Antineoplastic activity of GP-2250 in-vitro and in mouse xenograft models

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This study examined the antineoplastic effects of GP-2250 (misetionamide), an oxathiazine derivative with broad activity, in multiple cancer cell lines and mouse xenograft models. Antineoplastic activity of GP-2250 was tested in >300 cancer cell lines using the OncoPanel cytotoxicity assay. GP-2250 activity was further tested in mouse xenograft models, in which GP-2250 or vehicle (10 ml/kg) was administered daily for 28 days by intraperitoneal injection in the lower right abdomen of CrTac:NCR-Foxn1nu mice with tumor volumes of 100 to 200 mm^3. In the in-vitro models, GP-2250 increased cytotoxicity readings with IC50 and EC50 as well as indications of cell cycle blockage in pancreatic and ovarian cell lines. In mouse xenograft models, a reduction of 30–40% in tumor volume occurred in the GP-2250 group versus the vehicle group. On the final day of the study, tumor progression was significantly reduced in 4 tumor types: HT-29 in the GP-2250 500 and 1000 mg/kg groups, SKOV-3 in all GP-2250 treatment groups, Cal-27 in the GP-2250 1000 mg/kg group, and Hs-695T in the GP-2250 250 and 1000 mg/kg groups. Tumor regression in Cal-27 tumors was dose-dependent. GP-2250 demonstrated cytotoxic activity *in vitro* and reduced the tumor volume in a variety of human cancer cell lines in a xenograft mouse model. Given these results, as well as evidence of synergism with other anticancer drugs, GP-2250 shows promise as a new therapeutic agent for treating human cancers and is being evaluated in a phase 1 dose-escalation study (NCT03854100). *Anti-Cancer Drugs* XXX: XXXX–XXXX

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Introduction

GP-2250 (misetionamide), an oxathiazine derivative, is a novel structural analog of taurultam (Fig. 1). GP-2250 has demonstrated in-vitro activity in five pancreatic cancer cell lines (AsPC-1, Bx-PC-3, MiaPaca-2, Panc-1, and Panc-Tul) as a monotherapy both before and after the administration of the radical scavenger N-acetylcysteine in an MTT assay. In the 5-bromo-2'-deoxyuridine (BrdU) assay, GP-2250 increased the proportions of viable, apoptotic, and necrotic cells, as determined by fluorescence-activated cell sorting analysis with Annexin V-FITC and propidium iodide [1].

In addition, data from an in-vitro cell line assessment and a patient-derived xenograft model showed that GP-2250, when used in combination with other anticancer drugs, synergistically stabilized or regressed tumor growth [2,3]. The synergistic effect of GP-2250 with multiple anticancer agents provides strong proof of concept for GP-2250 as a promising new chemotherapeutic.

The goal of the present study was to further explore the antineoplastic effects of GP-2250, using both in-vitro and in-vivo assessments, in a number of tumor cell lines and sarcoma, pancreatic, colon, and ovarian human tumor cell lines in nude mice.

Materials and methods

In-vitro cytotoxicity assay

To screen multiple cancer cell types to the sensitivity of GP-2250 as a potential antineoplastic agent, the OncoPanel multiplexed cytotoxicity assay (ONCOPANEL Trademark, EUROFINS PANLABS, Inc.. Serial Number:97337567: Trademark Elite Trademarks), along with individual experiments with other cell lines, were employed. OncoPanel measures multiple parallel responses of cancer cell lines to drug treatments through high-content fluorescence imaging.

Cells grown in RPMI-1640, 10% FBS, 2 mM L-alanyl-L-glutamine, 1 mM Na pyruvate, or a special medium were seeded in 384-well plates and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The following day, a time-zero untreated cell plate was generated, and GP-2250 or vehicle was added to the cells. Cells were incubated for 3 days, fixed, and stained with fluorescently labeled antibodies and nuclear dye to allow imaging of nuclei, apoptotic cells, and mitotic cells.

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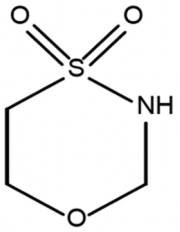
Fig. 1



GP-2250

(misetionamide)

(1,4λ⁶, 3-Oxathiazinane-4,4-dione)

GP-2250 chemical structure, name, and generic name.

GP-2250 was serially diluted in half-log steps from the highest test concentration (2000 μM) and assayed over nine concentrations, with a maximum assay concentration of 0.1% DMSO. Automated fluorescence microscopy was performed using a Molecular Devices ImageXpress Micro XL high-content imager, and images were collected with a 4X objective. 16-bit TIFF images were acquired and analyzed using MetaXpress 5.1.0.41 software.

Cell proliferation was assessed by measuring the fluorescence intensity of the incorporated nuclear dye. The output was referred to as the relative cell count, where the measured nuclear intensity was transformed to the percentage of control using the following formula:

$$\mathrm{POC} = \mathrm{I_x}/\mathrm{I_o}\mathrm{x}100$$

Where I_x is the nuclear intensity at concentration x, and I_o is the average nuclear intensity of the untreated vehicle wells.

Cellular response parameters were calculated using nonlinear regression to a sigmoidal single-site dose-response model.

$$\mathrm{y} = A + \left(\mathrm{B\ A}/1 + [C/\mathrm{x}]^{\mathrm{D}}\right)$$

where y is the response measured at concentration x; A and B are the lower and upper limits of the response, respectively; C is the concentration at the response midpoint (EC_{50}); and D is the Hill slope.

Time-zero non-treated plates were used to determine the number of doublings during the assay period, using the following formula:

$$\mathrm{Doublings} = \log_2 (\mathrm{N}/\mathrm{N_{TO}})$$

Where N is the cell number in untreated wells at the assay endpoint, and N_{T0} is the cell number at the time of compound addition.

The four primary data collections were concentrations (μM) at which the IC_{50}, EC_{50}, apoptosis induction at 5 times baseline levels ('5X baseline apoptosis'), and induction of a cell cycle G1/S or G2/M block occurred. Multiple cell lines were tested in the major cancer categories of colon, lung, breast, pancreas, lymphoma, sarcoma, brain, prostate, bladder, skin, head and neck, gynecological, liver, endocrine, and leukemia. The specific cell lines used in the pancreatic evaluations were AsPC-1, Bx-PC-3, MiaPaca-2, Panc-1, Panc-Tul, YAPC, SU.86.86, PSN-1, HuP-T4, Hs 766T, HPAF-II, CFPAC-1, Capan-1, and Capan-2. The ovarian cancer cell lines used were SKOV-3, CaOV3, CaSki, ME-180, MS751, OVCAR3, and PA-1.

Antibodies against activated caspase-3 were used to label cells undergoing early- to late-stage apoptosis [4]. The output is shown as a fold increase in apoptotic signal over the vehicle background normalized to the relative cell count in each well. The concentration of GP-2250 that caused a 5-fold increase in caspase-3 signal has been reported.

An antibody against phosphorylated histone H3 was used to label mitotic cells [5]. The output is shown as the fold induction of mitotic signal over the vehicle background normalized to the relative cell count in each well. The concentration of GP-2250 that caused a 2-fold increase or decrease in the phospho-histone H3 signal has been reported. A > 2-fold decrease indicated a G1/S block.

Xenograft models

Tumor cell lines and cultures

Eight different human cancer cell lines were tested: HT-1080 (sarcoma), AsPC-1 (pancreas), HT-29 (colon), SKOV-3 (ovary), NCI-H-596 (lung), SU-DHL-5 (lymphoma), Cal-27 (head and neck), Hs 695T (melanoma), HT-1080, AsPC-1, HCl-H-596, and SU-DHL-5 tumor cells were cultured in RPMI-1640. HT-29 and SKOV-3 tumor cells were cultured in McCoy's 5a medium. Cal-27 tumor cells were cultured in Dulbecco's Modified Eagle's medium. Hs-695T tumor cells were cultured in Eagle's Minimal Essential Medium (MEM). All media were supplemented with 10% fetal calf serum (FCS). After culture, the cells were washed in a medium containing FCS, resuspended in 1 : 1 PBS:Matrigel, and kept on ice until use.

Mice and tumor injection

A total of forty-five 8-week-old female CrTac:NCR-Foxn1nu mice were obtained from Taconic Biosciences and acclimated to a controlled 12-hour light cycle environment with food and water ad libitum for 15 days. The mice were injected with approximately 2×10^6 tumor cells, subcutaneously into the scuff of the neck with approximately 2 × 106 tumor cells. The mice were placed in the study when the tumors in the majority of animals reached a volume of 100 to 200 mm^3 and were randomized based on tumor volume.

GP-2250 treatment

Fresh test article solutions were prepared on each day of dosing by dissolution of GP-2250 in Lactated Ringers to concentrations of 25, 50, and 100 mg/ml, and the pH was adjusted with sodium hydroxide. The animals were dosed within 2 h of preparation completion. Dose groups of 10 mice each were administered the test vehicle, 250, 500, and 1000 mg/kg GP-2250 at 10 ml/kg by intraperitoneal injection in the lower right abdomen daily for 28 days or until the size of the tumor became too large for the viability of the animal. Intraperitoneal administration of GP-2250 has been successfully employed in previous studies.

Table 1 In-vitro cytotoxicity assay results

Cancer cell line	Cell lines used in-vitro testing, n	IC50 range, μM	EC50 range, μM	5X baseline apoptosis range, μM
Ovarian	7	9.7–93.8	21.7–86.6	12.8–115.3
Pancreatic	13	11.0–124.0	10.4–124	11.9–66.4
GI track	36	8.14–92.8	8.3–9.3	7.37–133.0
Lung	23	15.5–101.0	15.2–101.0	17.1–143
Lymphoma	32	3.68–54.0	3.43–78.4	6.56–45.7
Sarcoma	25	8.15 > 2000	7.96–144.0	10.8–189
Melanoma	25	21.0 > 2000	11.4 > 2000	28.9–124
Head and neck	10	10.0–59.2	9.9–99.1	14.6–62.0

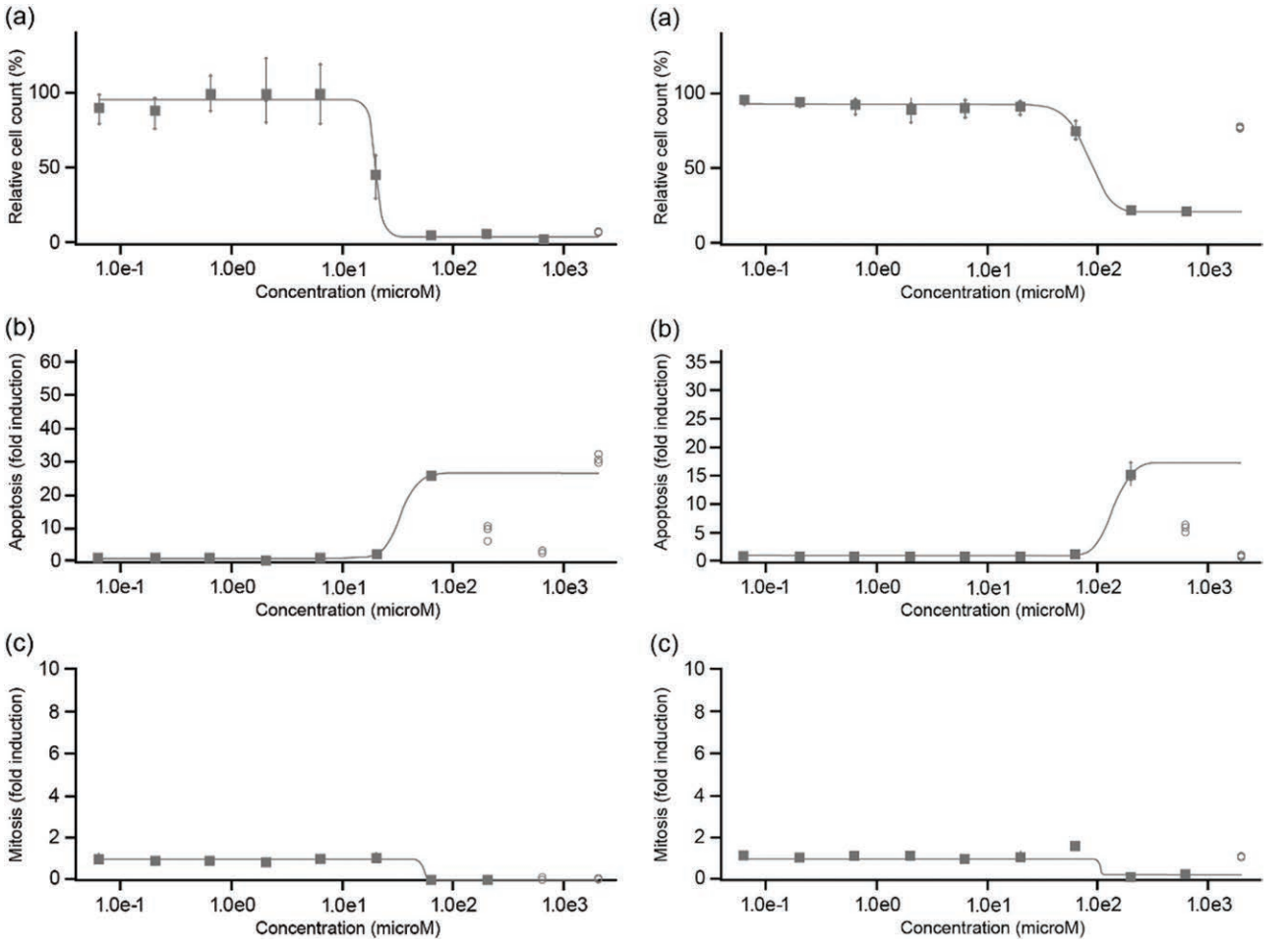
Tumor assessment

The tumor volume was measured twice weekly using calipers, and the volume was determined using the following formula:

$$V = \left(L * W^2\right) * 0.5$$

where L is the longest diameter of the tumor and W is the diameter measured at a right angle to L. Once the majority of tumors in each group reached 100–200 mm^3,

Fig. 2



Representative assay results for (a) IC_{50}, (b) EC_{50}, (c) and apoptosis range using Mia PaCa-2 pancreatic cancer cells (left panel) and OVCAR3 ovarian cancer cells (right panel).

Table 2 Mean body weight data

Group No.	n	Tumor	Treatment	Dose (mg/kg)	Mean body weight (g)	
					Day 0	Day 28
1	8	NCI-H-595	Vehicle	0	24.0±0.57	25.6[a] ± 0.53
2	8		Geistlich -2250	250	24.6±0.60	25.5±0.60
3	8		Geistlich -2250	500	24.4±0.84	26.6[a] ± 0.87
4	8		Geistlich -2250	1000	24.0±0.68	25.3[a] ± 0.49
5	7	SU-DHL-5	Vehicle	0	24.4±0.61	25.4±0.75
6	7		Geistlich -2250	250	24.4±0.37	25.3±0.36
7	7		Geistlich -2250	500	24.3±0.57	25.1±0.46
8	7		Geistlich -2250	1000	25.0±0.98	25.0±1.18
9	10	Cal-27	Vehicle	0	25.1±0.46	25.2±0.66
10	10		Geistlich -2250	250	26.0±0.79	26.9±0.86
11	10		Geistlich -2250	500	26.0±0.65	27.3[a] ± 0.67
12	10		Geistlich -2250	1000	25.4±0.50	26.8±0.60
13	10	HS 695T	Vehicle	0	23.8±0.33	23.5±0.56
14	10		Geistlich -2250	250	23.4±0.52	23.2±1.09
15	10		Geistlich -2250	500	23.7±0.56	24.0±0.75
16	10		Geistlich -2250	1000	24.5±0.52	23.9±0.61
17	10	HT-1080	Vehicle	0	22.7±0.62	24.8±1.16
18	10		Geistlich -2250	250	21.5±0.62	23.3±1.49
19	10		Geistlich -2250	500	21.7±0.58	23.4±1.72
20	10		Geistlich -2250	1000	21.8±0.77	22.4±1.43
21	10	AsPC-1	Vehicle	0	23.8±0.47	25.6[a] ± 0.34
22	10		Geistlich -2250	250	23.5±0.50	25.4[a] ± 0.54
23	10		Geistlich -2250	500	24.1±0.35	25.2±0.49
24	10		Geistlich -2250	1000	23.3±0.34	25.2[a] ± 0.44
25	10	HT-29	Vehicle	0	23.8±0.44	23.6±0.75
26	10		Geistlich -2250	250	23.3±0.63	23.9±0.89
27	10		Geistlich -2250	500	23.6±0.34	24.3±0.50
28	10		Geistlich -2250	1000	23.2±0.39	24.1±0.48
29	6	SKOV-3	Vehicle	0	23.0±0.26	24.8[a] ± 0.31
30	6		Geistlich -2250	250	23.5±0.76	25.0±0.78
31	6		Geistlich -2250	500	25.3±0.62	27.5[a] ± 1.31
32	6		Geistlich -2250	1000	24.3±0.67	25.7±0.67

Data presented as mean ± SEM.
Two-way repeated measure ANOVA followed by Bonferroni Multiple Comparison Test (SigmaStat, v.4.0).
There were no statistically significant changes compared to the respective vehicle control group.
[a]Statistically significant ($P \leq 0.05$) change compared to baseline (day 0) values.

the mice were randomized to one of four treatment groups: vehicle/control (lactated ringers), 250 mg/kg GP-2250, 500 mg/kg GP-2250, or 1000 mg/kg GP-2250. Mice received these treatments as once-daily intraperitoneal injections. Mice were euthanized via CO_2 asphyxiation at the end of the study or if their tumor volumes exceeded 1500 mm^3. All experiments were carried out at Calvert Laboratories, Inc., with the approval of the Calvert Intramural Animal Care and Use Committee.

Statistical analysis

The progression of tumor growth in the test groups was compared to that in the control group. The difference in tumor volume between the first dose and each measurement day for each mouse is reported. The equality of the means of each group on each measurement day was assessed via one-way ANOVAs using the F-distribution in Systat version 9.01. If statistically significant differences were found, Dunnett's test was used to determine the degree of significance of the control means. Additionally, one-sided *t* tests were performed on the difference between the mean tumor volumes in the treatment groups relative to the vehicle group on the last day of the study (day 27).

Results

In-vitro cytotoxicity assay

Three hundred standard commercially available cancer cell lines were used to test the in-vitro activity of GP-2250. The GP-2250 concentration ranges at which IC_{50}, EC_{50}, and induction of 5X baseline apoptosis are shown in Table 1. Representative assay results for IC_{50} (A), EC_{50} (B), and Apoptosis Range (C) using Mia PaCa-2 pancreatic cancer cells and OVCAR3 ovarian cancer cells are shown in Fig. 2, left panel, and right panel, respectively. In both pancreatic and ovarian cancer cell lines, at doses of GP-2250 between 10 and 100 μM, the cell count decreased (Fig. 2a), induction of 5X apoptosis over baseline increased (Fig. 2b), and cancer cell mitosis decreased (Fig. 2c).

Table S1, Supplemental digital content 1, *http://links.lww.com/ACD/A511* summarizes the changes in phospho-histone H3 signal levels and the implications of G1/S and G2/M blockage. The majority of the cell lines tested had an initial G1/S block induced, and five ultimately had a G2/M block.

Xenograft models

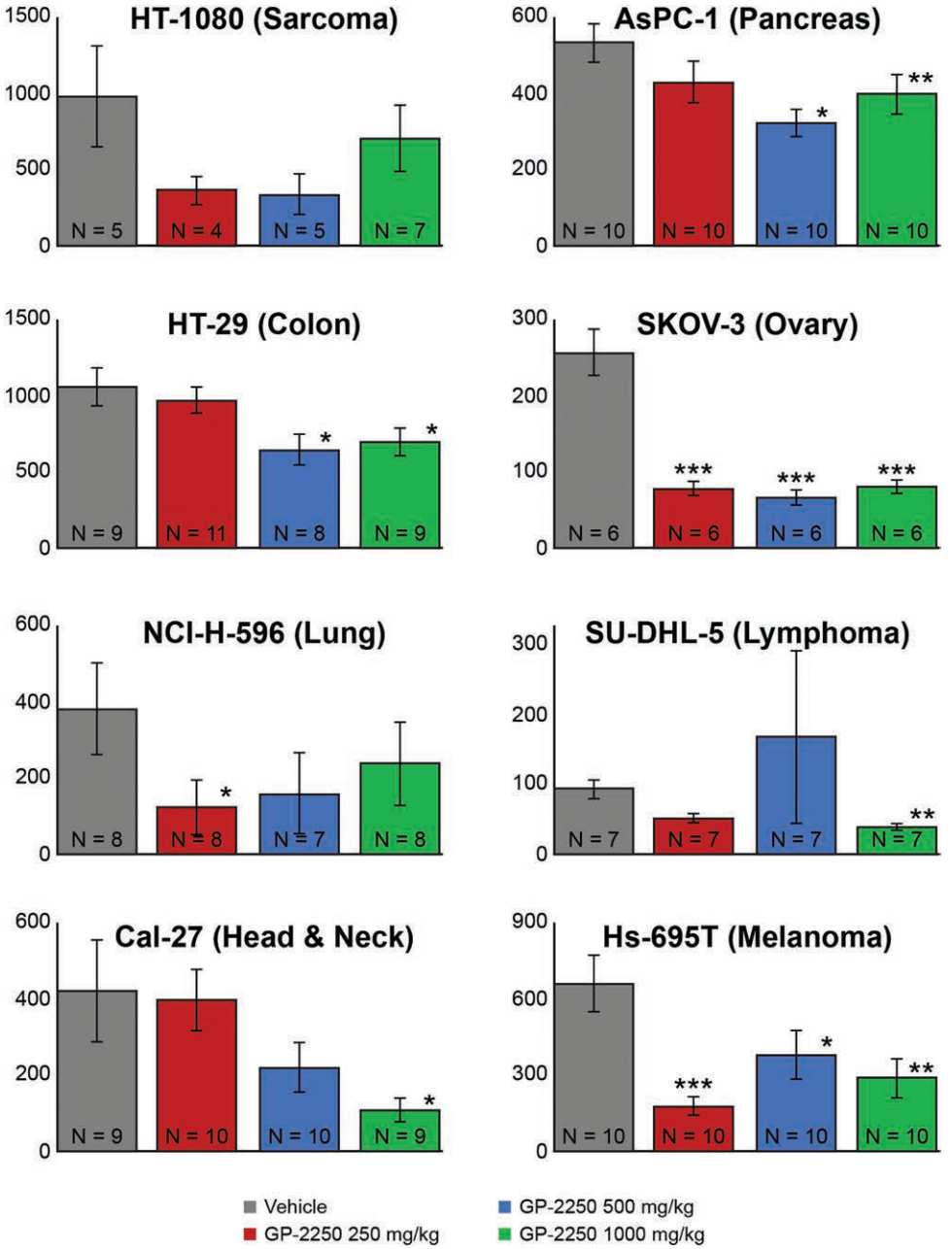
The animal weights for baseline and treatment periods are provided in Table 2.

Seven mice were found dead during the duration of the study, and 17 were euthanized prior to the completion of the study due to their tumor size. The disposition of the mice used in the experiment is shown in Table S2, Supplemental digital content 1, *http://links.lww.com/ACD/A511*.

The mean change from baseline in tumor volumes for each tumor cell line and treatment group is shown in Fig. 3. The mean tumor volumes in the vehicle group were consistently higher than those in the GP-2250 treatment groups, with the exception of SU-DHL-5 mice treated with 500 mg/kg GP-2250. However, there was high variability in tumor volumes in all treatment groups.

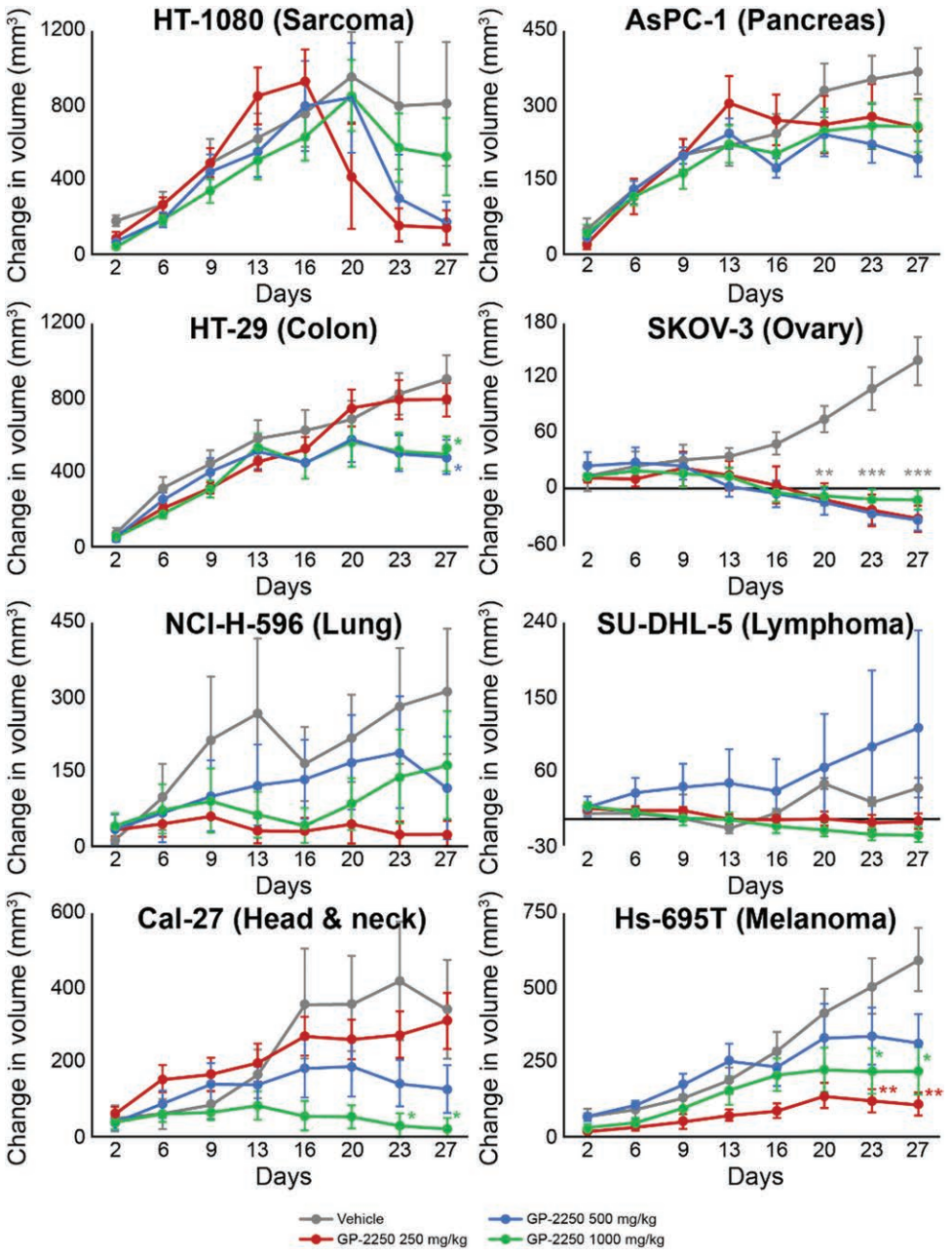
The mean change in tumor volume on each measurement day for each tumor cell line and each treatment group is shown in Fig. 4. Overall, there was a reduction in tumor volume progression in the treated

Fig. 3



Mean (SE) change from baseline in tumor volumes on day 27 by tumor cell line and treatment group.[a] SE, standard error. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control group. [a]Values on x-axis indicate dosage of GP-2250.

Fig. 4



Mean change in tumor volume over time by tumor type and treatment group.[a] Error bars indicate ± SEM. Differences compared to the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). [a]Values in legend indicate dosage of GP-2250.

groups relative to that in the control group, except for SU-DHL-5 treated with 500 mg/kg GP-2250. However, there was a high variability in the progression of tumor volume between mice and tumor types, and many changes were not statistically significant. On the final day of the study, the reduction in tumor progression in the GP-2250 treatment groups compared to the control group was statistically significant for four tumor types: HT-29 in the 500 and 1000 mg/kg groups, SKOV-3 in all treatment groups, Cal-27 in the 1000 mg/kg group, and Hs-695T in the 250 and 1000 mg/kg groups. Tumor regression in Cal-27 tumors may be dose-dependent.

Discussion

The cell cycle has several checkpoints at various phases of the cellular duplication process (Figure S1, Supplemental digital content 1, *http://links.lww.com/ACD/A511*). Two of the earliest checkpoints are used to check cell size, DNA damage and replication initiation,

and accurate and complete chromosomal replication. An error occurring at one of these checkpoints will stop the duplication process until the error can be repaired or the cell will be relegated to apoptosis. The in-vitro results showed that GP-2250 (misetionamide) is a broadly acting compound that induces apoptosis in cancer cells, resulting in decreased cell proliferation and viability. Cancer cell reproduction is also decreased by blocking the cell cycle at the G1/S phase or occasionally at the G2/M phase of cell division. The majority of cell lines tested had a G1/S block, and five cell lines had a G2/M block. These results are also shown graphically in Fig. 2c, where mitosis reduction was observed to occur at approximately the same concentrations of G-2250 as the concentrations inducing enhanced apoptosis and a reduction in relative cell counts.

Blocking the cell cycle at important checkpoints is a potentially important aspect of the mechanism of action of GP-2250, as checkpoint inhibition at the G1/S phases can cause an excess of cells to accumulate in the S phase of cell division, a stage at which cancer cells are known to have enhanced sensitivity to both chemotherapeutic drugs and radiation [6]. Such activity could potentially cause a combination chemotherapy to have enhanced activity against cancer cells if used in conjunction with GP-2250.

A previous study showed that GP-2250 exhibits a dose-dependent antineoplastic effect on both established malignant pancreatic cancer cell lines and xenograft models from patient tissues [1].

The goal of the present study was to investigate the effects of GP-2250 on a wider range of human cancer cell lines in a xenograft mouse model. The current study showed a reduction of 30%-40% of tumor volume in xenograft mouse models treated with GP-2250. and demonstrated that GP-2250 reduced the progression of tumor cell volume in multiple cancer cell lines. Although there is a high variability in the effect both within treatment groups and across cancer cell lines, these results suggest a strong antineoplastic effect, and for some treatment groups, this result was statistically significant. Notably, in the SKOV-3 tumor line, GP-2250 appeared to produce a regression in tumor volume, as evidenced by the negative change in tumor volume. Additionally, across all groups, it appears that the onset of GP-2250 activity began around 16 days after treatment initiation and when the tumor was well established.

Previous studies have shown that GP-2250 has a synergistic antineoplastic effect when administered in combination with anticancer agents in patient-derived xenograft models [2,3]. GP-2250 shows promise as an effective adjuvant to conventional chemotherapeutics in human cancers and is currently being evaluated in a phase 1 dose-escalation study in patients with advanced pancreatic cancer (NCT03854110).

In conclusion, GP-2250 demonstrated cytotoxic activity in ovarian and pancreatic cell lines, and reduced tumor volume in a variety of human cancer cell lines in xenograft mouse models. Given these promising results as well as other studies demonstrating strong synergism with other anticancer agents, including gemcitabine, bevacizumab, and PARP inhibitors, GP-2250 shows promise as a new therapeutic agent for human cancers.

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R. Duane Sofia contributed to the study conception and design, data acquisition, and data analysis and interpretation. Kathryn M. Martin contributed to data interpretation and writing of the draft and final manuscript. James C. Costin contributed to the conception and design of the study, data interpretation, and drafting of the manuscript. All authors have provided a critical review of, given the final approval for, and agree to be accountable for this article.

Conflicts of interest

RDS, KMM, and JCC are consultants of Geistlich Pharma, which supported the research.

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