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ORIGINAL ARTICLE

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Folate receptor specific anti-tumor activity of folate–mitomycin conjugates

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Abstract *Purpose:* Folate receptor (FR) targeted drug conjugates were prepared by covalently attaching the vitamin folate, to the potent anticancer drug, mitomycin C (MMC). One such conjugate, called EC72, was synthesized with an intramolecular disulfide bond, and it was found to exhibit efficacious anti-tumor activity against FR-expressing M109 tumors in a manner that yielded no gross or microscopic toxicity, even to FR-positive kidneys. *Methods:* EC72's specificity was demonstrated by two methods: (1) blocking EC72's activity with an excess of co-administered folic acid (FA) in M109 tumor bearing mice and (2) the absence of therapeutic activity in mice bearing FR-negative tumors. The importance of having a cleavable bond in the conjugate was also exemplified, since EC110 (a folate–MMC conjugate constructed with a more resilient amide bond) failed to produce anti-M109 tumor activity. EC72's therapeutic potential was found to decrease with respect to the increasing size of subcutaneous tumor. However, a combination therapy with paclitaxel reproducibly improved the anti-tumor efficacy relative to either agent alone at well tolerated dose levels and with no apparent increase in toxicity. A more advanced folate–MMC conjugate was also synthesized in an effort to improve activity. Thus, EC118, a molecule constructed with both a reducible disulfide bond and an acid-labile hydrazone bond in the linker region, was tested and found to produce a significantly greater number of tumor regressions of more established M109 tumors than that achieved with EC72. *Conclusion:* Overall, these data indicate that folate-targeted drug therapy alone, or in combination with paclitaxel, may be a novel and effective clinical approach towards treating FR-positive cancers.

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Introduction

In chemotherapy, an increase in the fraction of cells killed often requires an exponential increase in the administered dose of anti-cancer drug. However, because of their toxic side effects on actively proliferating non-malignant cells, only limited doses of these chemotherapeutic agents can be administered. Therefore, techniques are being developed to selectively target drug molecules to the tumor site in a manner that greatly reduces unwanted systemic toxicity. One such approach is to deliver potent anti-proliferative agents to the tumor by conjugating cytotoxic drugs to tumor-homing agents, such as that exemplified by Mylotarg™. Mylotarg™ was recently approved for the treatment of acute myeloid leukemia [1, 2], and it consists of an anti-CD33 monoclonal antibody (MAb) conjugated to calicheamicin, a highly potent DNA-alkylating agent, via a linker containing both an acid-labile hydrazone and trisulfide bonds. Other Mab–drug conjugates, such as anti-Lewis Y MAb BR96-doxorubicin [3, 4], anti-CanAg MAb C242-maytansinoid DM1 [5, 6], anti-CD56 MAb HuN901-maytansinoid DM1 [7], and anti-CD19 MAb B4-CC-1065 analogue DC1 [8] have also demonstrated impressive pre-clinical anti-tumor activities. However, due to their larger molecular size and restricted tumor penetration ability, Mab–drug conjugate therapy may yet be suboptimal [9]. Hence, it is expected that replacing the MAb "ligand" with a smaller but high affinity receptor targeting ligand could dramatically improve the tumor penetration and activity properties of such conjugates.

An example of one such small molecule targeting ligand system is the use of folic acid (FA) to deliver attached therapeutic and imaging agents to cells that express the folate receptor (FR) [10–12]. This high affinity membrane protein (Kd∼0.1–1 nM for FA) is

functionally expressed in high quantities by many primary and metastatic cancers [13–15], and it has been successfully exploited for drug delivery purposes by using a wide range of drug payloads [11, 12, 16–23]. Because FA-linked molecules are efficiently bound and internalized by FR-expressing cells, we have explored the possibility of using FA to target a small molecule drug to FR-enriched tumors. For this purpose, we elected to evaluate the in vivo performance of EC72, a previously described FA conjugate of the powerful DNA alkylating drug, mitomycin C (MMC) [24]. MMC is a well-known drug having a wide clinical anti-tumor spectrum; notably, MMC has proven to be efficacious in various tumor types, such as gastric cancer, pancreatic cancer, breast cancer, non-small cell lung cancer, cervical cancer, head and neck cancer and bladder cancer [25]. Unfortunately, the delayed cumulative myelosuppressive toxicity (in the form of thrombocytopenia and leukocytopenia) prevents the more widespread clinical use of this drug [26].

EC72 was found to produce dose responsive activity in vitro against a panel of FR-positive cell lines. Initial in vivo tests confirmed EC72's activity in both syngeneic and xenograft models, and this activity occurred in the apparent absence of gross or pathological toxicity. These latter results were deemed significant, because daily dosing of EC72 for more than 30 consecutive days yielded no evidence of myelosuppression or toxicity to major organs, including the FR-positive kidneys [24].

Herein we report on our continued in vivo investigation of EC72 along with a couple of structurally related FA–MMC conjugates. We tested these agents under more rigorous conditions than that previously reported, namely through the use of intravenous administration for treating subcutaneous tumors. Furthermore, since EC72 was again confirmed to be well tolerated over a wide dose range, we explored the potential for administering this targeted agent in combination with a clinically approved, non-targeted drug (paclitaxel) for the purpose of enhancing the overall therapeutic activity.

Materials and methods

Compounds

Paclitaxel (Hauser Inc., Boulder, CO) was initially dissolved in a vehicle containing equal volumes of absolute ethanol and polyoxyethylated castor oil (Cremaphor; Sigma, St. Louis, MO) and sonicated for 10 min. Just before use, the paclitaxel/Cremaphor solution was slowly diluted into 0.9% NaCl solution (90% of the final volume) with occasional stirring. The synthesis of EC72 has been previously described [24]. EC110 and EC118 were produced by using similar solid and solution phase methods as that used to synthesize EC23; a detailed synthesis, purification and analytical characterization of the two new agents will be described elsewhere (manuscript in preparation).

Tumor model and therapy

Six- to eight-week-old female mice (Balb/c strain) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN), maintained on a standard 12 h light–dark cycle and fed ad libitum with folate-deficient chow (Harlan Teklad, Madison, WI) for the duration of the experiment. Since normal rodent chow contains a high concentration of FA (6 mg/kg chow), mice used in these studies were maintained on the folate-free diet for 2 weeks before tumor implantation to achieve serum folate concentrations close to the range of normal human serum [27]. Syngeneic, FR-positive Madison 109 (M109) lung carcinoma cells were grown in folate-deficient RPMI 1,640 with 10% FBS at 37°C in a 5% CO_2 humidified atmosphere, whereas syngeneic 4T1 breast cancer cells were maintained in normal RPMI under similar conditions. For tumor cell inoculation, 1×10^6 M109 cells or 2×10^5 4T1 cells in 100 µl were injected in the subcutis of the dorsal medial area. Dosing solutions for the folate–MMC conjugates and FA were prepared in phosphate buffered saline (PBS) and administered through the lateral tail vein of the mice. Paclitaxel/Cremaphor solution was diluted into 0.9% NaCl saline and also administered intravenously. Tumors were measured in two perpendicular directions every 2–3 days using a caliper and their volumes calculated as $0.5\times L\times W^2$, where L is the measurement of longest axis in mm and W the measurement of axis perpendicular to L in mm. Treatments were administered via a lateral tail vein.

Drug toxicity was assessed by collecting blood via cardiac puncture and submitting the serum for independent analysis of BUN, creatinine, AST-SGOT and ALT-SGPT at Ani-Lytics, Inc. (Gaithersburg, MD). In addition, histopathologic evaluation of formalin-fixed heart, lungs, liver, spleen, kidney, intestine, skeletal muscle and bone (tibia/fibula) were done at Animal Reference Pathology Laboratories (ARUP; Salt Lake City, Utah).

Results

Folate receptor specific potency of EC72

In vivo therapy experiments were performed in Balb/c mice bearing subcutaneously implanted, FR expressing (95 pmol FR/mg protein [22]), syngeneic M109 lung carcinomas. EC72 (Fig. 1a) was first evaluated by administering the drug using a 1.8 µmol/kg dose level following a qdx5, 4-week schedule. As shown in Fig. 2, EC72 was found to produce a marked anti-tumor effect in animals (0.8 log cell kill; LCK) if dosing had been

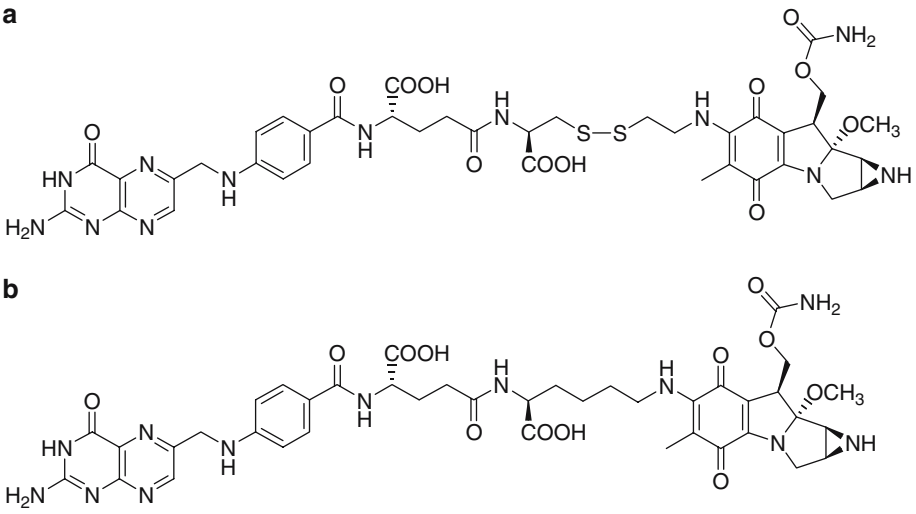

Fig. 1 Chemical structures of **a** EC72 (Folate-ss-MMC) and **b** EC110 (Folate-Lys-MMC)

initiated 4 days post-tumor cell inoculation. Furthermore, the EC72-treated animals actually gained weight throughout the dosing period, and no gross toxicity or pathological degeneration was noted in any major organ, including the bone marrow of the tibia/fibula (consistent with a previous report [24]). The latter observation was significant since the mitomycin class of drugs is known to be especially myelosuppressive [28]. Importantly, EC72's anti-proliferative effect was nearly abolished (0.2 LCK) when this agent was co-dosed with a mere five-fold molar excess of FA. This result indicated that EC72's activity was primarily dependent on binding to tumor-associated FRs.

To further demonstrate the targeted specificity of EC72, an additional study was conducted where Balb/c mice were inoculated with subcutaneous FR-negative

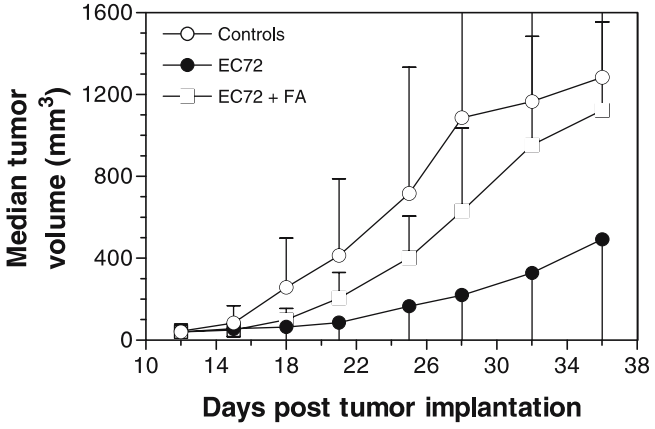

Fig. 2 Effect of EC72 on the growth of subcutaneous FR-positive M109 tumors. Four days post-s.c.-tumor implantation Balb/c mice were randomized and then treated intravenously with 1.8 μmol/kg/injection of EC72 given on a qdx5, 4-week schedule. The *open and filled circles* correspond to the untreated and EC72-treated cohorts, respectively. The *open squares* correspond to the cohort of animals treated with EC72 plus ~10 μmol/kg of FA. *Points* represent an average tumor volume from five animals

(< 2 pmol FR/mg protein [22]), syngeneic 4T1 cancer cells[1] and then subsequently treated with intravenous EC72 injections in an identical manner as that described above for M109 tumors. Once again, none of the EC72 treated animals lost weight or showed signs of toxicity. However, as shown in Fig. 3, the 4T1 tumors grew at a similar exponential rate in both the treated and untreated cohorts. These results supported our aforementioned conclusion that EC72's activity is specific for FR-expressing tumors.

EC72 was constructed with an intramolecular disulfide bond (see Fig. 1a) which was designed to release the potent mitomycin drug fragment following endocytic entry into the cell [24]. To better understand the importance of EC72's disulfide linker in regards to its overall pharmacology, a non-releasable folate–MMC counterpart was synthesized and tested in vivo for anti-tumor activity. The structure of the non-releasable conjugate, referred to as EC110, is shown in Fig. 1b. Compared to EC72's activity, EC110 at an equivalent dose and schedule failed to produce meaningful anti-tumor activity (data not shown). We concluded from this study that the mitomycin drug fragment of a folate–MMC conjugate must be hydrolyzed/released intracellularly to yield measurable anti-tumor activity, and that the disulfide bond in EC72 is one such example of an effective biologically releasable linker.

Combination therapy

Throughout the course of our investigation, we noted that EC72's anti-proliferative activity in the syngeneic M109 lung carcinoma subcutaneous tumor model had actually declined with the length of delay in EC72 administration. Thus, as summarized in Table 1, performance was maximal if treatment began 1 day post-tumor implantation (PTI), where two of five complete

[1] 4T1 cells are sensitive to MMC

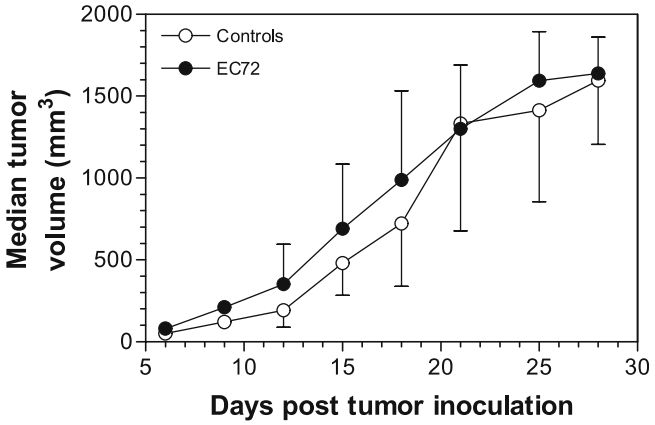

Fig. 3 Effect of EC72 on the growth of subcutaneous FR-negative 4T1 tumors. Four days post-s.c.-tumor implantation Balb/c mice were randomized and then treated intravenously with 1.8 μmol/kg/injection of EC72 given on a qdx5, 4-week schedule. The *open and filled circles* correspond to the untreated and EC72-treated groups, respectively. *Points* represent an average tumor volume from five animals

responses resulted and three of five animals had a 133% increase in lifespan (ILS). In contrast, delaying EC72 treatment until day 12 PTI produced only an 8% ILS with zero of five complete responses. These results suggested that EC72 might not be an effective chemotherapeutic agent when administered as a stand-alone agent, particularly if used to treat solid, s.c. tumors. This realization, however, prompted us to next explore the use of EC72 in combination with an approved agent for the purpose of identifying possible synergistic regimens, especially since EC72 monotherapy was determined to be extremely tolerable [24].

A modest review of the literature revealed that cells exposed to sub-toxic dose levels of MMC experience a profound decrease in p-glycoprotein (pgp) expression with a concomitantly dramatic reduction in multi-drug resistance [29, 30]. Knowing that (1) EC72 delivers a MMC moiety into FR-expressing tumor cells via a folate-targeted approach [24], (2) EC72's activity is reduced to sub-toxic levels in advanced solid tumors (Table 1) and (3) paclitaxel is a very active anti-tumor agent (but also a substrate for the pgp), we speculated that the combination of EC72 and paclitaxel therapy might produce a synergistic anti-tumor effect by the virtue of EC72 (MMC)-mediated reduction of pgp expression. To investigate this possibility, s.c. M109 tumors were first formed in Balb/c mice for 12 days (i.e., a time when EC72 monotherapy is essentially ineffective; see Table 1). The animals were then treated with paclitaxel with or without EC72, and the primary tumor volumes were measured. As shown in Fig. 4, paclitaxel administered at 15 and 20 mg/kg in mice bearing 30–60 mm^3 tumors resulted in significant tumor growth inhibition and rendered 33 and 40% CRs, respectively. When combined with 1.8 μmol/kg/injection of EC72, 15 and 20 mg/kg paclitaxel therapy resulted in a significant increase in tumor growth inhibition relative to EC72 or paclitaxel alone, as detailed in Table 2. Basically, the paclitaxel-15 + EC72 combination produced four of six CRs relative to only two of six CRs for paclitaxel-15 alone. Likewise, the paclitaxel-20 + EC72 combination produced three of five CRs and two of five PRs relative to only two of five CRs for the paclitaxel-20 alone. Since it was previously established that EC72 does not have significant activity against the more established subcutaneous M109 tumors (Table 1), these results clearly show that the EC72 plus paclitaxel combination therapy produced superior anti-tumor activities to that of both the agents when dosed alone. Notably, there was no clear evidence of toxicity (no lethality, weight loss or lethargy) in mice treated with this particular EC72/paclitaxel combination regimen. In contrast, dosing mice with a combination of free MMC and paclitaxel at similar dose levels proved to be a lethal combination (data not shown).

EC118 combination therapy

Because our folate-targeted, bio-releasable MMC conjugate (EC72) was found to be active both as a single agent against less established s.c. tumors and in combination with paclitaxel against more established tumors, we questioned whether enhanced activity might result if a second but distinct releasable linker were included in the conjugate's design. Analogous to Mylotarg™, we elected to construct EC118, a folate–MMC conjugate containing both an acid-labile hydrazone bond and a

Table 1 Effect of treatment delay time on EC72-mediated anti-proliferative activity of M109 tumors

Study summary Animals/cohort Initiation of treament (Days PTI)	$n=5$ Day 1	$n=5$ Day 3	$n=5$ Day 7	$n=5$ Day 12	$n=8$ Untreated controls
Median survival (days)	56	54	28	26	24
%ILS	133	125	17	8	n/a
Partial responses	3	5	5	5	n/a
Complete responses	2	0	0	0	0

Animals were treated qdx5, 4 week with 1,800 nmol/kg of test article administered i.v. beginning on the designated day PTI. Animal survival was measured daily. Animals showing complete response were those designated to be tumor free past day 60 PTI. *%ILS*, percent increase in lifespan

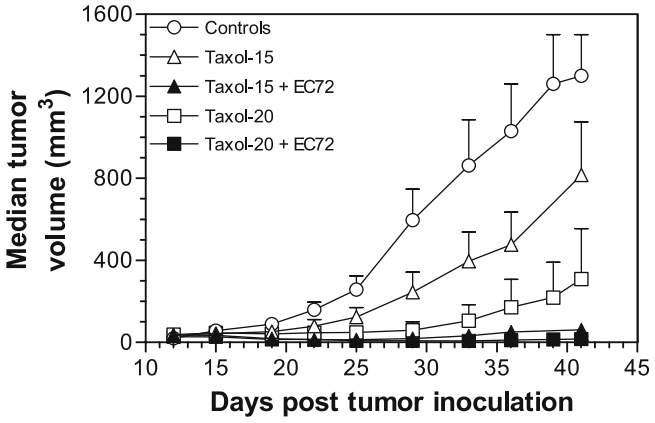

Fig. 4 Effect of EC72 combined with paclitaxel on the growth of subcutaneous M109 tumors. Eight days post-s.c.-tumor implantation (tumor volume range: 30–60 mm^3), Balb/c mice were randomized and then treated intravenously with the designated formulations. Paclitaxel was administered as a single agent administered at 15 mg/kg (*open triangle*) or 20 mg/kg (*open square*) on days 12, 15, 19, 22 and 26. Combination therapy included EC72 (1.8 μmol/kg/injection, qdx5, 4 weeks) and paclitaxel at 15 mg/kg (*filled triangle*) or 20 mg/kg (*filled square*) and on days 12, 15, 19, 22 and 26. The *open circles* correspond to the untreated group. *Points* represent an average tumor volume from five animals

reducible disulfide bond in the linker moiety (see Fig. 5). EC118 was found to be acid sensitive, and it rapidly (within minutes) liberated the mitomycin-containing moiety from the folate moiety at pH 5, a condition typically found in intracellular vesicles [31]. In addition, mild reducing agents cleaved the disulfide bond in EC118's linker to similarly release the mitomycin fragment (assessed by HPLC; data not shown). In cell culture studies, EC118 displayed a similar cytotoxicity profile as EC72, with IC_{50} values ranging between 5 and 10 nM [24]. Thus, using a more established tumor model (average subcutaneous size ~80 mm^3), the anti-tumor activity of 1.8 μmol/kg/injection EC118 combined with paclitaxel (20 mg/kg) was tested; and as shown in Fig. 6, this regimen was found to be superior to the corresponding paclitaxel-20 + EC72 combination. In this new study, treatment with the paclitaxel-20 + EC72 combination produced partial regressions (83%) but no CRs (see Table 3). In contrast, the EC118 + paclitaxel-20 combination produced a 100% response rate with 40% CRs and 60% PRs.

A critical question that needed to be addressed was whether Folate–MMC in combination with paclitaxel would cause severe toxicity. We addressed this question by carefully monitoring the mice by observing motor and feeding behavior, measurement of body weight, complete blood count and liver enzyme analysis. Motor activity and feeding behavior of the treated mice were normal. The body weight of the mice remained stable throughout the experiment, and there were no major gross differences among the treatment groups. Complete blood counts did not reveal any significant myelosuppression in any of the cohorts; however, liver enzyme analysis demonstrated a slight rise in aspartate and alanine aminotransferase levels in all of the treatment groups (presumably due to paclitaxel since such changes are not observed with single agent EC72 therapy; data not shown). Overall, preliminary surveillance of morbidity and mortality suggested that the EC72/paclitaxel or the EC118/paclitaxel drug combination therapy was well tolerated by our test animals.

Discussion

Knowing that folate conjugation enables a drug molecule to target and become endocytosed into FR-expressing tumor cells, we initiated a project to construct and evaluate the activities of various folate–MMC conjugates. Our first conjugate, EC72, was built with a disulfide linker, and it was found to produce anti-tumor activity specifically towards FR expressing M109 tumors, but not when it was co-dosed with an excess of free FA. In addition, EC72 did not exert any activity towards a FR-negative tumor model. Taken together, these results clearly showed that (1) the anti-proliferative activity of EC72 was dependent on functional FR expression and (2) tissues that do not express appreciable levels of the FR (i.e., most normal tissues) would not likely be affected by its activity. By comparing EC72, which contains an endosome-cleavable disulfide bridge [24], to its amide linked counterpart (EC110), we further demonstrated that release of free MMC was required to produce measurable anti-tumor activity. However, it was also apparent from our in vivo data that tumor regrowth can occur after EC72 therapy is stopped, possibly because a population of FR-negative tumor cells

Table 2 Response parameters to EC72 ± paclitaxel combination therapy in Balb/c mice ($n = 5$) 8 days post-M109 s.c. tumor implantation

Regimen	CR	PR		
	Response (%)	Response (%)	%T/C	LCK
Paclitaxel-15	33	0	135	0.6
EC72 + Paclitaxel-15	66	0	155	1.5
Paclitaxel-20	40	0	169	1.3
EC72 + Paclitaxel-20	60	40	194	1.8

LCK, log cell kill; *%T/C*, percentage increase in survival of treated cohort over untreated cohort; *CR*, complete response; *PR*, partial response

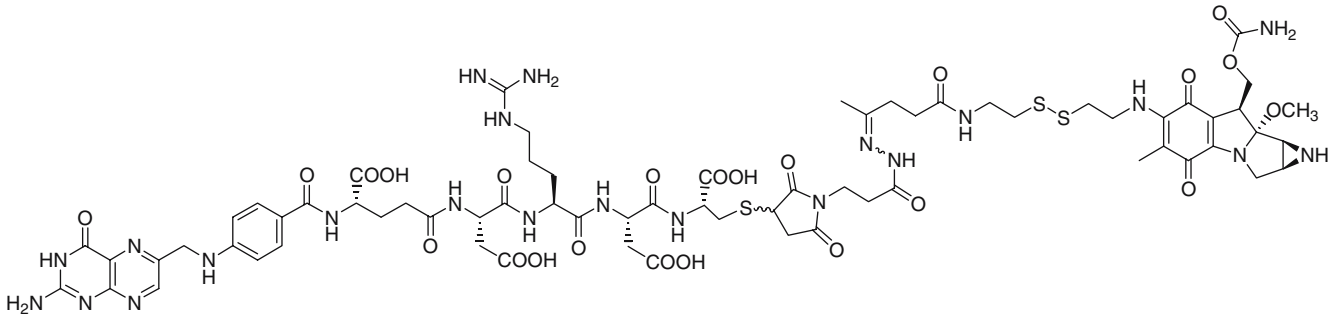

Fig. 5 Chemical structure of EC118 (Folate-hydrazone-ss-MMC)

were not targeted/killed by EC72 and were able to proliferate. Since EC72 therapy did not produce any obvious deleterious effects in our test animals, this latter explanation suggested that combination therapy (folate–MMC + free drugs) might prove to be a worthy new approach.

Paclitaxel has demonstrated significant activity against many solid tumors as a single agent as well as in combination with other chemotherapeutic agents [32, 33]. However unlike MMC, paclitaxel has a very different mode of action by binding to tubulin and inhibiting microtubule depolymerization [34, 35]. Because of the differences in mechanism between MMC and paclitaxel, we looked at the use of EC72 in combination with paclitaxel. In studies employing tumor sizes too large for EC72 to have appreciable activity as a single agent, and where paclitaxel displayed moderate activity at the chosen dose levels, the combination of EC72 plus paclitaxel was found to produce a significant increase in the tumor regression rate over that seen with an equivalent dose of paclitaxel alone. Furthermore, it was found that by adding an intramolecular hydrazone bond to the linker portion of an EC72-like construct, a new conjugate (EC118) was found to exhibit superior activity in combination with paclitaxel relative to EC72. Notably, both EC72 and EC118 are targeted and enter the cell through the same FR-mediated endocytic pathway. However, internalization of conjugates by this pathway exposes these agents to the intracellular acidic milieu of endosomal vesicles, where only EC118 (due to its acid sensitive hydrazone bond) could perhaps release its MMC drug moiety more efficiently than EC72. EC118 may have another advantage over EC72, based on the functional biology of FRs. Essentially, the rate of internalization of FRs is somewhat slow when compared to the rates of other endocytosing receptors [36]. Because of this, and perhaps due to the fact that the FR can also act as an extracellular storage receptor, a sizable fraction of receptor-bound folate–drug conjugates can remain externally bound at the tumor site for extended periods of time. In regards to EC118, its acid labile hydrazone bond would also be expected to hydrolyze (albeit at a much slower rate) at physiological pH 7.4 to release the MMC moiety within the tumor microenvironment. Should this occur, it would lead to an enhancement in net anti-tumor activity over related conjugates that lacked the hydrazone linker (i.e., EC72). While plausible, this explanation must be scrutinized further.

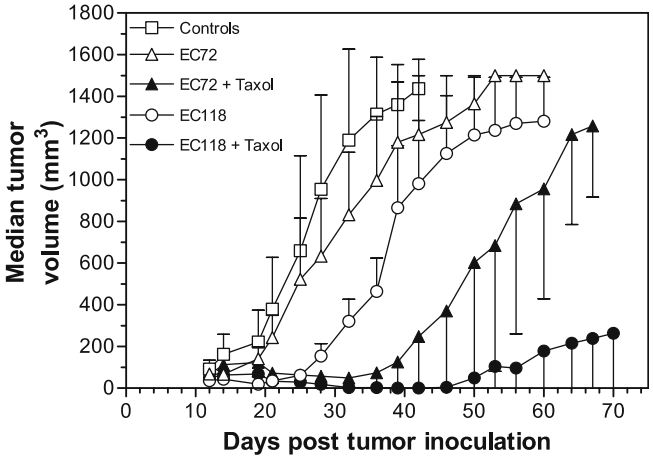

Fig. 6 Effect of EC72 or EC118 combined with paclitaxel on the growth of subcutaneous M109 tumors. Twelve days post-s.c.-tumor implantation (average tumor volume ~80 mm^3), Balb/c mice were randomized and then treated intravenously with the designated formulations. Combination therapy included EC72 (1.8 μmol/kg/injection, qdx5, 3 weeks; *open triangle*) or EC118 (1.8 μmol/kg/injection, qdx5, 3 weeks; *filled circle*) and paclitaxel at 15 mg/kg on days 12, 15, 19, 22 and 26. The *open circles* correspond to the untreated groups. *Points* represent an average tumor volume from five animals

Table 3 Response parameters to EC72 or EC118 ± paclitaxel combination therapy in Balb/c mice ($n = 5$) 12 days post-M109 s.c. tumor implantation

Regimen	CR	PR		
	Response (%)	Response (%)	%T/C	LCK
EC72 + Paclitaxel-20	0	83	200	1.6
EC118 + Paclitaxel-20	40	60	252	2.5

LCK, log cell kill; *%T/C*, percentage increase in survival of treated cohort over untreated cohort; *CR*, complete response; *PR*, partial response

We found it curious that the anti-tumor activity of paclitaxel appeared to be substantially enhanced against larger sized tumors with the co-administration of folate–MMC conjugates. However, this significant increase in anti-tumor activity might be explained by the fact that cells exposed to sub-toxic dose levels of MMC experience a marked decrease in pgp expression with a concomitantly dramatic reduction in multi-drug resistance [29]. Since paclitaxel is a substrate for the pgp, it is entirely possible that for the more established tumors, folate–MMC conjugates target the tumor, get endocytosed within its FR-positive cells, and release MMC at a level that effectuates a pharmacological reduction in pgp expression and activity without fully inducing apoptosis. Although it is beyond the scope of our current report, understanding pgp's role in folate–MMC/paclitaxel combination therapy will be pursued further by these investigators.

Like any chemical-based therapy, concern was raised about the potential toxicity associated with administering folate–drug conjugates. Although most normal tissues in the body express very low or non-detectable levels of the FR [37], the proximal tubules of the kidney do express an appreciable amount of FR [13, 38]. However, since FR expression is limited to the apical membrane of these polarized epithelial cells (i.e., facing the lumen of the tubule), its function is believed to be for capturing folates prior to their urinary excretion and then returning them back into circulation via a transcellular re-absorption process [38–40]. The degree to which the kidney or any other organ would be expected to be harmed by parenteral administration of folate–drug conjugates was, therefore, not apparent. During the in vivo evaluation of EC72 and EC118, we found no evidence of myelosuppression or kidney damage. Conversely, equivalent doses of MMC treatments resulted in extensive myelosuppression (which is a characteristic, dose-limiting side effect of the parent drug's therapy) [24]. Examination of blood collected from folate–MMC treated animals indicated normal blood–urea–nitrogen and creatinine levels. Overall, these observations support the hypothesis that the FRs in the kidney proximal tubules function primarily to shuttle scavenged folates (or small pteroate–drug conjugates) back into systemic circulation rather than to consume them for localized biological functions [38, 39].

Our choice of using 1.8 μmol/kg dose level is based on previous biodistribution studies using radiolabeled folate conjugates which have determined that ~1.8 μmol/kg is an adequate dose to saturate tumor-associated FRs following a single i.v. injection [36]. When translated (or scaled) to the human-equivalent dose based on body surface area conversions, the mouse dose level of 1,800 nmol/kg EC72 used for the aforementioned studies would be approximately 5.6 μmol/m^2. Interestingly, the human dose level for MMC is 60 μmol/m^2 given in 6–8-week intervals [28]. Considering that EC72 is inherently less toxic than MMC, we estimate that a cancer patient could be safely treated >10 times in 6–8-week intervals with "receptor-saturating" dose levels of EC72. Importantly, such a dose density, or metronomic, style of dosing may produce greater therapeutic outcomes, especially if given in combination with paclitaxel.

Finally, it is well known that >90% of human ovarian carcinomas express high levels of the FR protein [41], which consequently are in a similar quantitative range as our M109 tumor model [37]. Likewise, ovarian carcinoma patients are typically treated with a combination drug regimen that includes both a taxane and a platin-containing compound. Alternative combinations are continually being explored clinically, but some prove to be too toxic for humans to withstand. For instance, in a Japanese-sponsored clinical trial, MMC was added to a docetaxel/cisplatin regimen [42]. Unfortunately, this regimen produced intolerable toxicities and the trial was ended. However, considering that (1) ovarian carcinomas have >90% chance of being FR-positive, (2) the present evidence showing that MMC can be effectively targeted to FR-positive tissues to produce anti-proliferative properties in a safer manner and (3) enhanced anti-tumor activity of folate–MMC conjugates in combination with taxanes, it is possible that the addition of EC72 or EC118 to the standard taxane/platin therapeutic regimen may provide an opportunity to increase the clinical outcomes of patients that suffer from advanced ovarian (and other FR-positive) cancers.

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