Anti-Cancer Properties of a Novel Class of Tetrafluorinated Thalidomide Analogs

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ABSTRACT

Thalidomide has demonstrated clinical activity in various malignancies affecting immunomodulatory and angiogenesis pathways. The development of novel thalidomide analogs with improved efficacy and decreased toxicity is an ongoing research effort. We recently designed and synthesized a new class of compounds, consisting of both tetrafluorinated thalidomide analogs (Gu973 and Gu998) and tetrafluorobenzamides (Gu1029 and Gu992). In this study, we demonstrate the anti-angiogenic properties of these newly synthesized compounds. We examined the specific anti-angiogenic characteristics *in vitro* using rat aortic rings with carboxyamidotriazole as a positive control. Additionally, further *in vitro* efficacy was evaluated using HUVECs and PC3 cells treated with 5μM and 10μM doses of each compound. All compounds were seen to reduce microvessel outgrowth in rat aortic rings as well as inhibit HUVECs to a greater extent, at lower concentrations than previously tested thalidomide analogs. The anti-angiogenic properties of the compounds was also examined *in vivo* in *fli*1:EGFP zebrafish embryos, where all compounds were seen to inhibit the extent of outgrowth of newly developing blood vessels. In addition, Gu1029 and Gu973 reduced the anti-inflammatory response in *mpo*:GFP zebrafish embryos, while Gu998 and Gu992 showed no difference. The compounds anti-tumor effects were also explored *in vivo* using the human prostate cancer PC3 xenograft model. All four compounds were also screened *in vivo* in chicken embryos to investigate their teratogenic potential. This study establishes these novel thalidomide analogs as a promising immunomodulatory class with anti-cancer effects that warrant further development to characterize their mechanisms of action.

INTRODUCTION

When thalidomide was originally made available in the 1950s, it was used as a non-addictive, non-barbiturate sedative and soon after as an anti-emetic, alleviating the symptoms of morning sickness in pregnant women. Shortly thereafter, it was found that thalidomide had adverse effects upon fetal development, leading to multiple severe deformities in newborns, including phocomelia (shortening of the long bones of the limbs), as well as causing miscarriages (1-3). The mechanism for these teratogenic effects has since been linked to thalidomide having anti-angiogenic characteristics (4, 5). Thalidomide was removed from the market in late 1961 and never received FDA approval in the US for the purpose of treating morning sickness. However, interest in thalidomide has resurfaced over the past two decades as the drug possesses potent anti-inflammatory and anti-angiogenic properties.

Thalidomide is currently FDA approved for the treatment of erythema nodosum leprosum (6, 7) and multiple myeloma (8-15). Furthermore, thalidomide has shown some activity in acute myeloid leukemia (16), metastatic renal cell carcinoma (17), high-grade gliomas (18), prostate cancer (19, 20) and Kaposi's sarcoma (21). Recent studies have also shown the efficacy of two thalidomide analogs, lenalidomide and pomalidomide, for the treatment of select malignancies (22). When compared to thalidomide, these compounds show increased response rates and less toxicity, however, patients still suffer from related side effects, and eventually many will develop resistance to these drugs (23, 24). The development of novel thalidomide analogs that are safer, and with improved activity has been an ongoing research effort. A previous collaboration between the National Cancer Institute and the Gütschow lab in Germany, led to the synthesis of 118

N-substituted and tetrafluorinated thalidomide analogs, where seven of these compounds (four N-substituted and three tetrafluorinated) displayed anti-angiogenic activity when tested *in vitro* and one also displayed anti-angiogenic activity *in vivo* in chicken and zebrafish embryos (5, 25). These analogs were found to be active at much lower concentrations than thalidomide. Based on these results, a novel collection of tetrafluorinated compounds has been synthesized, comprised of both tetrafluorinated thalidomide analogs and tetrafluorobenzamides. The structure of these analogs is based upon 5′-OH-thalidomide, a biologically active hydroxylated metabolite of thalidomide, which has been shown to have anti-angiogenic activity *in vitro* (25, 26). We screened this library of novel compounds with *in vitro* and *in vivo* assays to determine their anti-angiogenic and anti-inflammatory effects, evaluated their *in vivo* anti-tumor effects in the prostate cancer xenograft model and studied effects on teratogenesis in the chicken embryo model. The current study has examined the therapeutic potential of the four lead tetrafluorinated thalidomide analog compounds consisting of two tetrafluorobenzamides (Gu1029 and Gu992) and two tetrafluorinated thalidomide derivatives (Gu973 and Gu998).

METHODS

Thalidomide Analogs

Analogs were synthesized by Dr. Agnieszka Ambrozak in the group of Dr. Michael Gütschow at the University of Bonn, Germany. US patents of these analogs have been filed (Patent No. US 8,143,252 B2, Mar. 27, 2012). Their chemical structures are shown in Figure 1.

Cell lines and reagents

Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich (St. Louis, MO). All water used was ultra filtered by a MilliQ system (MilliPore, Billerica, MA). Human umbilical vein endothelial cells (HUVEC; Clonetics) were purchased from Lonza (Allendale, NJ). PC3 cells were purchased from American Type Culture Collection (Manassas, VA).

Rat Aortic Ring Assay

A rat aortic ring assay was performed to determine the extent of the anti-angiogenicity of the four lead compounds based on previous similar assays (5, 25, 26). Briefly, 12-well tissue culture plates were covered with 250 μL Matrigel (Becton-Dickinson, Bedford, MA) and allowed to gel for 30-45 min at 37 °C and 5% CO_2. Sections of thoracic aorta were removed from 8- to 10-week old male Sprague Dawley rats. Following excision of fibroadipose tissue, the aortic sections were cut into 1 mm-long cross sections, placed on Matrigel-coated wells, and layered with an additional 250 μL of Matrigel. These were then allowed to set, after which the cross-sectional rings were covered with endothelial cell growth media (EGM-II) and incubated under 5% CO_2 at 37

°C overnight. The EGM-II was comprised of endothelial cell basal medium (EBM-II; Lonza, Allendale, NJ), in addition to endothelial cell growth factors. The culture medium was then traded for EBM-II that was supplemented with 2% fetal bovine serum, 0.25 μg/mL amphotericin B, and 10 μg/mL gentamicin. The aortic rings were treated daily with vehicle (0.5% DMSO), carboxyamidotriazole (CAI; 12 μg/mL; positive control), Gu973, Gu992, Gu998, or Gu1029, each at a dose of 50μM, for four days. This was replicated four times using aortas from four different rats. The area of angiogenic sprouting, reported in square pixels, was quantified using Adobe Photoshop. Data was presented as percent growth based on the negative control (vehicle), which was normalized to 100% growth.

Antiangiogenic and anti-inflammatory Zebrafish embryo assays

Zebrafish were maintained in an approved aquarium habitat at 28°C and all necessary ethical and legal approvals were obtained for embryo work. Sexually mature males and females were crossed and the progeny were screened for the expression of GFP or EGFP. Chorions were removed at 24 hours post fertilization (hpf) using forceps. All screening and imaging was performed using a Nikon SMZ1500 stereo dissecting microscope and camera.

fli1:EGFP zebrafish (27) were used to assess anti-angiogenic properties of the compounds. Embryos were exposed to either a vehicle control at 24 hpf, or a Gu compound (5-100 μg/mL) for 24 hours. Embryos were anaesthetized in 0.1% tricaine and the length and number of intersegmental vessels were quantified as previously described (28).

mpo:GFP zebrafish (29) (also known as Tg(*mpo*:GFP)^{114}) were used to assess the anti-inflammatory properties of the compounds. Adult fish were crossed, embryos were obtained and incubated until 72 hpf, whereupon they were sedated in 0.1% tricaine then given a small cut in the dorsal third of the tail fin. They were then incubated with a vehicle control (0.1% DMSO) or a compound of interest (5-100 μg/mL) in aquarium water for 24 h as previously described (28). The number of fluorescent neutrophils migrating to the wound site was quantified as described previously (28).

NCI 60 Cell Line Screening

Gu973, Gu992, Gu998, and Gu1029 were each tested in the NCI60 cell line screen at five different concentrations using previously published and standardized methodology (30). This screen assesses the ability of a compound to inhibit growth in 59 different human tumor cell lines.

Cell Proliferation Assay

The four lead compounds were tested for their ability to inhibit cell proliferation *in vitro*, using a CCK-8 assay. HUVEC cells and PC3 (prostate cancer cell line) were plated in 12-well plates with a density of 30,000 cells/well and allowed to attach overnight at 37 °C in 5% CO_2. The media was then removed and replaced with media containing vehicle (0.5% DMSO), Gu973, Gu992, Gu998, or Gu1029, either at 5 or 10μM for each compound.

***In Vivo* Mouse Xenograft Study**

The *in vivo* efficacy of the four lead compounds was determined using a variation of a previously described prostate cancer xenograft mouse model (31). PC3 cells were allowed to grow in culture and then injected (~3 million cells) into a mouse flank subcutaneously. Tumors were allowed to grow for 9 days before daily intraperitoneal (IP) injections of vehicle (5% DMSO in saline), Gu973 (1.55 mg/kg), Gu992 (2.34 mg/kg), Gu998 (0.42 mg/kg), or Gu1029 (4 mg/kg) were begun. These doses were the maximum tolerated doses determined in a previous study and based upon drug solubility (data not shown). Treatment lasted for 4 weeks, except for Gu973, which was stopped after 3 weeks due to adverse effects. All animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, DC). The study design and protocol were approved by the NCI Animal Care and Use Committee (Bethesda, MD).

Chicken Embryo Teratogenicity Analysis

Fertilized White Leghorn chicken embryos were incubated for 3 days in an incubator at 37°C, 5% CO_2. The eggs were windowed and embryos staged according to the Hamburger-Hamilton (HH) stages of chicken embryo development (33). Embryos at HH St 18 (E3) were retained for experimentation as at this stage the embryo is undergoing organogenesis and rapid growth. Drug administration was carried out as previously described (5, 28). Briefly, embryonic membranes were removed and the compound of interest was applied over the embryo. After the procedure, the window was sealed with tape and returned to the incubator. All embryos were treated with 100 μL of

dosing solution. Initially, compounds were applied to developing chick embryos at varying concentrations 50, 100 and 200 μg/ml (5-20 μg/embryo) in order to establish a dose-response relationship. These doses were tested as they are within the ranges of previous thalidomide and thalidomide analogs screens (5, 28, 34). Once a dose-response relationship had been determined for each compound, the concentrations allowing the best survival rates were re-tested in a new cohort of embryos. Gu973, Gu998, Gu1029 were applied at 10 μg/embryo (concentrations of 258.2 μM, 241.1 μM and 266.5 μM respectively) and Gu992 was tested at 20 μg/mL (513.7 μM). The compounds were given as a single dose and the development of each embryo was noted at 24 and 48 hours after drug application. Developmental defects and the survival of the embryos were recorded each day.

Statistical considerations

All results were presented as mean ± standard error of the mean (SEM). Comparisons were made with one-way analysis of variance (ANOVA), followed by Dunnett's test, with a $p < 0.05$ as the criterion for statistical significance. Statistical analysis was performed using GraphPad Prism.

RESULTS

Effect of Lead Compounds on Rat Aortic Angiogenesis

Rat aortic microvessel outgrowth was normalized to 100% based on vehicle (0.5% DMSO) control. All lead compounds, each at 50μM, demonstrated approximately 90% inhibition of angiogenesis, comparable with CAI (Figure 2). Gu1029 and Gu973 attenuated microvessel outgrowth to 9.02% and 6.23% of control, respectively. Gu992 and Gu998 were also effective anti-angiogenic compounds, each demonstrating inhibition of outgrowth to 10.2% and 6.19% of control, respectively ($p < 0.001$, $n = 5$).

In Vitro Cancer Cell Proliferation

We assessed the anti-cancer capabilities of the compounds in the NCI60 screen, as well as the PC3 prostate cancer cell line. In the NCI60 screen, three compounds significantly reduced growth at a single dose of 10 μM, and went on to be tested at 5 different doses. All three compounds had a mean 50% growth inhibition (GI50) of all cell lines tested over 5 different doses. Gu973, Gu992, and Gu998 had mean GI50 values of 5.12 μM, 36.7 μM, and 2.12μM, respectively. Gu1029 did not induce significant growth inhibition in the initial NCI60 cell screen (at a single dose of 10 μM) and thus testing of this compound in the NCI60 cell lines was not continued.

PC3 cells exhibited a decrease in cell viability with each lead compound. Gu973 and Gu998 inhibited PC3 proliferation in a dose-dependent manner (Figure 3), where Gu973 inhibition decreased to 26.0% and 10.9% of control at 5 and 10 μM, respectively, and Gu998 inhibited growth to 31.0% and 9.7% of control at 5 and 10 μM, respectively ($p < 0.0001$, $n = 8$). However, at 10 μM, Gu1029 and Gu992 demonstrated almost no

decrease in proliferation in PC3 cells, at 87.7% and 95.5% of control ($p > 0.05$ $n = 8$), consistent with the NCI60 cell line testing at 10 μM (Gu1029: 95.2%; Gu992: 93%).

In Vitro Endothelial Cell Proliferation

We then investigated the compounds' effect on endothelial cell proliferation by using the HUVEC cell line. HUVEC cells showed significant reduction in cell viability when treated with each compound ($n = 8$). Gu973 and Gu992 both demonstrated dose-dependent inhibition of HUVEC proliferation at 5μM and 10μM (Figure 3), where Gu973 reduced proliferation to 27.2% and 9.0% of control, respectively, and Gu992 reduced to 27.1% and 10.7% of control, respectively. Gu1029 significantly reduced HUVEC cell proliferation by >85% at both 5 μM and 10μM. Gu998 was the most effective of the compounds, potently inhibiting HUVEC proliferation by >97% at 5 μM and 10 μM ($p < 0.0001$).

Gu compounds exhibit anti-angiogenic activity in Zebrafish embryos

Thalidomide exhibits anti-angiogenic properties *in vitro* (4), *in vivo* (36) and when assessed with *in ovo* chicken embryo assays (38). Moreover, anti-angiogenic analogs of thalidomide cause teratogenic effects by inhibiting the growth of newly forming vessels (2, 3, 5). *fli*1:EGFP zebrafish, which are known to be sensitive to teratogens (5, 27, 28), were used to assess the ability of Gu compounds to affect the development of the forming vasculature. We used the formation of the inter-somitic vessels (ISVs) as markers of vessel outgrowth. At 24 hpf, ISVs are present in the anterior of the embryos, forming in the spine of the embryos and are yet to form in the posterior

of the embryos. Compared to embryos treated with a vehicle control (0.1% DMSO, Figure 4A, F, n = 33), treatment with Gu973 reduced the outgrowth of the ISVs at 1 μg/mL ($p < 0.05$, n = 9), 10 μg/mL ($p < 0.01$, n = 9) and at 20 μg/mL (Figure 4B, F, $p < 0.05$, n = 17). Gu992 decreased outgrowth of forming vessels at all concentrations tested (Figure 4F, 1 μg/mL, $p < 0.001$, n = 15; 10 μg/mL, $p < 0.001$, n = 20; 20 μg/mL, $p < 0.001$, n = 11; 50 μg/mL, Figure 4C, $p < 0.01$, n = 5). Gu998 decreased outgrowth (Figure 4F) at 1 μg/mL ($p < 0.05$, n = 10) and10 μg/mL (Figure 4D, $p < 0.05$, n = 7). Gu1029 decreased outgrowth at all concentrations tested (Figure 4F,1 μg/mL, $p < 0.05$, n = 10; 10 μg/mL, $p > 0.05$, n = 9; 20 μg/mL, $p < 0.001$, n = 18; 50 μg/mL, Figure 4E, $p < 0.001$, n = 11) however the extent of inhibition was not significant at 10 μg/mL. The number of sprouting ISVs was also quantified (Figure 4G). Gu973 decreased vessel number, but only significantly at 10 μg/mL ($p < 0.01$, n = 9) and 20 μg/mL ($p < 0.05$, n = 17). Gu992 inhibited sprouting significantly at 50 μg/mL ($p < 0.001$, n = 5). Gu998 showed a non-significant decrease in sprouting vessels. Gu1029 was the only compound that had no effect on the vessel number. We found that testing at higher concentrations (100 μg/mL) killed all the embryos, possibly due to toxicity, although we could not rule out the embryos died due to systemic vessel and circulatory failure.

Gu compounds action upon the inflammatory response in zebrafish embryos

Thalidomide exhibits anti-inflammatory actions (1-3) and its analog, lenalidomide, is known to be clinically effective in the treatment of multiple myeloma via its anti-inflammatory properties (39). We therefore tested the Gu compounds to determine if they have an effect on the inflammatory response. We tested the compounds

on *mpo*:GFP zebrafish embryos, where neutrophils express GFP under the control of the myeloperoxidase promoter, and are recruited to the wound site following tissue injury/damage which allows the study of the inflammatory response (28, 29). Treatment with Gu1029 caused a reduction in neutrophil migration to the wound site in a concentration dependent manner (Figure 4H), where the response at 10 μg/mL (n = 13) 15 μg/mL (n = 8), 20 μg/mL (n = 13), 40 μg/mL (n = 8), 50 μg/mL (n = 9) and 100 μg/mL (n = 7) were all significant ($p < 0.001$). Gu973 showed a significant inhibition of neutrophil numbers and migration at 2.5 μg/mL ($p < 0.01$, n = 5) 5 μg/mL ($p < 0.05$, n = 5) and 6.25 μg/mL ($p < 0.01$, n = 8) compared to embryos with a tail fin cut but without a drug. Gu992 (5 μg/mL and 10 μg/mL, n = 16 and n = 8 respectively) and Gu998 (5 μg/mL, n = 10) gave no significant response, and neutrophil behavior was similar to the controls.

***In Vivo* Efficacy of Gu compounds in the prostate cancer xenograft model**

Finally, the behavior of the compounds in mouse models of cancer was evaluated. Mice (n=29) were divided into five groups and treated daily for four weeks with a vehicle control or one of the four lead compounds. Before day 21 of the study, no compound was observed to have a statistically significant decrease in tumor volume size compared to vehicle control (Figure 5). However, from day 21 and continuing through the end of the study (day 39), Gu1029 showed statistically significant decreases in tumor volume size (compared to vehicle) at each time point ($p < 0.05$). By day 36 of the study, tumor growth inhibition was 48.19% compared to control. Treatment lasted 4 weeks for all compounds except Gu973, which was stopped after 3 weeks due to animals experiencing abdominal

swelling, with moderate weight loss of approximately 10% of baseline body weight. The weight loss of mice treated with Gu973 was significant but stable over the course of the experiment, and supplementation with transgel did not reverse condition.

Given there was no statistical response from Gu992 and Gu998, these *in vivo* results indicate Gu1029 is a potent compound at inhibiting prostate tumor growth.

Teratogenicity of lead compounds in the developing chicken embryo

Screening of these compounds during chicken embryo development was conducted to assess their potential for teratogenesis (Figure 6). The results are indicative of the concentration at which embryos survived the treatment and exhibited defects.

After establishing a concentration at which embryos could survive the treatment (266.5 μM, 10 μg/embryo), ten embryos were treated with Gu1029, of which eight survived the treatment. All embryos at 24 hours had hemorrhaging (Figure 6A). Of the eight surviving embryos, three of these had twisted spines and six were developmentally delayed. Gu973 administration caused defects at 258.2 μM (10 μg/embryo) where ten embryos were treated at this concentration. Two of the eight surviving embryos presented with microopthalmia, 5/8 were developmentally delayed and 3/8 embryos exhibited hemorrhaging. Gu992 exposure was tested at 513.77 μM (20 μg/embryo) in twelve embryos, of which eleven survived the treatment. Three embryos had twisted spines, two had growth retardation and one embryo had a limb reduction injury. Gu998 was administered over developing chicken embryos at 241.1 μM (10 μg/embryo) in eleven embryos, seven of which survived the treatment. Gu998 caused a range of complications

including two limb defects, microopthalmia, three embryos with spinal defects and two embryos that were underdeveloped.

Defects were seen following drug exposure in every instance (Figure 6E) and every treatment caused hemorrhaging in the bodies and heads of the embryos, as well as constriction of the vasculature and necrosis of the chorioallantoic membranes (CAM) surrounding the embryos. This data indicates all four compounds are teratogenic to the developing embryo yet the severity of the injuries caused varies amongst them.

DISCUSSION

These results show that the four lead tetrafluorinated thalidomide analogs tested demonstrated varying degrees of anti-angiogenic, anti-inflammatory, anti-tumor and teratogenic characteristics. Gu973 displayed only moderate efficacy in all assays, as well as causing unacceptable abdominal swelling, pain, and weight loss in week 3 of the mouse xenograft assay. Gu998 was the most effective compound in the *in vitro* tests, yet it showed no significant tumor volume reduction in the *in vivo* efficacy study. Gu992 was seen to have similar effects as Gu998 in most assays excluding the *in vitro* cell proliferation, where Gu992 demonstrated low potency in the NCI60 cell screen and minimal activity in inhibiting PC3 cell proliferation. Gu1029 exhibited anti-tumor effects in the prostate xenograft study, significant activity against HUVEC proliferation and in anti-angiogenic assays but displayed no potency against cancer cell lines. Previous studies have found a number of compounds to also be inactive in the NCI60 screen at the concentrations tested, which include thalidomide, lenalidomide, aminolevulinic acid and levamisole (41). Because the Gu compounds were not cytotoxic in this screen, but did

inhibit proliferation in endothelial cells, the results are indicative of the specificity of the anti-angiogenic action of the compounds. However, the lack of response from the NCI60 screen may also be attributed to the fact that the efficacy of at least some of the drugs depends on their effect on the immune system or components of the tumor microenvironment (40), which would not be detectable in a cell line screen such as the NCI60 (38).

The anti-angiogenic properties of these compounds compared favorably to tetrafluorinated thalidomide analogs previously reported by Ng *et al.* (31) and Lepper *et al*. (32). Multiple cell types are involved in the formation of the microtubules from the rat aortic rings, and the order in which they form the sprouts emulates the succession of formation in humans, making this assay an excellent indicator of the effect of drugs on angiogenesis (42). Analyzing microvessel growth in the rat aortic ring assay, all compounds performed equally well or better at 50 μM than the previously synthesized N-substituted class of thalidomide analogs at the same dose. Additionally, each compound tested here showed greater HUVEC lattice network inhibition at lower concentrations (5 μM) than similar thalidomide analogs tested previously at 12.5 μM (25). When comparing results from the *in vitro* PC3 cell proliferation assay, Gu992 and Gu1029 had no effect at 10 μM. However, Gu973 and Gu998 at 5 μM were both more effective at inhibiting PC3 cells than all previously tested compounds at 12.5 μM (25).

The tetrafluorinated Gu compounds were designed around the structures of CPS thalidomide analogs previously found to be efficacious in prostate cancer xenograft models and anti-angiogenic (25, 31). Several studies have shown tetrafluorinated compounds to exhibit increased bioactivity compared to non-fluorinated compounds (25).

The increased activity of Gu973 and Gu998 *in vitro* compared to the CPS compounds (25) may be due to the combination of additional fluorine substituents as well as a more appropriate substituent at the phthalimide nitrogen, *i.e.* a dialkyl barbituric acid (Figure 1). It has already been shown that non-fluorinated CPS compounds display less anti-angiogenic activity than their tetrafluorinated counterparts (25). Compounds Gu1029 and Gu992 also possess better anti-angiogenic activity than CPS compounds *in vitro*, but less so than Gu973 and Gu998. Scission of one phthaloyl CN bond of the tetrafluorophthalimide structure (Gu973 and Gu998) and the loss of one CO unit would likely result in increased degrees of conformational freedom of the tetrafluorobenzamide structure (Gu1029 and Gu992). This may be a contributing factor towards explaining a reduced bioactivity of the tetrafluorobenzamides in the *in vitro* assays. Similarly, a replacement of the methylene bridge by CO in the phthaloyl ring have been shown to increase the potency of thalidomide analogs *in vitro*. Perhaps retaining this structural element of thalidomide is a requirement for improved anti-angiogenic activity; structure-activity relationship studies are currently ongoing in our laboratory. Other thalidomide analogs reported in the literature have also demonstrated varying degrees of antiangiogenic and/or anti-tumor properties, for example, lenalidomide and pomalidomide. Thus, it should be noted that structural differences between thalidomide analogs and thalidomide itself may affect binding to distinct target protein(s) such as cereblon and associated downstream factors hence subsequently modifying their mechanisms of action.

Despite these promising *in vitro* findings, only Gu1029 performed as well in the human prostate cancer xenograft assay as previously tested tetrafluorinated analogs (31).

When examined by Ng *et al.,* (31), the three chosen compounds (CPS45, CPS49, CPS11) previously synthesized and described demonstrated reductions in tumor size of 51%, 68%, and 90%, respectively. In the current study, Gu1029 was the only compound that significantly inhibited prostate tumor growth. Further studies comparing the actions of Gu1029 with previously synthesized CPS compounds would be useful to determine the tolerability and *in vivo* efficacy of these compounds, and may allow for the synthesis of new compounds with more potent properties.

Given the teratogenic nature of thalidomide, the lead compounds in this study were tested for any effects upon embryonic development using the chicken embryo assay. The chicken embryo is a well-established system to study teratogenesis and the effects of drugs upon development (2, 3, 5, 28, 34). Gu998 and Gu992 were shown to be teratogenic, causing limb, spinal and head defects as well as causing a high death rate. Embryos treated with Gu973 and Gu1029 did not produce limb defects, however surviving embryos did show hemorrhaging, growth retardation and necrosis in the surrounding CAM as well as twisted spines (Gu1029) and microopthalmia (Gu973) at equivalent concentrations as Gu998. Full interpretation of this data would require further study in higher species. Additionally, these studies were conducted to assess for the potential for teratogenic effects and further testing would be required to establish the effects of these compounds at lower concentrations. However, these compounds are potent and act outside the activity window of the parent molecule thalidomide in the chicken embryo, which needs to be applied much earlier in development to induce developmental defects (33). The concentrations of compounds used in our *in vitro* studies are comparable to those used in previous work by other groups (25 36). The

concentrations used in the chicken embryo *in vivo* testing are lower (Gu973, Gu998, Gu1029) or equivalent (Gu992) to concentrations previously used to assess the teratogenicity of thalidomide, and its analog lenalidomide, at doses that induce maximal anti-inflammatory responses in zebrafish embryos and TNFalpha analysis in RAW cells (28). The compounds were applied once directly over the body of the embryo, and the chicken embryo was left to bathe in the solutions, however how much of the drug is able to enter the embryo is unclear. Given that this amount is likely small, but the concentrations applied are lower or equivalent to concentrations of thalidomide and lenalidomide that induce teratogenesis from previous studies (28, 34), the likelihood of these compounds possessing teratogenic abilities is highly probable.

We also assessed the anti-angiogenic and anti-inflammatory effects of these compounds on development of the vasculature in *fli*1:EGFP and *mpo*:GFP zebrafish embryos. We found that all the compounds reduced the extent of outgrowth of the blood vessels, although only Gu992 significantly reduced the number of forming blood vessels. Gu973 and Gu1029 had a significant decrease in the number of neutrophils migrating to the area of the cut. Gu992 and Gu998 did not, however at high concentrations embryos were not able to survive the treatments. This indicates the toxicity of these compounds in the zebrafish embryo.

In summary, this study demonstrated that Gu973, Gu992, Gu998, and Gu1029 possess significant *in vitro* anti-angiogenic effects with potential anti-tumor activity. Of these compounds, Gu998 proved to be the most effective in the greatest number of experimental designs. However, Gu1029 displayed the most potential in the human prostate cancer xenograft assay, as well as exhibiting anti-inflammatory properties. Given

its potency in both angiogenic and inflammatory assays, Gu1029 appears to be a prime candidate molecule for further screening as a potential anti-cancer compound. Studies are currently underway to determine the downstream targets and effector molecules of Gu1029. Preclinical assays which evaluate the efficacy of Gu1029 in the treatment of multiple myeloma would be useful to assess the activity in comparison to the currently used analogs lenalidomide and pomalidomide. Future studies on the compound's *in vivo* efficacy are also warranted, including optimization of dose and schedule, improved delivery and formulation, to decipher if Gu structural analogs could be more effective or safer versions of thalidomide.

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fli1:EGFP zebrafish were obtained from the Zebrafish International Research Center, USA (27). *mpo*:GFP zebrafish {also termed Tg(MPO:GFP)^{114} } zebrafish were obtained from Dr Stephen Renshaw, University of Sheffield, UK (29).

Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Beedie, Peer, Chau, Vargesson, Figg

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Figure legends

Figure 1. Structures of the tetrafluorobenzamides, Gu1029 and Gu992, and the tetrafluorinated thalidomide analogs Gu973 and Gu998.

Figure 2. In vitro screening was conducted in the Rat Aortic Ring (RAR) angiogenesis assay following the standard protocol. CAI was used as a positive control and shows little to no outgrowth. The Gu compounds were dosed at a concentration of 50μM. In comparison to the control outgrowth was inhibited in every instance.

Figure 3. *In Vitro* testing of Gu compounds. Each compound reduced cell proliferation in HUVEC cells at 5μM and 10μM. Compounds Gu1029 and Gu992 were tested in PC3 cells at 10μM and exhibited no effect, while Gu973 and Gu998 were tested at both 10μM and 5μM causing a reduction in cell proliferation.

Figure 4. The effect of Gu compounds on transgenic zebrafish. Vessel length is decreased by Gu compounds. After incubation with vehicle control (0.1% DMSO) for 24 hours embryos had normal blood vessel outgrowth (Figures A, F). Treatment with Gu973 reduced the outgrowth at 1 and 10 μg/mL, with a slight increase at 20 μg/mL (Figures B, F). Gu992 decreased outgrowth at all concentrations tested (Figures F, C). Gu998 decreased outgrowth (Figure F) at 1 and 10 μg/mL (Figure D). At higher concentrations all embryos were dead. Gu1029 decreased outgrowth at all concentrations tested (Figure F) however the extent of inhibition was not significant at 10 μg/mL. The number of

sprouting vessels was also quantified (Figure G). Gu1029 was the only compound that had no effect on the vessel number. Gu998 showed a non-significant decrease in sprouting vessels. Gu973 decreased vessel number, but only significantly at 10 μg/mL. Gu992 inhibited sprouting significantly at 50 μg/mL. (Figure H) Gu compounds effects upon the inflammatory response in *mpo*:GFP zebrafish. Compared to control embryos, Gu973 & Gu1029 significantly reduce neutrophil cell migration. In contrast Gu992 and Gu998 did not affect the inflammatory response at concentrations tested. Scale bar represents 100 μm.

Figure 5. *In vivo* efficacy of Gu compounds. (A) The tumor volume in mice inoculated with 3 million PC3 cells (n = 29) was monitored over time. All compounds were compared to vehicle (5% DMSO in saline). Significance was determined by both Student-t and Mann-Whitney tests, with results being similar in both instances. (B) Weight loss of treated mice. Weight loss of Gu973 treated mice was significant but stable. Supplementation with transgel did not abrogate the weight loss. Severe weight loss after the treatment period was seen in some mice with larger tumors.

Figure 6. Application of Gu compounds at HH St 18 (E2.5) causes developmental defects in the chicken embryo. Examples of phenotypical outcomes with application of Gu compounds at HH St 18. (A) Application of Gu1029 induces hemorhaging in the embryo (black arrow head; A) and necrosis in the CAM *in ovo* (white arrow head; A) (n = 10/10). (B) Gu998 causes hemoragging in the limb of the treated embryo (arrowhead; B) (n = 2/10). The embryo also shows has growth retardation (n = 5/10). (C) Application of

Gu992 causes retardation of limb growth (arrow head; C) (n = 1/12). (D) Gu973 causes hemoragging through the body of the embryo (n = 3/10). The limbs in particular are affected (arrow head; D). Scale bar represents 1000 μm. (E) Application of compounds Gu1029 (n = 10), Gu992 (n = 12), Gu973 (n = 10) and Gu998 (n = 11) induces variable defects in the developing chick embryo as well as having adverse effects on the vasculature within the CAM, and causing premature embryonic death.