von Karstedt, J Fritsch, et al., TRAIL induces nuclear translocation and chromatin localization of TRAIL death receptors, *Cancers.* 11 (2019), E1167. <https://doi.org/10.3390/cancers11081167>.
- [41] V Haselmann, A Kurz, U Bertsch, S Hübner, M Olempska–Müller, J Fritsch, R Häsler, A Pickl, H Fritsche, F Annenwarter, et al., Nuclear death receptor TRAIL-R2 inhibits maturation of Let-7 and promotes proliferation of pancreatic and other tumor cells, *Gastroenterology* 146 (2014) 278–290, <https://doi.org/10.1053/j.gastro.2013.10.009>.
- [42] M de Loeff, S de Jong, FAE Kruyt, Multiple interactions between cancer cells and the tumor microenvironment modulate TRAIL signaling: Implications for TRAIL receptor targeted therapy, *Front. Immunol.* 10 (2019) 1530, <https://doi.org/10.3389/fimmu.2019.01530>.
- [43] D. Sag, Z.O. Ayyildiz, S. Gunalp, G.Wingender, The role of TRAIL/DRs in the modulation of immune cells and responses, *Cancers (Basel).* 11 (2019) 1469. <https://doi.org/10.3390/cancers11101469>.