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Short communication

Clinically and orally compatible formulation-manufactured DDX5 (p68)-targeting molecular glue FL118 products exhibit low toxicity but high efficacy against human cancer

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Our recent breakthrough discovery demonstrated that the anticancer drug FL118 tightly binds to and then dephosphorylates and degrades the oncogenic protein DEAD-box helicase 5 (DDX5), leading to the inhibition of DDX5 downstream targets (e.g., survivin, myeloid cell leukemia 1 (Mcl-1), X-linked inhibitor of apoptosis (XIAP), c-Myc, mutant Kras, etc.) [1]. FL118 is a molecular glue (MG) that can alter the interactomes of two or more non-interacting proteins [2]. Thus, FL118 exhibits high efficacy against colorectal and pancreatic cancer xenograft tumors [1,3]. However, moving FL118 into clinical trials requires a clinically compatible FL118 drug product (DP) that possesses high antitumor efficacy and low toxicity via oral (ideal) or intravenous (iv) administration. Here, we report the development and characterization of a clinically and orally compatible FL118 DP. We show that (1) FL118 drug substances (DS) exhibit high chemical stability under various test conditions; (2) a clinically and orally compatible FL118 DP can be manufactured through the formulation of FL118 DS with 2-hydroxypropyl-β-cyclodextrin (HPβCD) using mixed solvents of glacial acetic acid (GAA) with ethanol through microfluidizer-mediated spray dried dispersion (M-SDD); (3) as revealed from toxicology studies in rats and dogs, the clinically compatible FL118 DP exhibits much lower toxicity with higher maximum tolerated dose (MTD) than preclinically/non-clinically compatible FL118 DP; and (4) this clinically and orally compatible FL118 DP displays exceptionally high efficacy against human colorectal cancer, pediatric osteosarcoma, castration-resistant prostate cancer, and pancreatic cancer.

The method used to determine the stability (purity and impurity) of FL118 active pharmaceutical ingredient (API) over time was described in Section 2.1 of the Supplementary data, and the potential impurities in FL118 API were presented in Fig. S1. The method used to produce the preclinically/non-clinically compatible manufacturing of FL118 DP was described in Section 2.2 of the Supplementary data. The method used to produce the clinically compatible manufacturing of FL118 DP was described in Section 2.3 of the Supplementary data. The method that was used to analyze FL118 DP was described in Section 2.4 of the Supplementary data. The method used to determine and analyze the toxicology and MTD of the preclinically compatible FL118 DP in rats was described in Section 2.5 of the Supplementary data, and the experimental design was outlined in Table S1. The CRO, Covance, performed the studies using a rat protocol approved by the Covance IACUC. The method used to determine and analyze the toxicology and MTD of the clinically compatible FL118 DP using a limited number of rats was described in Section 2.6 of the Supplementary data, and the experimental design was outlined in Table S2. The method used to determine and analyze the toxicology and MTD of the clinically

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compatible FL118 DP using the standard number of rats was described in Section 2.7 of the Supplementary data, and the experimental design was outlined in Table S3. The method used to determine and analyze the toxicology and MTD of the clinically compatible FL118 DP using the standard number of dogs was described in Section 2.8 of the Supplementary data, and the experimental design was outlined in Table S4. The method used to determine the antitumor efficacy of the clinically compatible FL118 DP with human tumor animal models was described in Section 2.9 of the Supplementary data. It was determined in the Roswell Park Animal Center following the mouse protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Roswell Park Comprehensive Cancer Center. The IACUC-approved animal protocol is consistent with the National Research Council's Guide for the Care and Use of Laboratory Animals.

The CRO WuXi AppTec (Wuhan, China) has manufactured three batches of FL118 DS (i.e., API; Table S5) which exhibited high chemical stability in various test conditions including (i) stress test at 60 °C (Table S6) or at 25 °C/92.5% relative humidity (RH) (Table S7) for 0–30 days, and light stress test at 25 °C/60% RH for 0–10 days (Table S8); (ii) accelerated test at 40 °C/75% RH for 0–180 days (Tables S9–S11); and (iii) ambient long-term test at 25 °C/60% RH for 0–730 days (Tables S12–S14). Independently, prior to the manufacture of clinically compatible FL118 DP using the GMP FL118 API (C180402127–BF18001, Table S5) in the non-GLP condition in a small scale, the CRO BioDuro (San Diego, CA, USA) also performed high performance liquid chromatography (HPLC) analysis of FL118 API purity and impurity. After storing FL118 API at room temperature for more than 2 years, no changes in purity (Fig. S2A) and impurity were observed (Fig. S2B).

X-ray powder diffractometer (XRPD) and differential scanning calorimetry (DSC) analysis of FL118 DS indicated that FL118 is in a weak crystal status (Fig. S3) with no clear melting point (Fig. S4). We formulated the FL118 DS with HPβCD using anhydrous ethanol followed by a spray dried dispersion (SDD) process. XRPD analysis of the formulation of FL118 DS into ethanol-HPβCD solution (1 h, 6 h, 24 h) without the use of microfluidizer before processing SDD indicated that FL118-HPβCD complex (i.e., DP) are mainly in a co-amorphous status (Fig. S5) and exhibited similar modulated DSC (mDSC) profiles (Figs. S6–S8), which are distinct from the mDSC profile of HPβCD without FL118 (Fig. S9). We repeated using a larger batch of ethanol-mediated formulation to manufacture FL118 DP, which obtained similar XRPC (Fig. S10) and mDSC (Fig. S11) results. Importantly, ethanol-mediated manufacture of FL118 DP was highly consistent and mainly in a co-amorphous status in the repeated studies (Fig. S12), which was very similar to the results as shown inFig. S5 Figs. S5 and S10Fig. S10.

Our screening of more than 30 Food and Drug Administration (FDA)-recommended Class 3 organic solvents led to the discovery of GAA being the best organic solvent for FL118 formulation with HPβCD. Through trial and error, 10% GAA with 90% ethanol was found to be the optimal ratio to be used in the M-SDD equipment to reduce the FL118-HPβCD particle size from 60–800 μm (via shake homogenization) to 0.12–11.3 μm before the SDD process (Fig. S13). Analysis of the FL118-HPβCD complex (i.e., DP) manufactured using the GAA/ethanol (10:90, *V/V*) formula indicated that the FL118 DP is in co-amorphous status mixed with micro co-crystal (Figs. S14 and S15). Detailed methodology information can be found in Sections 2.3 and 2.4 of the Supplementary data.

Formulation is presented in Section 2.2 of the Supplementary data. Studies indicated that rats were sensitive to this FL118 DP, showing high toxicity (Tables S15–S17) and exhibiting the highest non-severely toxic dose (HNSTD, a dose slightly higher than MTD) at 1.65 mg/kg weekly (Table 1A, left).

Studies from a limited number of rats indicated that with the same schedule and route as used in the preclinically/non-clinically-compatible FL118 DP, the clinically compatible FL118 DP in the dose range of 0.8–4.77 mg/kg weekly × 7 total administrations (described in Section 2.6 of the Supplementary data) did not show significant toxicity to rats (Tables S18 and S19). The estimated MTD was ≥2.44 mg/kg for male rats and ≤4.77 mg/kg for female rats (Table 1A, right). Next, we studied doses of 3.0 → 3.0, 3.75 → 2.5 and 4.69 → 2 mg/kg using more rats (described in Section 2.7 of the Supplementary data). The 3.75 → 2.5 and 4.69 → 2 mg/kg doses negatively affected some hematopoietic (Tables S20–S22) and serum chemistry (Tables S23–S25) parameters and significantly decreased body weight in male rats, while the body weight change in female rats was negligible (Figs. S16 and S17). Death was observed in some male rats in the 4.69 → 2 mg/kg dose (Table 1B, right). The estimated MTD is 2.5 mg/kg for male rats and 3.0 mg/kg for female rats, and the estimated HNSTD is 3.0 mg/kg for male rats and 3.75 mg/kg for female rats (Table 1B, right). Next, doses of 2.5 → 9.9, 3.3 → 6.6, or 4.4 → 4.4 mg/kg were studied in the FDA-recommended number of dogs (described in Section 2.8 of the Supplementary data). Dogs were dosed on Days 1 and 8 and then observed over a 14-day period. During the observation period, no significant toxicities including body weight changes (Figs. S18A and S19A), food consumption (Figs. S18B and S19B) and other clinical and pathological parameters were noted. However, the treatment did negatively affect some hematopoietic (Tables S26–S28) and serum chemistry (Tables S29–S31) parameters. The estimated MTD is 5.0 mg/kg for both male and female dogs, and the estimated HNSTD is ≥ 6.0 mg/kg for both male and female dogs (Table 1C, right).

Table 1
Summary of FL118 DP toxicology studies in rats and dogs, and comparison of toxicity profiles of preclinically/non-clinically compatible FL118 DP versus clinically compatible FL118 DP.

Animal models		Preclinically compatible FL118 DP (mg/kg)			Clinically compatible FL118 DP (mg/kg)		
		Dose range, weekly	HNSTD	MTD	Dose range, weekly	HNSTD	MTD
Rats (A)	Male	1.65; 3.3[a]; 6.6[a]	1.65	<1.65	0.8–1.56; 1.95–3.05; 3.81; 4.77[b]	Not reached	≥2.44
	Female	1.65; 3.3[a]; 6.6[a]	1.65	<1.65	0.8–1.56; 1.95–3.05; 3.81; 4.77	Not reached	≤4.77
Rats (B)	Male	Not applicable (N/A)	N/A	N/A	3.0 → 3.0; 3.75 → 2.5; 4.69 → 2.0[c]	3.0	2.5
	Female	Not applicable (N/A)	N/A	N/A	3.0 → 3.0; 3.75 → 2.5; 4.69 → 2.0	3.75	3.0
Dogs (C)	Male	0.55; 1.1; 2.2	>2.2	2.2[d]	2.5 → 9.9; 3.3 → 6.6; 4.4 → 4.4	≥6.0	~5.0
	Female	0.55; 1.1; 2.2	>2.2	2.2[d]	2.5 → 9.9; 3.3 → 6.6; 4.4 → 4.4	≥6.0	~5.0

HNSTD: highest non-severely toxic dose. MTD: maximum tolerated dose.
[a] Doses induce rats in a moribund condition and/or death.
[b] Male rats at the highest 3 doses (3.05, 3.81, 4.77 mg/kg) temporarily experience mild adverse clinical signs.
[c] Induce male rat death.
[d] Reported in [3].

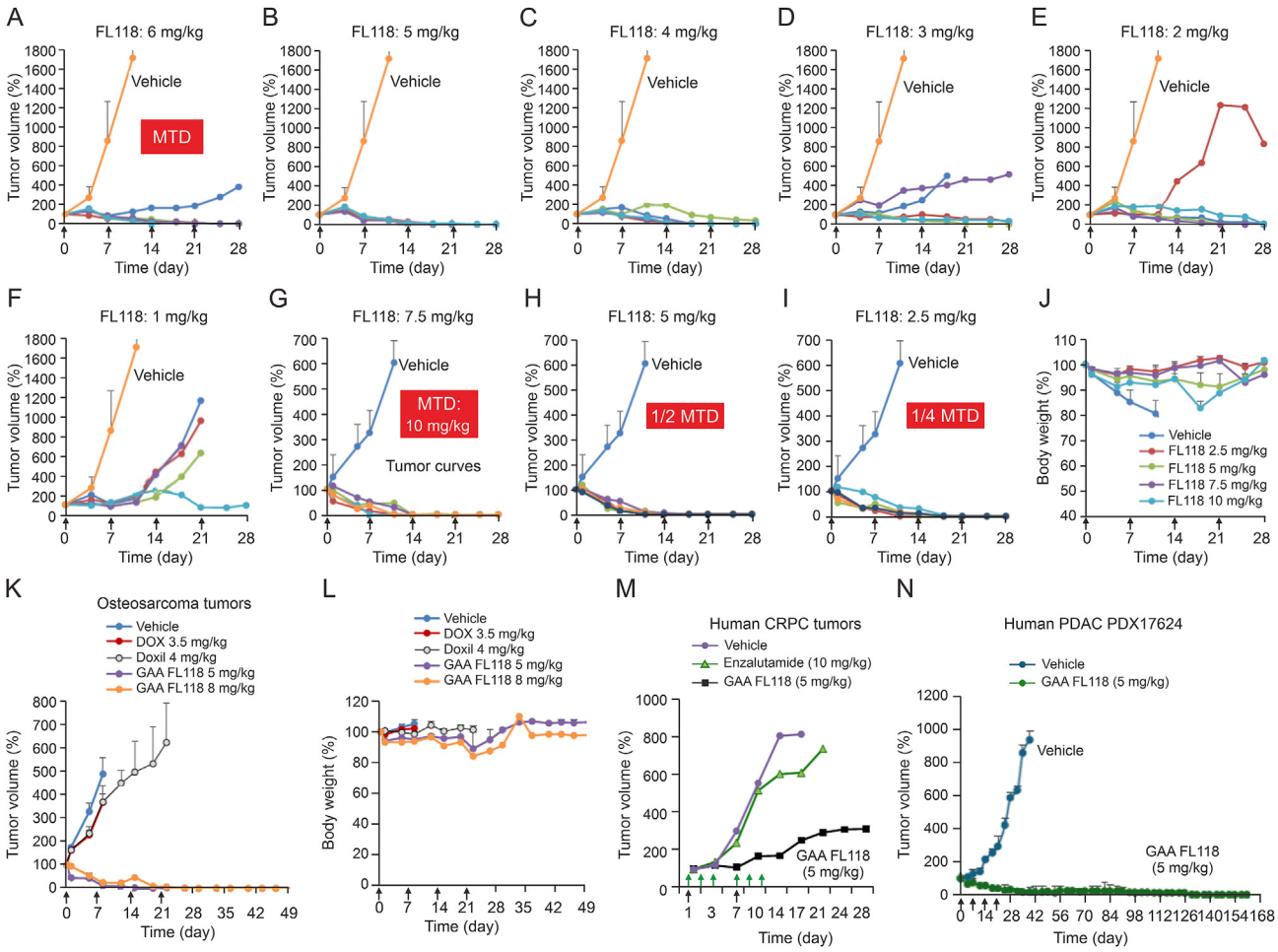

Fig. 1. FL118 DP antitumor efficacy studies—Clinically compatible FL118 DP exhibits low toxicity and high efficacy. (A–F) Ethanol-processed clinically compatible FL118 DP (Lot No.: FR00535-05-191104-01) improved FL118 efficacy. Severe combined immunodeficiency (SCID) mice were implanted subcutaneously at the flank area with 25–50 mg non-necrotic SW620 xenograft tumors with triple mutations in *Kras, p53* and *APC* genes. When tumors reached 100–200 mm^3 (day 0), each group of mice (5 mice per group) were orally dosed with FL118 DP at doses of 6, 5, 4, 3, 2 and 1 mg/kg of FL118 API once per week for 4 weeks as a cycle/course (arrows), respectively. The vehicle-treated tumor curve is the mean ± standard deviation (SD) from 5 mice. FL118-treated individual tumor curves from 5 mice in each group are shown. (G–J) Glacial acetic acid (GAA)-processed FL118 products (Lot No.: CGT SDC LOT 2020-252-023) further improved the FL118 efficacy-toxicity ratio. SCID mice implanted with SW620 tumors were treated in the same way as in (A–F). When tumors reached 100–200 mm^3 (day 0), each group of mice (5 mice per group) were orally dosed with FL118 DP at 10, 7.5, 5, and 2.5 mg/kg of FL118 API once per week for 4 weeks as a cycle/course (arrows). The vehicle-treated tumor curves represent the mean ± SD from 5 SCID mice whereas individual tumor growth curves are shown for the FL118-treated mice (G–I). Changes in body weight of mice (mean ± SD; $n = 5$/group). Only a transient decrease in body weight, within the acceptable range of ≤20%, was observed at the 10 mg/kg of FL118 after dose 3 on day 14 (J). (K) FL118, in contrast to doxorubicin (DOX) or liposome-formulated DOX (Doxil), effectively eliminates pediatric osteosarcoma (OS) xenografts in SCID mice. The SK-ES-1 OS cells (2×10^6 per tumor site) mixed with 50% matrigel were subcutaneously injected into the flank area of 2–3 SCID mice to establish xenograft tumors. The established OS tumors were implanted into experimental SCID mice for drug efficacy studies. Treatment with vehicle, DOX, Doxil, or GAA/ethanol (10:90, *V/V*) formulated, clinically compatible FL118 DP at the indicated doses of FL118 API was started when tumors reached 150–200 mm^3. The schedule and route were weekly x 4 via oral administration (arrows). Each curve represents the mean tumor size + SD from 3 to 4 SCID mice. (L) Changes in body weight of mice after vehicle, DOX, Doxil, or FL118 treatment. Each curve represents the mean + SD from 3 to 4 SCID mice. (M) Antitumor efficacy of FL118 and enzalutamide on human LAPC9-CRPC in SCID mice. Mice bearing the $AR^{-/lo}$ LAPC9-CRPC were treated with enzalutamide (10 mg/kg, i.p 3x/week for 2 weeks; green arrows) or with vehicle or FL118 (5 mg/kg, oral, 1x/week for 2 weeks; black arrows). Three female tumor-bearing SCID mice were used with tumor curve variation ≤10%. (N) Antitumor efficacy of FL118 on human pancreatic ductal adenocarcinoma (PDAC) patient-derived xenograft (PDX) tumor, PDX17624 in SCID mice. Implantation of PDX17624 tumors on SCID mice was performed as described above. When tumors reached 100–200 mm^3 (day 0), mice were treated orally with vehicle or FL118 (5 mg/kg) once per week for 4 weeks as a cycle/course (arrows) to the two groups of mice (5 mice per group), respectively. The tumor curves are the mean + SD derived from 5 individual tumors on 5 mice in each group. MTD: maximum tolerated dose.

First, we studied the ethanol-processed FL118 DP's antitumor activity. As shown, FL118 DP regressed CRC tumors below its MTD (Figs. 1A–F). Next, we studied the antitumor effects of FL118 DP formulated in GAA/ethanol (10:90, *V/V*), which displayed further improved tumor-inhibitory effects with increased MTD (Figs. 1G–J) in comparison with the pure ethanol-processed FL118 DP (Figs. 1A–F), although both formulations of FL118 DP demonstrated impressive antitumor efficacy (Figs. 1A–J).

Recent literature studies have revealed that DDX5 plays a critical role in oncogenic signaling pathways of osteosarcoma (OS) [4,5] and castration-resistant prostate cancer (CRPC) [5,6]. Since DDX5 is the direct physical and functional target of FL118 [1], we studied the potential antitumor effects of the GAA/ethanol (10:90)-processed FL118 DP in OS and CRPC. Doxorubicin (DOX) is known to be the most used chemotherapeutic drug for soft tissue sarcoma (STS) and OS. In this regard, our previous studies indicated that DOX at its MTD only exhibited a minimal inhibition of STS whereas FL118 below its MTD regressed STS xenografts [5]. Consistent with these earlier results, our new studies revealed that while DOX or liposome-formulated DOX (Doxil) failed to regress SK-ES-1-established OS xenografts, FL118 regressed OS tumors below its MTD levels (Fig. 1K) with acceptable toxicity evidenced by various clinical observations (e.g., animal movement, behavior, fur shining and diarrhea

statuses, etc.) and mouse body changes (Fig. 1L). The human LAPC9-CRPC tumor model was used in CRPC therapeutic studies. LAPC9-CRPC tumors possess negative/low androgen receptor ($AR^{-/lo}$), and showed very subtle response to enzalutamide, the standard-of-care clinical drug for CRPC. In contrast, LAPC9-CRPC tumors displayed exquisite sensitivity to FL118 (Fig. 1M). Notably, enzalutamide was administered six times (green arrows), while FL118 was dosed only twice (black arrows), instead of 4 times as usually done as a cycle/course (Fig. 1M). Furthermore, our previous studies indicated that the human pancreatic ductal adenocarcinoma (PDAC) patient-derived xenograft 17624 (PDX17624) could only be transiently eliminated [3] or transiently repressed [1] by the preclinically/non-clinically compatible FL118 DP, and the transiently eliminated/regressed PDX17624 tumors relapsed after such treatment [1,3]. In contrast, the PDX17624 tumor exhibited high sensitivity to the GAA/ethanol (10:90)-processed, clinically compatible FL118 DP, and the eliminated PDX17624 tumor showed no relapse after this treatment (Fig. 1N).

In conclusion, these *in vivo* studies (Table 1 and Fig. 1) demonstrated that the 10%GAA/90%ethanol-processed, clinically compatible FL118 DP possesses superior antitumor efficacy with favorable low toxicity profiles in comparison with the preclinically/non-clinically compatible FL118 DP or with the commonly used chemotherapeutics such as DOX, Doxil and enzalutamide tested here. Thus, the studies presented in this report have overcome a critical hurdle in our drug development efforts and laid a foundation for scaling up FL118 DP manufacturing under GLP/GMP conditions for the upcoming FL118 clinical trials.

CRediT authorship contribution statement

Xiang Ling: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Wenjie Wu:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Li Yan:** Formal analysis, Methodology, Software, Validation, Writing – review & editing. **Leslie Curtin:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Melanie M. Farrauto:** Data curation, Methodology, Writing – review & editing. **Sandra Sexton:** Methodology, Project administration, Resources, Writing – review & editing. **Anmbreen Jamroze:** Data curation, Investigation, Methodology, Writing – review & editing. **Changjun Yu:** Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Christos Fountzilas:** Methodology, Project administration, Visualization, Writing – review & editing. **Dean G. Tang:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Fengzhi Li:** Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Validation, Visualization.

Declaration of competing interest

FL118 and FL118 core structure-based analogs will be further developed in Canget BioTekpharma LLC (www.canget-biotek.com), a Roswell Park Comprehensive Cancer Center (www.roswellpark.org)-spinoff company. Xiang Ling and Fengzhi Li are two of the 17 initial investors of Canget for development of FL118 and FL118-derived analogs. All other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2024.101001.

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