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ORIGINAL ARTICLE

Identification, characterization and potent antitumor activity of ECO-4601, a novel peripheral benzodiazepine receptor ligand

Henriette Gourdeau · James B. McAlpine · Maxime Ranger · Bryan Simard · Francois Berger · Francis Beaudry · Pierre Falardeau

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Abstract
Purpose ECO-4601 is a structurally novel farnesylated dibenzodiazepinone discovered through DECIPHER® technology, Thallion's proprietary drug discovery platform. The compound was shown to have a broad cytotoxic activity in the low micromolar range when tested in the NCI 60 cell line panel. In the work presented here, ECO-4601 was further evaluated against brain tumor cell lines. Preliminary mechanistic studies as well as in vivo antitumor evaluation were performed.
Methods Since ECO-4601 has a benzodiazepinone moiety, we first investigated if it binds the central and/or peripheral benzodiazepine receptors. ECO-4601 was tested in radioligand binding assays on benzodiazepine receptors obtained from rat hearts. The ability of ECO-4601 to inhibit the growth of CNS cancers was evaluated on a panel of mouse, rat and human glioma cell lines using a standard MTT assay. Antitumor efficacy studies were performed on gliomas (rat and human), human breast and human prostate mouse tumor xenografts. Antitumor activity and pharmacokinetic analysis of ECO-4601 was evaluated following intravenous (IV), subcutaneous (SC), and intraperitoneal (IP) bolus administrations.
Results ECO-4601 was shown to bind the peripheral but not the central benzodiazepine receptor and inhibited the growth of CNS tumor cell lines. Bolus SC and IP administration gave rise to low but sustained drug exposure, and resulted in moderate to significant antitumor activity at doses that were well tolerated. In a rat glioma (C6) xenograft model, ECO-4601 produced up to 70% tumor growth inhibition (TGI) while in a human glioma (U-87MG) xenograft, TGI was 34%. Antitumor activity was highly significant in both human hormone-independent breast (MDA-MB-231) and prostate (PC-3) xenografts, resulting in TGI of 72 and 100%, respectively. On the other hand, IV dosing was followed by rapid elimination of the drug and was ineffective.
Conclusions Antitumor efficacy of ECO-4601 appears to be associated with the exposure parameter AUC and/or sustained drug levels rather than C_{max}. These in vivo data constitute a rationale for clinical studies testing prolonged continuous administration of ECO-4601.

Keywords Dibenzodiazepine · Peripheral benzodiazepine receptor (PBR) · Cancer · Glioma · Xenografts

Abbreviations

AAALAC	International Association for Assessment and Accreditation of Laboratory Animal Care
AUC	Area under the curve
CCAC	Canadian Council on Animal Care
CBR; $GABA_A$	Central benzodiazepine receptor
D5W	5% dextrose

H. Gourdeau (✉) · J. B. McAlpine · M. Ranger · P. Falardeau
Thallion Pharmaceuticals Inc., 7150 Alexander-Fleming, St Laurent, QC, Canada H4S 2C8
e-mail: hgourdeau@thallion.com

B. Simard · F. Berger
INSERM U318, Grenoble, France

F. Beaudry
Department of Veterinary Biomedicine, Faculty of Veterinary Medicine, University of Montreal, St Hyacinthe, QC, Canada J2S 2M2

q3d × 7	Every 3 days for 7 cycles
IV	Intravenous
IP	Intraperitoneal
MPTP	Mitochondrial permeability transition pore
q1d × 5	Once daily for 5 consecutive days
qod	Once every other day
PBR	Peripheral benzodiazepine receptor
SC	Subcutaneous

Introduction

ECO-4601 (4,6,8-trihydroxy-10-(3,7,11-trimethyldodeca-2,6,10-trienyl)-5,10-dihydrodibenzo[*b,e*] [1, 4] diazepin-11-one) is a structurally novel farnesylated dibenzodiazepinone (MW 462.58) discovered using Thallion's genomic platform for analysis of actinomycete gene loci encoding pathways leading to bioactive compounds [33, 51]. The compound was also isolated and characterized by Wyeth Laboratories [11]. Initial in vitro assessment by the US National Cancer Institute (NCI) showed that ECO-4601 had broad cytotoxic activity in the low micromolar range inhibiting the growth of hematological and solid tumor cell lines.

Since ECO-4601 has a benzodiazepine moiety, we first investigated if it had affinity for the central ($GABA_A$; CBR) and/or peripheral benzodiazepine receptors (PBR). The CBR is restricted to the central nervous system and mediates the anxiolytic and anticonvulsant properties of benzodiazepines [36, 43]. In the late 1970s an alternative binding site for the benzodiazepine, diazepam (valium®), was identified and referred to as the PBR [7]. The PBR is a critical component of the mitochondrial permeability transition pore (MPTP) (reviewed in [17, 20, 37, 38]). This multiprotein complex is located at the contact site between inner and outer mitochondrial membranes and is involved in the initiation and regulation of apoptosis. Although present in most tissues, the PBR is highly abundant in glandular and steroid-producing tissues such as adrenal glands and gonads [6, 8, 53]. The PBR are involved in steroidogenesis, heme biosynthesis, immune and stress responses, cell growth, differentiation, and mitochondrial respiratory control [6, 20, 26, 37]. Increase in PBR is documented in many tumor types compared to normal tissues [22]. Indeed, binding of PBR-specific ligands, such as PK11195 [1-(2-chlorophenyl[e1]l)-*N*-methyl-(1-methylpropyl)-3-isoguinoline carboxamide], is highly increased in several solid tumor types including colon [25, 29], brain [15], breast [4, 10, 24], prostate [22], ovary [3] and liver [47, 48]. Moreover, PBR ligands have been used as imaging tools in the diagnosis of brain tumors [9, 31] and have been shown to inhibit proliferation and induce apoptosis of rat C6 glioma cells [12].

In this study, we have therefore extended our preliminary in vitro results to evaluate the cytotoxicity activity of ECO-4601 on a panel of brain tumor cell lines, including the rat C6 glioma cell line. We have also investigated the antitumor activity of ECO-4601 against glioma tumors and other solid tumors known to over-express the PBR. The antitumor efficacy data were compared with the pharmacokinetic properties of the drug given by different administration routes. Taken together, the results presented here demonstrate the potent antitumor activity of ECO-4601, a novel agent with PBR binding activity.

Materials and methods

Materials

ECO-4601 (4,6,8-trihydroxy-10-(3,7,11-trimethyldodeca-2,6,10-trienyl)-5,10-dihydrodibenzo[*b,e*] [1, 4] diazepin-11-one; MW = 462.58 Da) was discovered through analyses of over 500 actinomycete loci encoding bioactive compounds using Ecopia's proprietary DECIPHER® technology platform [2]. The molecular structure is shown in Fig. 1a.

For in vitro studies, a 20 mM DMSO stock solution was prepared, and aliquots were stored frozen at –20°C. At time of testing, aliquots were thawed and the stock solution was further diluted in cell culture media containing 10% fetal bovine serum (FBS; Gibco-BRL).

For in vivo studies in mice, the compound was prepared in 15% w/v polysorbate 80 (PS80), 5% w/v polyethylene glycol 400 (PEG400), and 5% v/v ethanol in sterile 5% dextrose (D5W) at final concentrations of 6 and 10 mg/ml depending on treatment doses. Treatment and control groups thus received equivalent amounts of the different vehicle components. All other reagents were of analytical grade and were obtained from commercial sources.

Cell culture and cell lines

Rat glioma C6, human glioma U-87MG, human breast MDA-MB-231 and human prostate PC-3 cell lines were obtained from the American type culture collection (Manassas, VA, USA). Rat gliosarcoma 9L, human astrocytoma GHD and U373, and human oligodendroglioma DN and GHA were from INSERM U318 (Grenoble, France).

Exponentially growing tumor cells (5,000–10,000 cells per well depending on their doubling time; cell number

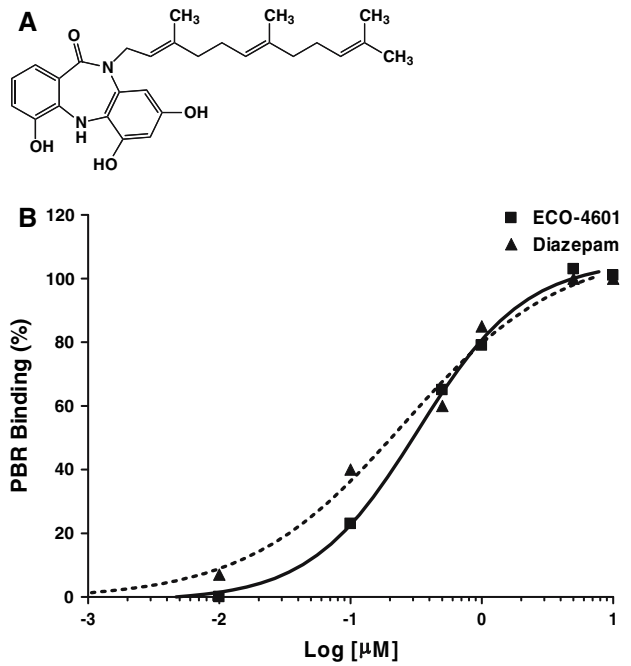

Fig. 1 Inhibitory binding curves of ECO-4601 and diazepam on PBR. **a** ECO-4601 molecular structure. ECO-4601 was expressed in the fermentation broth of an actinomycete (*Micromonospora* sp.) and purified by a series of chromatographic methods as described previously [2]. The compound is virtually neutral and very lipophillic. **b** Interaction of ECO-4601 on PBR was evaluated in a radioligand binding assay using PBR obtained from Witsar rat hearts and displacement of [^{3}H]-PK11195. ECO-4601 was tested at 0.01, 0.1, 0.5, 1, 5 and 10 μM and the concentration of [^{3}H]-PK11195 was 0.3 nM

determined with a hemocytometer) were seeded in 96-well flat-bottom plates and allowed to attach overnight. Tumor cells were then incubated for 72 h with seven different concentrations of ECO-4601: 50, 10, 1, 0.5, 0.1, 0.05, and 0.01 μM. The in vitro cytotoxic activity was determined by a standard MTT assay [41]. All measurements were done in quadruplicate and each experiment was performed 2–3 times. IC_{50} values were calculated with the PrismPad computer program (GraphPad Software Inc., San Diego, CA, USA). The IC_{50} was estimated from individual inhibition curves and represents the concentration of drug that inhibits 50% of the cell growth as compared to non-treated control cells.

Receptor binding assays

The effect of ECO-4601 on CBR ($GABA_A$) and PBR was evaluated in a radioligand-binding assay at MDS Pharma Services (Taipei, Taiwan). The CBR and PBR were obtained from rat brain and heart membrane-fractions, respectively. Displacement assays were done in the presence of 1 nM [^{3}H]-flunitrazepam (CBR; $GABA_A$) or 0.3 nM of [^{3}H]-PK11195 (PBR). ECO-4601 was tested at 0.01, 0.1, 0.5, 1, 5 and 10 μM. Non-specific binding was estimated in the presence of 10 μM diazepam (CBR) or 100 μM dipyrimadole (PBR) and assays were performed according to previous described methods [16, 28].

Antitumor efficacy studies

The rat C6 glioblastoma and the human U-87MG glioblastoma antitumor efficacy studies were performed at INSERM U318 (Grenoble, France). These animal studies were done in accordance with the recommendations of the French Ethics Committee and under the supervision of authorized investigators. Both the human breast MDA-MB-231 and human prostate PC-3 antitumor efficacy studies were done at Piedmont Research Center (Morrisville, NC, USA), which is accredited by AAALAC International. On each dosing day, ECO-4601 bulk formulations (24 and 40 mg/ml in 20% v/v ethanol, 20% w/v PEG400 and 60% w/v PS80) were diluted with D5W to prepare final dosing solutions of 6 and 10 mg/ml of ECO-4601 in a vehicle composed of 5% v/v ethanol, 5% w/v PEG400, 15% w/v PS80, and 80%v/v D5W. Control groups received an equal volume of vehicle only, that is 5 ml/kg of a solution consisting of 5% v/v ethanol, 5% w/v PEG400, 15% w/v PS80, and 80%v/v D5W.

For the rat glioma antitumor efficacy study, female athymic swiss (nu/nu) nude mice (6–7 weeks of age; Charles River Laboratories, Arbresle, France) were inoculated SC with 5×10^6 C6 cells (day 0). Tumor bearing animals were randomized (ten per group) when tumors were palpable (day 6). Group 1 (control group) received drug-free vehicle IP once daily on days 6–18 (q1d × 13). Group 2 received ECO-4601 (drug concentration: 6 mg/ml) IP at 20 mg/kg, once daily on days 6 through 13 and then at 10 mg/kg once daily on days 14 through 18 (q1d × 13). Group 3 received ECO-4601 (6 mg/ml) SC at 30 mg/kg, once daily on days 6 through 13 and then at 15 mg/kg once daily on days 14 through 18 (q1d × 13). Subcutaneous injections were administered at a site distant from the tumor. Group 4 received ECO-4601 (10 mg/ml) IV at 100 mg/kg q1d × 5 for two cycles. Each animal was euthanized when its tumor reached the predetermined endpoint size (~2,500 mm^3) or at the end of the study (D18).

For the human glioblastoma antitumor efficacy study, female athymic swiss (nu/nu) nude mice (6–7 weeks of age; Charles River Laboratories, Arbresle, France) were inoculated SC with 5×10^6 U-87MG cells (day 0). Tumor bearing animals were randomized (ten per group) when tumors were palpable (day 24). Group 1 (control group) received drug-free vehicle SC, once daily q1d × 5 for two cycles (days 24–28 and 32–35). Group 2 received ECO-4601 (drug concentration: 6 mg/ml) SC at 30 mg/kg,

q1d × 5 for two cycles (days 24–28 and 32–35). Group 3 (positive control group) received temozolomide PO at 150 mg/kg, q4d × 3. Each animal was euthanized when its tumor reached the predetermined endpoint size (~2,500 mm^3) or at the end of the study (D40).

The human breast carcinoma cells used for implantation were harvested during log phase growth and resuspended in PBS at 2.5×10^7 cell/ml. Female Hsd:athymic nude-foxn1^{nu}/foxn1$^+$ (8–9 weeks old; Harlan, Indianapolis, IN, USA) were injected SC in the right flank with 5×10^6 MDA-MB-231 cells (0.2 ml cell suspension). Mice were randomized into seven groups of ten mice once tumors had reached 80–120 mm^3 (day 1 of study; 8 days post tumor cell inoculation). Group 1 (control group) received drug-free vehicle, SC, once daily on days 1–21 (q1d × 21). Group 2 received ECO-4601 IV at 100 mg/kg (drug concentration: 10 mg/ml), q1d × 5 for three cycles. Group 3 received ECO-4601 SC at 20 mg/kg (6 mg/ml), q1d × 21. Group 4 received ECO-4601 SC at 30 mg/kg (6 mg/ml), q1d × 5 for 3 cycles. Group 5 received ECO-4601 IP at 30 mg/kg (6 mg/ml), once daily on days 1, 4, 7, 10, 13, 16 and 19 (q3d × 7). Group 6 (positive control group) received paclitaxel IV at 30 mg/kg, once daily on days 1, 3, 5, 7 and 9 (qod × 5). All SC doses were injected into the left flank. Each animal was euthanized when its tumor reached the predetermined endpoint size (1,000 mm^3) or at the end of the study (56 days).

The human PC-3 prostate carcinoma line utilized for these studies was maintained in athymic nude mice by serial engraftment. A tumor fragment (1 mm^3) was implanted SC into the right flank of male Nude-Foxn1^{nu}/Foxn1$^+$ mice (8–9 weeks old; Harlan, Indianapolis, IN, USA). When tumor volumes reached 80–120 mm^3, mice were randomized and treatment began (day 1 of study; 19 days following tumor fragment inoculation). Group 1 received drug-free vehicle, SC, q1d × 5 for three cycles. Group 2 (positive control group), received cyclophosphamide IP at 90 mg/kg, q1d × 5. Group 3 received ECO-4601 SC at 30 mg/kg (drug concentration: 6 mg/ml), q1d × 5 for three cycles. Group 4 received ECO-4601 SC at 50 mg/kg (10 mg/ml), q3d × 7. Group 5 received ECO-4601 IV at 100 mg/kg (10 mg/ml), q1d × 5 for 3 cycles. All SC doses were injected into the left flank. Each animal was euthanized when its tumor reached the predetermined endpoint size (1,500 mm^3) or at the end of the study (71 days).

Determination of antitumor activity

In all the models, tumor growth was followed every other day by measuring tumor length (L) and width (W) using a calliper. Measurements were converted to tumor volumes (TV; mm^3) using the standard formula, $TV = (L \times W^2)/2$. Tumor volume at day n was expressed as relative tumor volume (RTV) according to the following formula $RTV = TV_n/TV_0$, where TV_n is the tumor volume at day n and TV_0 is the tumor volume at day 0. The percentage of tumor growth inhibition (%TGI) was determined by 1 (mean RTV of treated group/mean RTV of control group) × 100. According to the NCI standards, a % TGI of ≥58% (T/C ≤ 42%) is indicative of antitumor activity [1, 14]. Statistical analysis was calculated by the two-tailed unpaired t test using the Prism software. Animals were weighed at least twice weekly during and after treatment until completion of the study. The mice were examined frequently for overt signs of any adverse drug-related side effects. Animals were euthanized if they showed more than 15% body weight loss for three consecutive days or more than 20% body weight loss on a single day.

In some studies, the time to endpoint (TTE) for each mouse was also calculated by the following equation:

$$TTE = \frac{\log10(\text{endpoint volume}) - b}{m}$$

where TTE is expressed in days, endpoint volume is in mm^3, b is the intercept, and m is the slope of the line obtained by linear regression of a log-transformed tumor data set. This value was used to determined % tumor growth delay (%TGD), defined as the increase in median TTE for a treatment group compared to the control group.

Pharmacokinetics

Female Crl:CD1 (ICR) mice (6 weeks old, Charles River Laboratories; Montreal, Canada) were randomized on day 0 according to individual body weight and were administered a single bolus dose of ECO-4601 by IV, SC, or IP injection at 30 mg/kg (four mice per group per time point). These experiments were performed at NucroTechnics (Scarborough, Canada), which is fully accredited by both the AAALAC and CCAC. Blood was collected by cardiac puncture into tubes containing K_2–EDTA as an anticoagulant at 3, 5, 15, and 30 min, 1, 2, 4, and 8 h following compound administration. Samples were centrifuged at 10,000×g for 10 min and plasma samples were collected and stored at –80°C until analysis. Mouse plasma (50 μL) was mixed with 500 μL of acetone containing 100 ng/ml of the internal standard (ECO-4614, a methyl analogue of ECO-4601). The mixture was vortexed for 20 s, incubated 10 min at RT and centrifuged at 12,000×g for 10 min. The supernatants were transferred into HPLC injection vials and 20 μL were analyzed by tandem liquid chromatography/mass spectrometry. The chromatography was achieved on a Luna C18 column (30 × 4.6 mm, 5 μm particles; Phenomenex, Torrance, CA, USA) with a mobile phase

consisting of 100% methanol: 0.5% formic acid in water (85:15) at a flow rate of 1 ml/min. Flow was split 1:10 prior to introduction into the ESI source. The nebulization was assisted by an octagonal jet stream of nitrogen heated at 350°C and set at a flow of 4 l/min. The ion source voltage (ISV) was at 4,000 V in positive mode, declustering potential (PD-QO) was set to 20 V, and the collision energy (Elab) was set to 25 V. The mass transition monitored for ECO-4601 and the internal standard were *m/z* 463 → 271 and 477 → 273, respectively. Standard curve in mouse plasma ranged from 25 to 10,000 ng/ml, with seven calibration points.

Results

Cytotoxicity activity of ECO-4601 on brain tumor cell lines

ECO-4601 was screened twice at the NCI against 55 tumor cell lines originating from different tumor types. The compound showed activity in vitro against leukemia (GI_{50} range 1.1–8.1 μM), non-small cell lung carcinoma (GI_{50} range 0.23–13.6 μM), melanoma (GI_{50} range 1.3–46.7 μM), prostate carcinoma (GI_{50} of 3.9–12.9 μM), breast carcinoma (GI_{50} range 0.11–18.3 μM), ovarian carcinoma (GI_{50} range 0.71–30.7 μM), renal carcinoma (GI_{50} range 1.5–22.3 μM), colon carcinoma (GI_{50} range 1.3–20.7 μM) and malignant central nervous system (CNS; gliomas, GI_{50} range 1.4–13 μM) tumor cell lines (data not shown). Following the "flat" pattern of activity of ECO-4601 across the 55 cell lines tested, no significant correlation was observed using the COMPARE algorithm. The ability of ECO-4601 to inhibit the growth of CNS cancers was further examined on a panel of mouse, rat and human glioma cell lines. Gliomas have been defined pathologically as tumors that display histological, immunohistochemical, and ultrastructural evidence of glial differentiation. According to the World Health Organisation (WHO), gliomas are classified according to their putative line of differentiation, i.e., whether they arise from astrocytes, oligodendrocytes, and ependymal cells. They are then graded on a scale of I to IV according to their degree of malignancy judged by various histological features. The brain tumor cells used in this study originated from rat tumors induced by *N*-nitroso-*N*-methylurea (C6 and 9L) [5] or human brain tumor cell lines from both astrocytic (U-87MG, GHD and U373) and oligodendrocytic (GL26, DN and GHA) origin. The IC_{50} values of ECO-4601 against different representative types of brain tumor cell lines were similar to NCI data, ranging from 1.6 to 10.9 μM (Table 1). These results confirmed the activity of ECO-4601 against different brain cancer cell lines including rat C6 and human U-87MG glioma cell lines, which originate from the most malignant form of brain cancer, type IV glioblastoma multiform. The antitumor activity of ECO-4601 against rat C6 and human U-87MG glioblastomas was further investigated in xenograft models in nude mice.

Table 1 In vitro cytotoxic activity of ECO-4601 against a panel of CNS tumor cell lines

Tumor type	Origin	Cell line	IC_{50} at 96 h (μM)
Oligodendroglioma	Human	GHA	1.6 ± 0.7 (n = 10)
		DN	3.0 ± 0.7 (n = 4)
Astrocytoma	Human	U373	3.8 ± 1.4 (n = 4)
		GHD	6.5 ± 2.9 (n = 8)
Glioblastoma	Mouse	GL26	8.9 ± 1.1 (n = 4)
	Human	U-87MG	10.9 ± 0.5 (n = 3)
		SNB-19	5.3 ± 1.3 (n = 3)
Gliosarcoma	Rat	C6	4.3 ± 2.3 (n = 5)
	Rat	9L	8.3 ± 3.8 (n = 4)

PBR binding activity of ECO-4601

Because of structural dominance of the benzodiazepine moiety found in ECO-4601, we determined if the compound could bind the benzodiazepine receptors. ECO-4601 was tested on CBR obtained from Wistar rat brain membranes in a radioligand-binding assay using 1 nM of [^{3}H]-flunitrazepam as the specific ligand. ECO-4601 did not displace flunitrazepam when tested at concentrations up to 10 μM (data not shown). On the other hand, ECO-4601 was as potent as diazepam in displacing [^{3}H]-PK11195 from the PBR. Results obtained from the PBR binding studies indicated that ECO-4601 had an IC_{50} value of 0.291 μM and Ki of 0.257 μM (Fig. 1b).

Antitumor efficacy studies

Since ECO-4601 was able to inhibit growth of several brain tumor cell lines (Table 1) and to cross the blood brain barrier [19], the in vivo antitumor efficacy of ECO-4601 was first evaluated against a subcutaneous rat C6 glioblastoma tumor. Peripheral benzodiazepine receptors are highly expressed in rat C6 gliomas and specific PBR ligands have been shown to inhibit proliferation and induce apoptosis in this cell line [12]. ECO-4601 was administered via three different routes, SC, IP or IV. Toxicity and tolerability of ECO-4601 varies depending on the administration route. For this reason, different doses were given, which were dependent on the administration route. Maximum body weight loss of 15% was observed on day 13 for the IP group receiving 20 mg/kg (q1d × 8) followed by 10 mg/kg

(q1d × 7) and 11% for the SC group receiving 30 mg/kg (q1d × 8) followed by 15 mg/kg (q1d × 7). The doses were decreased by half during the study due to loss of appetite and body weight. The drug was much better tolerated following IV administration and no significant body weight loss was observed in this treatment group although the dose was 3–5 times greater. The effect of the different treatment routes on tumor growth inhibition was analyzed at day 18, at which time some animals from the vehicle control group had to be killed due to tumor burden. The efficacy data (Fig. 2a) indicated that daily bolus administrations of ECO-4601 either IP or SC had significant antitumor efficacy, resulting in TGIs of 66 and 60% ($P < 0.0001$). No significant difference in tumor volume relative to the vehicle control group was noted for IV bolus administration of ECO-4601 at 100 mg/kg (q1d × 5) for two cycles.

ECO-4601 in vivo antitumor efficacy was further evaluated against a second glioma model, using the human U-87MG glioblastoma cancer xenograft in nude mice. The U-87MG is a cell line derived from a brain glioblastoma of a 44-year-old Caucasian female. ECO-4601 has demonstrated in vitro activity in this cell line with an IC_{50} of 10.9 μM (Table 1) and U-87MG cells are known to express intermediate levels of the *PBR* gene [46]. ECO-4601 antitumor activity in this model was tested following SC bolus injection (Fig. 2b). The dose and regimen (30 mg/kg q1d × 5 for two cycles) was well tolerated with no significant body weight loss observed throughout the study. Tumor growth inhibition was calculated at day 34, time at which some animals from the vehicle control group had to be killed due to tumor burden. Moderate antitumor efficacy (TGI = 36%; $P = 0.05$) was observed when ECO-4601 was administered on a daily basis.

The in vivo antitumor efficacy of ECO-4601 was evaluated against other solid tumor types know to over-express PBR. The human MDA-MB-231 is a breast adenocarcinoma cell line derived from the pleural effusion of a 51-year-old Caucasian female. ECO-4601 is active in the micromolar range against this cell line in vitro (1.4/2.7 μM; NCI data) and MDA-MB-231 expresses PBR [24]. In this study, four regimens were evaluated to determine the antitumor efficacy of ECO-4601, and the results are presented in Fig. 3a. The ECO-4601 IP and SC dose regimens led to body weight loss observed up to day 21, but were tolerated and mice regained weight after treatment stopped (Fig. 3b). Paclitaxel was used as a positive control. Administration of vehicle control daily for 21 consecutive days had minimal effect on mice body weight and growth of MDA-MB-231 xenografts (mean tumor volume of 918 mm^3 at day 21). ECO-4601 resulted in highly significant antitumor efficacy ($P < 0.0001$) when mice were dosed SC at 30 mg/kg (q1d × 5 for three cycles, TGI of 72%) and at 20 mg/kg (q1d × 21, TGI of 66%). Mean tumor volumes at day 21 were 318 and 366 mm^3, respectively. Less frequent administrations given every three days were effective following IP administration (TGI of 56%, $P < 0.001$) but not following SC administration (data not shown). No-significant antitumor activity ($P = 0.5$) was observed when ECO-4601 was administered by the bolus IV route, even if doses were 3–5 times greater. Median TTE was 20.7 days for the vehicle control group while this value was 32.1 days (30 mg/kg SC q1d × 5 for three cycles), 31.2 days (20 mg/kg SC q1d × 21), 26.4 (30 mg/kg IP q3d × 7), and 22.2 days (100 mg/kg IV q1d × 5 for three cycles), resulting in TGDs of 55, 51, 27.5 and 7%, respectively.

ECO-4601 in vivo antitumor efficacy was also evaluated against a human PC-3 prostate cancer xenograft in nude mice. PC-3 is a cell line derived from a bone metastasis of a grade IV prostate adenocarcinoma from a 62-year old

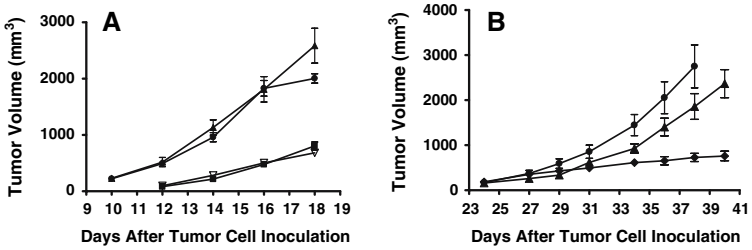

Fig. 2 In vivo antitumor activity of ECO-4601 against the glioma tumor xenografts. **a** Female athymic nude mice 6–7 weeks old were inoculated SC with 5×10^6 rat C6 glioma cells (day 0). Treatment was initiated when tumors were palpable (day 6). ECO-4601 was given IP at 20 mg/kg (days 6–13) followed by 10 mg/kg (days 14–18) (*inverted open triangle*); SC at 30 mg/kg (days 6–13) followed by 15 mg/kg (days 14–18) (*filled square*); and IV at 100 mg/kg (days 6–10 and 13–17) (*filled triangle*). Control group (*filled circle*) received 5 ml/kg of drug-free vehicle (15% PS80/ 5% PEG 400/ 5% EtOH/ 80% D5W) IP (days 6–18). **b** Female athymic nude mice 6–7 weeks old were inoculated SC with 5×10^6 human U-87MG glioma cells (day 0). Treatment was initiated when tumors were palpable (day 24). ECO-4601 (30 mg/kg) (*filled triangle*) and drug-free vehicle (5 ml/kg) (*filled circle*) were given SC q1d × 5 for two cycles. Temozolomide (*filled diamond*), used as positive control, was given PO at 150 mg/g every 4 days (total of three treatments). Results are expressed as tumor volume growth curves of the different groups (mean ± SEM)

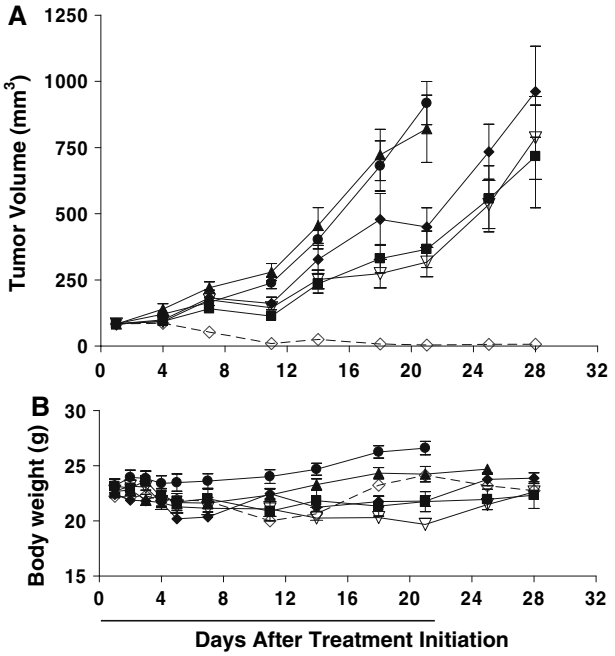

Fig. 3 In vivo antitumor activity of ECO-4601 against human breast MDA-MB-231 tumor xenograft. Female athymic nude mice 9–10 weeks old were inoculated SC with 5×10^6 MDA-MB-231 cells (day 0). Treatment was initiated when tumor volumes reached 80–120 mm^3 (day 1 of study). ECO-4601 was given IV at 100 mg/kg (*filled triangle*) and SC at 30 mg/kg (*inverted triangle*) on days 1–5, 8–12 and 13–17); SC at 20 mg/kg (*filled square*) once a day for 21 days; IP at 30 mg/kg (*filled diamond*) every third day for a total of seven treatments (days 1, 4, 7, 10, 13, 16, and 19). Control group (*filled circle*) received 5 ml/kg of vehicle (15%PS80/5%PEG400/5%EtOH/80%D5W) SC (once a day for 21 days). Paclitaxel (*open diamond*), used as positive control, was given IV at 30 mg/g every other day (days 1, 3, 5, 7 and 9). **a** Tumor volume growth curves of the different groups (mean ± SEM). **b** Body weights curves of the different groups (mean ± SEM). *Solid horizontal bar*, treatment period for ECO-4601

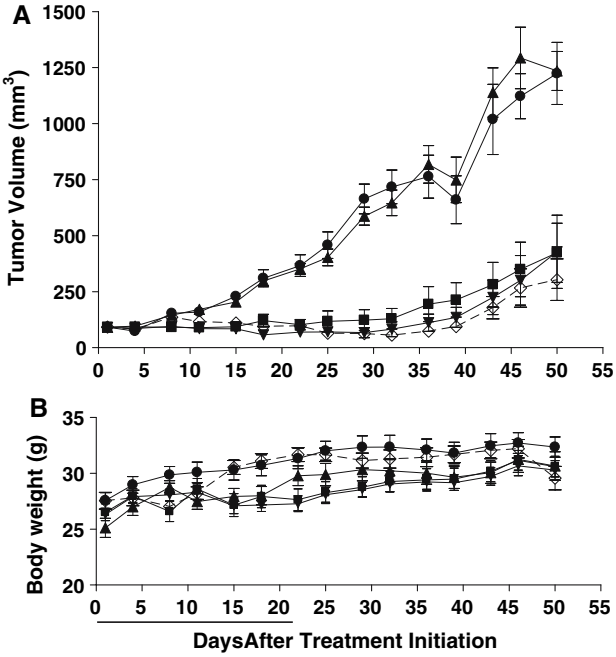

Fig. 4 In vivo antitumor activity of ECO-4601 against human prostate PC-3 tumor xenograft. Male athymic nude mice 8–9 weeks old were implanted SC with a tumor fragment (~1 mm^3) (day 0). Treatment was initiated when tumor volumes reached 80–120 mm^3 (day 1 of study). ECO-4601 was given IV at 100 mg/kg (*filled triangle*) and SC at 30 mg/kg (*filled inverted triangle*) on days 1–5, 8–212 and 13–17; SC at 50 mg/kg (*filled square*) every third day for a total of seven treatments (days 1, 4, 7, 10, 13, 16, and 19). Control group (*filled circle*) received 5 ml/kg of drug-free vehicle (15% PS80/5% PEG400/5% EtOH/80%D5W) SC on days 1–5, 8–12 and 13–17. Cyclophosphamide (*open diamond*), used as positive control, was given IP at 90 mg/g daily for 5 days (days 1–5). **a** Tumor volume growth curves of the different groups (mean ± SEM). **b** Body weights curves of the different groups (mean ± SEM). *Solid horizontal bar*, treatment period for ECO-4601

Caucasian male. ECO-4601 has demonstrated in vitro activity in this cell line with IC_{50} values of 3.9 and 3.0 μM (NCI data). Three different schedules and doses of ECO-4601 were used. Cyclophosphamide was used as a positive control. ECO-4601 resulted in highly significant antitumor activity (TGIs of 85 and 97%; $P < 0.0001$), with PC-3 tumor stabilization and in some instances regression observed (days 8–36) when given by the SC route at doses of 30 mg/kg (q1d × 5 for three cycles) and 50 mg/kg (q3d × 7), respectively (Fig. 4a). At day 43, the time at which animals in the control group had to be killed due to tumor burden, TGIs were 80 and 85.6%, respectively. The dose regimens were well tolerated in all groups, with no significant body weight loss observed (Fig. 4b). At the end of the study (day 71), seven mice remained in the 30 mg/kg (q1d × 5 for three cycles) treatment group, with a mean tumor volume of 700 mm^3 (0–1,476 mm^3). The 50 mg/kg (q3d × 7) treatment resulted in five mice remaining at day 71 with a mean tumor volume of 371 mm^3 (63–726 mm^3). In contrast, IV bolus treatment was ineffective. Median TTE was 52.5 days for the negative control group (dextrose) and 71 days for the positive control group (cyclophosphamide). Median TTE was 71 days for both SC groups and 50.2 days for the IV treated group.

Pharmacokinetics

We were somewhat puzzled by the lack of activity following IV administration. The drug was better tolerated following this route of administration and 3–5 times more drug was administered on a daily basis, yet, it did not translate into activity when compared to lower doses given by the SC and IP route. The plasma drug concentration levels were therefore evaluated following a single bolus and equal administration given by these three different routes. A dose of 30 mg/kg was chosen since it was well tolerated by all three routes of administration and resulted

into antitumor activity following SC administration. The results showed marked differences in plasma pharmacokinetic parameters when comparing IV bolus to SC or IP bolus injections. Bolus SC and IP bolus injections of ECO-4601 demonstrated similar pharmacokinetic profiles. The C_{max} values were 197,025 ng/ml (425.5 μM); 1,049 ng/ml (2.3 μM); and 3,602 ng/ml (7.8 μM) for the IV, SC, and IP groups, respectively (Fig. 5). Thus, the SC and IP groups had ~188 and ~55 times lower plasma C_{max} compared to the IV group. Plasma concentrations reached during the antitumor efficacy studies (daily bolus administrations of 30 mg/kg (SC) or 20 mg/kg (IP)) thus approach mean in vitro IC_{50} values for ECO-4601-induced cytotoxicity determined in PC-3, MDA-MB-231, U-87MG and rat C6 cell lines, which were 3.5, 2.1, 10.9 and 4.3 μM, respectively. Moreover, ECO-4601 plasma concentrations remained at approximately 1.8 μM for the SC 30 mg/kg group and 0.95 μM for the IP 30 mg/kg group for up to 8 h indicating that plasma concentrations approaching antitumor efficacy target levels were sustained over this extended period. The C_{max} attained for 30 mg/kg bolus IV administration was significantly higher, yet due to the rapid initial decline, ECO-4601 plasma concentrations could not be sustained at the target drug concentration of ≥2 μM for more than 1 h. These PK data can explain why bolus IV administration was not effective in producing tumor growth inhibition. The antitumor activity thus appears to be associated with the exposure parameter AUC and/or sustained drug levels rather than C_{max}

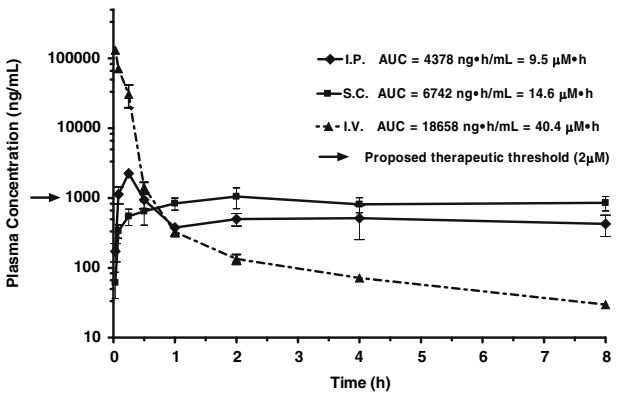

Fig. 5 Plasma concentration-time curves of ECO-4601 following IV, SC and IP injections. The pharmacokinetic profile of ECO-4601 was evaluated following a single bolus IV (*filled triangle*), IP (*filled diamond*) or SC (*filled square*) administration at a concentration of 30 mg/kg. Four mice per group were sacrificed at each of 3, 5, 15, and 30 min, 1, 2, 4 and 8 h time points. Blood was collected into EDTA containing tubes and plasma samples were analyzed by LC/MS/MS (limit of quantitation, 25 ng/ml). The therapeutic threshold refers to the sustained plasma concentration that resulted in antitumor efficacy in nude mice

Discussion

Like well-known chemotherapies such as doxorubicin and mitomycin C, ECO-4601 is derived from microorganisms that live in common soil. However, unlike these drugs that were discovered several decades ago, ECO-4601 represents a new chemical class that is the fruit of a very unique and proprietary drug discovery platform developed at Thallion, called the DECIPHER® technology. Furthermore, ECO-4601 has a type of structure that has not previously been shown to possess anticancer activity and, as a new pharmacophore, is a very promising agent.

"Structure prediction" deducted from genomic and bioinformatics analysis of actinomycete gene loci identified ECO-4601. As this compound is a natural product and represents a unique molecular class, its biological target was unknown at the time of discovery. The core moiety of the molecule being a dibenzodiazepinone, we first investigated its binding affinity towards the benzodiazepine receptors. Benzodiazepines (e.g. diazepam; Valium®), besides their well-known anxiolytic central action, have been shown to inhibit proliferation and induce differentiation of several cell lines [13, 27, 32]. These cellular effects of benzodiazepines are thought to occur following binding to a peripheral receptor, the PBR [50]. The PBR is pharmacologically, anatomically, structurally and functionally distinct from the CBR [39, 53]. It is a mitochondrial 18 kDa protein associated with the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT), all of which contribute to the formation of the mitochondrial permeability transition pore [34, 35]. Not surprisingly specific PBR ligands, such as PK11195 (isoquinoline carboxamide derivative), FGIN-27 (indoleacetamide), RO5–4864 (4′-chlorodiazepam), and diazepam all induce apoptosis and result in inhibition of cell proliferation [12, 18, 30, 40, 44]. ECO-4601 binding was tested on both the central and peripheral benzodiazepine receptors. Results obtained from these binding studies indicated that ECO-4601 did not bind the CBR (IC_{50} > 10 μM) while the binding affinity for the PBR was ~0.3 μM. The binding affinity of ECO-4601 to the PBR is similar to the concentration required to inhibit cell proliferation (1–10 μM, depending on cell lines). This contrasts with current specific PBR ligands, which bind the PBR with nanomolar affinity but inhibit cellular proliferation and induce apoptosis in the micromolar range.

Since ECO-4601 had a good in vitro cytotoxic profile, we were interested to see if ECO-4601 featured antitumor properties as a single agent. The tumor cell lines we used for xenograft studies where previously documented to express PBR and were sensitive to ECO-4601 in vitro (this study). ECO-4601 was shown to have moderate antitumor activity against a human glioblastoma xenograft model

(TGI of 36%) and significant antitumor activity against rat glioma tumors (TGI of 60–66%), resulting in reduction of tumor growth. Both these tumors are quite aggressive, with tumor doubling times of 3 and 3.5 days, respectively. ECO-4601 was also highly effective against human breast and prostate hormone-independent tumors, resulting in TGI values of 56–72% and 80–97%, respectively. In the prostate (PC-3) tumor xenograft, ECO-4601 produced tumor regressions in six of ten animals at 30 mg/kg (q1d × 5 for three cycles) and eight of ten animals at 50 mg/kg (q3d × 7) that extended beyond treatment period. For efficacy, daily administration of ECO-4601 was required in fast growing tumors, such as the rat C6. Higher doses given less frequently (every third day) were efficacious in slower growing tumors, such as the human PC-3 (doubling time of 12 days).

Our antitumor studies also indicated that ECO-4601 activity was dependent on the route of administration. Indeed, while IV dosing was better tolerated and higher doses could be administered, it did not result in antitumor activity. On the other hand, SC and IP bolus administrations, while not as well tolerated (IP dosing resulted in some intestinal occlusion and SC dosing in swelling and thickening of the skin), were effective. Intraperitoneal and SC daily administration treatment doses were 3–5 times lower than IV treatment doses. We thus evaluated the PK profile of ECO-4601 following these different administration routes. Plasma concentration of ECO-4601 fell rapidly within 1 h from ~425 to 1.3 μM following IV injection ($t_{1/2}$ α of 4.7 min). At the 4 h time point, the concentration of ECO-4601 in plasma further decreased to ~0.2 μM and continued to decrease to ~0.1 μM at 8 h. SC and IP administration resulted in similar profiles with peak concentrations of 2.3 and 7.8 μM reached after 1 h and 15 min, respectively. Furthermore, the plasma concentration of ECO-4601 remained at approximately 1.8 and 0.95 μM for up to 8 h. In xenograft antitumor studies, daily bolus administrations of 30 mg/kg (SC) or 20 mg/kg (IP), which are shown to results in sustained plasma concentrations of ~1–2 μM, are effective. The C_{max} attained for 30 mg/kg bolus IV administration was significantly higher, yet due to the rapid initial decline, ECO-4601 plasma concentrations would not be sustained at the target drug concentration of ~1–2 μM for more than 1 h. These PK data can explain why bolus IV administration was not effective in producing tumor growth inhibition. The antitumor activity of ECO-4601 is thus dependent on the maintenance of plasma concentrations approaching the average in vitro IC_{50} values of ≥ 1 μM against tumor cell lines, rather than high C_{max} levels followed by rapid elimination.

The cellular target of ECO-4601 responsible for its potent antitumor activity is still unknown. Although the compound binds the PBR, more potent and specific PBR ligands, RO5-4864 and PK11195, have been tested in vivo and are devoid of antitumor activity as single agents [18, 49]. For both studies, the PBR ligands were dissolved in PS80/ethanol and were administered SC. No PK information was given. While PBR ligands have been reported to inhibit cell proliferation and to induce apoptosis, no clear antitumor activity resulting from their direct interaction with PBR per se has been observed [18, 23, 49, 52]. To our knowledge, ECO-4601 is the first PBR ligand to result in antitumor activity when given as a single agent. These data imply that other cellular targets must contribute to the potent antitumor activity of ECO-4601.

ECO-4601 could preferentially target tumors via its interaction with the PBR resulting in a selective increase of drug levels in the tumors. Interestingly, specific PBR ligands have been linked to melphalan and gemcitabine to enhance tumor selectivity and brain delivery [21, 45]. Transcriptome analysis indicated that ECO-4601 treatment resulted in changes in expression of genes involved in apoptosis, cell cycle, and cell signalling [42]. Experiments are underway to elucidate the relationship of these changes to the mechanism of action of ECO-4601. The potent antitumor activity of ECO-4601 and its low toxicity profile has led us to initiate a phase I clinical trial against solid tumors.

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