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Original Article

Dose-Response Relationship of Niclosamide and Metformin Combination in Apc$^{Min/+}$ Mice: An Integrated *In Vivo* and Pharmacokinetic Modeling Study

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Background/Aims: Familial adenomatous polyposis (FAP), a hereditary colorectal cancer syndrome caused by *APC* gene mutations, is characterized by the development of numerous colorectal polyps and cancer at young age. To determine an effective chemopreventive strategy, we investigated the combined effects of varying doses of niclosamide and metformin in Apc$^{Min/+}$ mice.

Methods: Apc$^{Min/+}$ mice were treated with metformin, niclosamide, or their combination at three doses (50, 100, and 200 mg/kg) for 16 weeks. The polyp burden was analyzed, and drug interactions were assessed by using the Bliss independence model to evaluate pharmacodynamic synergy and a physiologically based pharmacokinetic (PBPK) model to quantify the contribution of known pharmacokinetic interactions.

Results: Low-dose metformin (50 mg/kg), niclosamide (50 mg/kg), and their combination showed no significant effects on the total polyp numbers compared with those in the control group. Higher doses (100 and 200 mg/kg) of both agents and their combination significantly reduced the total polyp numbers. The Bliss independence model showed a significant additive effect at the 100 mg/kg combination dose, whereas at the 200 mg/kg combination dose, an antagonistic interaction was observed. PBPK modeling predicted that coadministration of niclosamide increased exposure to metformin. Notably, the predicted metformin plasma Cmax remained within a safe therapeutic window at the 100 mg/kg combination dose but exceeded a safety threshold at 200 mg/kg.

Conclusions: By integrating *in vivo* efficacy testing with quantitative modeling, our study identified the 100 mg/kg combination of niclosamide and metformin as the optimal dose for chemoprevention in a murine FAP model, providing a strong rationale for future clinical translation in FAP management. **(Gut Liver, Published online December 9, 2025)**

Key Words: Familial adenomatous polyposis; Metformin; Niclosamide; Drug interactions

INTRODUCTION

Colorectal cancer (CRC) is a heterogeneous malignancy of the colon and rectum. Molecular and genetic pathogenesis of colorectal tumors is a multistep process involving specific genes at each step.[1-4] Trials performed to suppress colon tumorigenesis by controlling the expressions of these genes have shown limited success.[5,6] In the causes of CRC development, hereditary CRC accounts for approximately 5% to 10% of all cases.[7] Among the hereditary CRC syndromes, familial adenomatous polyposis (FAP) is characterized by the presence of numerous adenomatous polyps in the colon caused by mutations in the APC gene located at 5q21.[8,9] As most patients with FAP develop CRC in their

late 20s or early 30s, efforts have been made to suppress or delay disease progression and tumorigenesis.[10-12] However, even after surgical treatment in intolerable cases, postoperative recurrence and desmoid tumors limit the success of FAP management.

Numerous preclinical and clinical studies have focused on the development of chemopreventive drugs that suppress polyps in patients.[13] A few repurposed medications, such as celecoxib, sulindac, aspirin, eflornithine, and erlotinib, showed limited or some significant effectiveness accompanied by significant adverse effects in FAP management.[12,14-17] Recently, other repurposed drugs such as curcumin and metformin showed no significant clinical effects.[18,19] To increase the effectiveness of chemoprevention and decrease the side effects of drugs, a combination of drugs may be a useful strategy for optimizing chemopreventive drugs for FAP.

Metformin, a well-known antidiabetic drug, exerts antitumor effects via the AMP-activated protein kinase (AMPK)-mechanistic target of rapamycin pathway in CRC.[19-25] Additionally, the anti-helminthic agent, niclosamide also shows tumor-suppressive effects in in vitro and *in vivo* preclinical experiments for many types of cancers, including CRC.[26-28] The combination of these two agents could be promising for FAP chemoprevention due to a compelling dual-mechanism rationale. From a pharmacodynamic perspective, our previous work established a clear synergistic interaction. We found that while niclosamide effectively suppresses Wnt signaling, it can paradoxically activate the oncogenic Yes-associated protein (YAP) pathway.[25] Critically, metformin counteracts this specific off-target effect by inhibiting YAP activation via an AMPK-dependent mechanism, thus creating a more targeted and potent antitumor effect when combined.[25]

In addition to this pharmacodynamic synergy, a significant pharmacokinetic (PK) interaction provides a second rationale. Recent studies report that niclosamide inhibits the renal transporter organic cation transporter 2, which is crucial for metformin excretion, thereby increasing metformin's bioavailability and local concentration at its site of action.[29]

Given this strong dual-mechanism rationale, the present study was designed to experimentally define the optimal therapeutic dose of this combination in the $Apc^{Min/+}$ mouse model. To achieve this, we integrated *in vivo* efficacy studies with quantitative synergy analysis (Bliss independence model)[30-32] and physiologically based pharmacokinetic (PBPK) modeling.[33-35] This integrated modeling approach is essential, as it allows for the deconvolution of pharmacodynamic synergy from PK-driven effects, enabling a deeper understanding of the dose-dependent interplay between the two drugs.

MATERIALS AND METHODS

1. *In vivo* experiments using $Apc^{Min/+}$ mice, a mouse model of FAP

$Apc^{Min/+}$ mice were produced by mating C57BL/6J wild-type female mice with C57BL/6J- $Apc^{Min/+}$ male mice (strain 002020; The Jackson Laboratory, Bar Harbor, ME, USA). The evaluated agents were used to treat 6-week-old $Apc^{Min/+}$ mice. Metformin only (50, 100, and 200 mg/kg), niclosamide only (50, 100, and 200 mg/kg), or a combination of both agents at different doses (low-dose combination of 50 mg/kg metformin and 50 mg/kg niclosamide, middle-dose combination of 100 mg/kg metformin and 100 mg/kg niclosamide, and high-dose combination of 200 mg/kg metformin and 200 mg/kg niclosamide) were orally administered for 16 weeks to each group, whereas phosphate-buffered saline (PBS) was administered for 16 weeks to the control group. In addition, for comparison of relative effects, oral celecoxib at 75 mg/kg or PBS were administered to 6-week-old $Apc^{Min/+}$ mice for 16 weeks. All mice were sacrificed, and their intestines were dissected.

Mouse experiments were approved by the Institutional Animal Care and Use Committee, Yonsei University Health System (IACUC number: 2017-0328) and performed according to institutional guidelines and policies.

2. Measurement of polyps and tissue staining

The small intestines were collected from the mice and residual feces were removed by washing with PBS. The small intestines were sliced lengthwise and placed on a sheet of paper. After spraying with methylene blue (Sigma-Aldrich, St. Louis, MO, USA; #03978), the number and size of polyps were measured. Polyps <1 mm were regarded as small, those between 1 and 5 mm as medium, and those >5 mm as large. Swiss rolls of the intestines were fixed in 4% paraformaldehyde (Biosesang, Seongnam, South Korea; #PC2031) for 24 hours and embedded in paraffin blocks. The paraffin-embedded sections were deparaffinized in xylene substitue (Sigma-Aldrich; #A5597) and rehydrated with gradually decreasing concentrations of ethanol (Merk, Darmstadt, Germany; #1.00983.1011). After hematoxylin and eosin staining (Abcam, Cambridge, UK; #ab245880), the tissues were evaluated using light microscopy (Olympus, Tokyo, Japan; BX43).

3. Drug interaction analysis

The nature of the drug interaction (synergism, additivity, or antagonism) at each dose level was quantitatively assessed based on the mean response of total polyp numbers using the Bliss independence model. The expected fractional inhibition of the combination (E_{exp}) assuming

independent drug action was calculated as $E_{exp}=E_A+E_B-(E_A \times E_B)$, where E_A and E_B are the fractional inhibitions of each drug alone relative to the control group. A synergy score, calculated as the difference between the observed combination effect and the expected effect ($E_{obs}-E_{exp}$), was used to classify the interaction: a score >0.1 was considered synergistic, <–0.1 as antagonistic, and between –0.1 and 0.1 as additive.

4. PBPK modeling and simulation

To investigate a potential PK mechanism for the observed effects of the combination therapy, simulations were performed using a whole-body PBPK model implemented in R software (version 4.4.3; R Foundation for Statistical Computing, Vienna, Austria) with the deSolve package. The model consisted of six compartments representing the gut absorption site, central (plasma), gut tissue, liver, kidney, and a peripheral compartment. Key model parameters, including physiological data (organ volumes, blood flows) and drug-specific parameters (clearance, bioavailability, tissue partition coefficients), were derived from published literature.[33,34,36] Based on a literature review of $Apc^{Min/+}$ mouse feeding patterns,[37,38] the administration method in our study was expected to result in approximately 70% of the total daily dose being ingested during the 12-hour dark phase and 30% during the 12-hour light phase. The body weight parameter was set to 0.028 kg, the average weight of treated mice at day 35 from this study. The drug-drug interaction (DDI) was mechanistically modeled based on reports that niclosamide inhibits the organic cation transporter 2, which is crucial for metformin's renal excretion.[29] This was implemented as a saturated effect (maximum effect, Emax) of inhibitory function on metformin's renal clearance, dependent on the simulated niclosamide concentration in the kidney. To reflect the experimental conditions, drug administration was modeled as a continuous, two-phase, zero-order input into the gut absorption compartment. This input rate was designed to simulate the diurnal ingestion pattern resulting from the medicated feed, as detailed in the *in vivo* methods section.

5. Statistical analysis

Statistical analyses were performed using SPSS version 28.0 software (IBM Corp., Armonk, NY, USA). One-way analysis of variance and Student two-tailed t-tests were conducted to evaluate the differences between experimental groups. Tukey's post-hoc analysis was used to detect specific group differences. Statistical significance was set at $p<0.05$.

RESULTS

1. Effect of combination of low-dose niclosamide and metformin on intestinal polyps in $Apc^{Min/+}$ mice

As a preliminary dose for combined treatment, 50 mg/kg was used in accordance with the concentration of niclosamide used in several animal studies.[39-42] Body weight changes during the experimental period did not differ between the control and all drug treatment groups (metformin alone, niclosamide alone, and combination therapy) (Fig. 1A). The polyps were examined grossly and microscopically after hematoxylin and eosin staining (Fig. 1B and C). The reduction in the total number of polyps per mouse in the drug treatment groups was not significant compared with that in the control group (p=0.9013 for the metformin group, p=0.7495 for the niclosamide group, p=0.0548 for combination group) (Fig. 1D). However, evaluation of the drugs' effects based on polyp size revealed significant results only in the small polyp group (<1 mm) and not in the medium (1–5 mm) and large (>5 mm) polyp groups. The total number of small-sized polyps per mouse was reduced compared with that in the control group (metformin only, p<0.0001; niclosamide only, p=0.0016; combination, p<0.0001), although no significant effect was observed between the combination and metformin-only (p=0.5812) or niclosamide-only groups (p=0.2920). In contrast, the number of medium-sized polyps per mouse (1–5 mm) increased in the metformin-only (p=0.0005) and niclosamide-only (p=0.0113) groups (Fig. 1E).

2. Effect of combination of middle-dose niclosamide and metformin on intestinal polyps in $Apc^{Min/+}$ mice

Similar to the results observed in the 50 mg/kg group, body weight did not differ among mice in any of the experimental groups during the experiment (Fig. 2A). The tumor-suppressing effects were significant in all subgroups compared with those in the control (Fig. 2B and C). The total number of polyps per mouse in the drug treatment groups was significantly lower than that in the control group (metformin-only, p=0.012; niclosamide-only, p=0.0295; combination group, p=0.0009) (Fig. 2D). Although all regimens demonstrated a lack of significant polyp suppression for medium-sized (1–5 mm) and large polyps (>5 mm), combination treatment led to a significant decrease in the number of small polyps (<1 mm) per mouse compared with that in the control (p<0.0001), metformin-only (p=0.0032), and niclosamide-only (p=0.0107) groups (Fig. 2E). The number of small polyps (<1 mm) in the combination group at medium doses (100 mg/kg) was reduced by 34% compared with that in the control group.

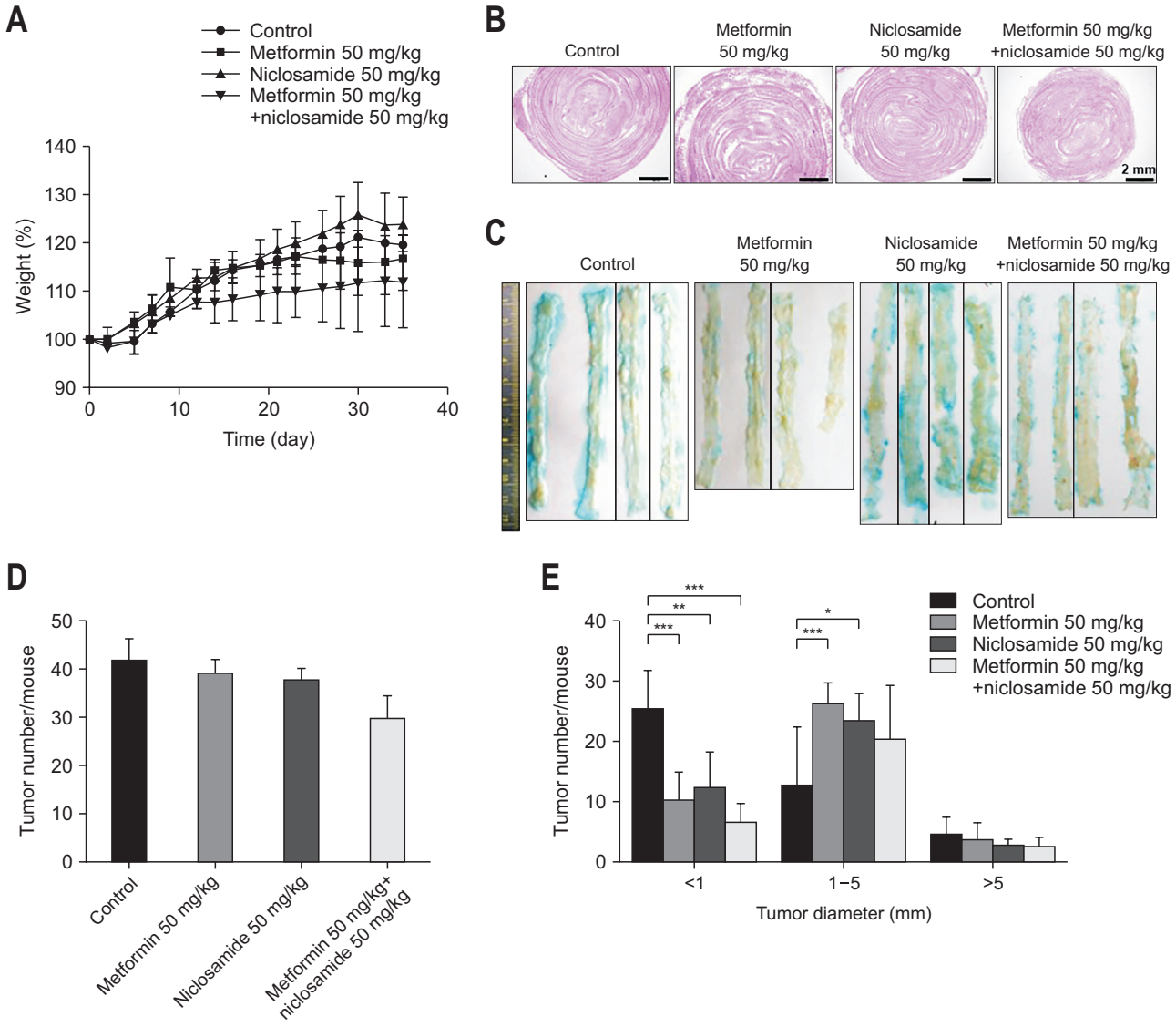


Fig. 1. Effect of the combination of low-dose niclosamide and metformin on intestinal polyps in Apc$^{Min/+}$ mice. Four groups of Apc$^{Min/+}$ mice received a 50 mg/kg dose of metformin (n=5), a 50 mg/kg dose of niclosamide (n=4), a combination of metformin (50 mg/kg) and niclosamide (50 mg/kg) (n=6), or a phosphate-buffered saline vehicle solution (n=3) for 16 weeks. The mice were sacrificed, and the numbers and sizes of intestinal polyps were analyzed. (A) Weight changes in the control and treatment groups. (B) Representative images of the mouse small intestine stained with hematoxylin and eosin (×12.5). (C) Representative gross images of the resected small intestine. Total number of polyps (D) and number of polyps by size (E). Data are presented as the mean±standard error of the mean. Significance was assessed by using one-way analysis of variance with Tukey's multiple comparison test. The results were considered significant at *p<0.05, **p<0.01, ***p<0.005.

3. Effect of combination of high-dose niclosamide and metformin on intestinal polyps in Apc$^{Min/+}$ mice

The body weights of the mice did not differ significantly between the control and high-dose treatment groups (Fig. 3A). Significant polyp reduction was observed following administration of metformin, niclosamide, and a combination of both drugs (Fig. 3B and C). The total number of polyps per mouse was also significantly reduced in the treatment groups (metformin, p=0.0188; niclosamide, p=0.0096; combination, p=0.0085) compared with that in the control group (Fig. 3D). A significant reduction in the polyp number was observed only for small polyps with a diameter <1 mm (Fig. 3E). Although metformin (p=0.0001), niclosamide (p=0.0003), and combination treatment (p<0.0001) reduced small polyps (<1 mm) compared with the number in the control group, combined treatment did not exhibit a more significant effect than either drug alone (Fig. 3E).

4. Effect of celecoxib on intestinal polyps in Apc$^{Min/+}$ mice

To evaluate the relative effects of niclosamide and metformin, a baseline experiment was conducted using

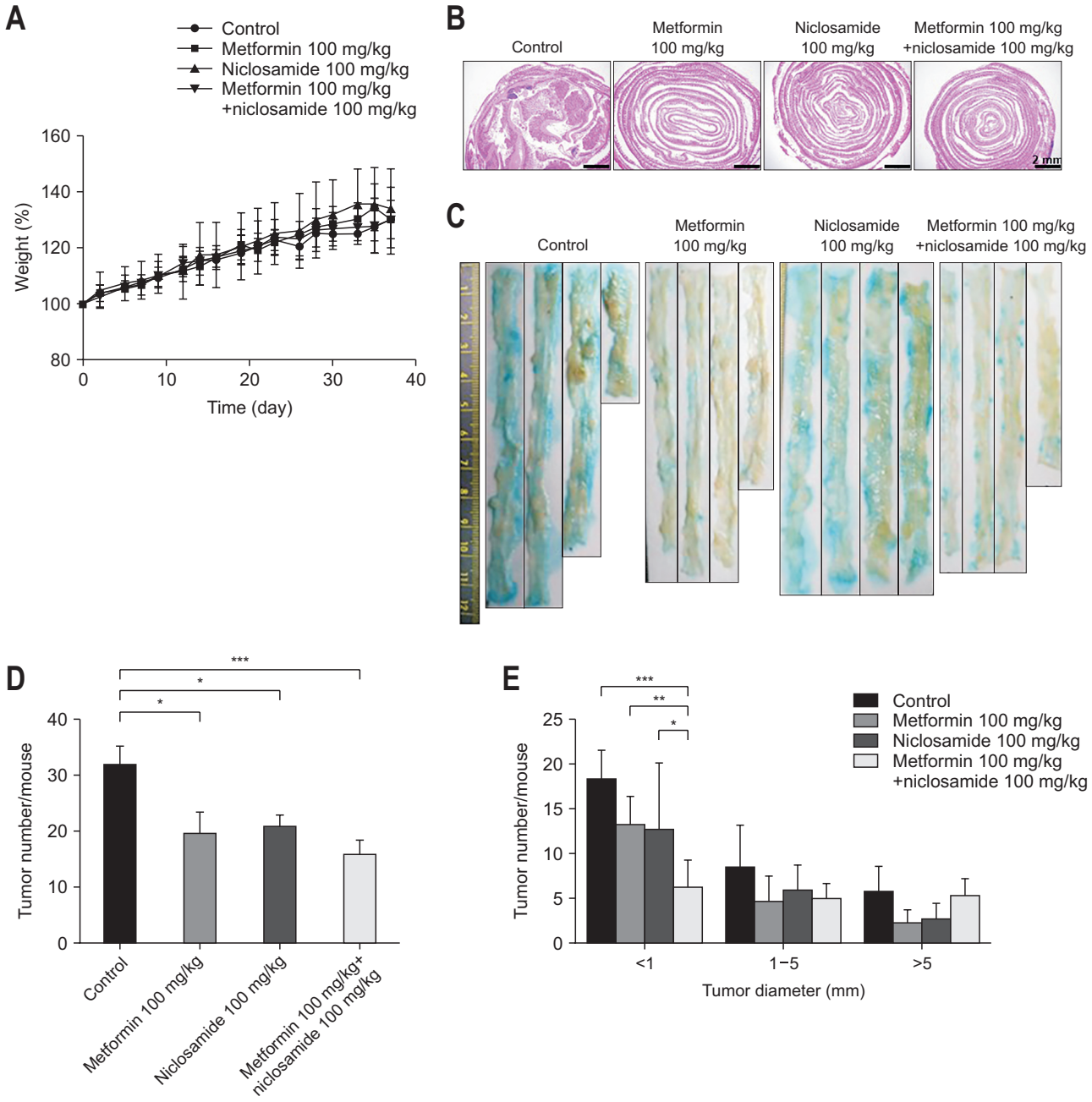


Fig. 2. Effect of the combination of medium-dose niclosamide and metformin on intestinal polyps in $Apc^{Min/+}$ mice. Four groups of $Apc^{Min/+}$ mice were administered a 100 mg/kg dose of metformin (n=5), a 100 mg/kg dose of niclosamide (n=4), a combination of metformin (100 mg/kg) and niclosamide (100 mg/kg) (n=6), or a phosphate-buffered saline vehicle solution (n=5) for 16 weeks. The mice were sacrificed and analyzed for the numbers and sizes of intestinal polyps. (A) Weight changes in the control and treatment groups. (B) Representative images of the mouse small intestine stained with hematoxylin and eosin (×12.5). (C) Representative images of the resected small intestine. Total number of polyps (D) and number of polyps by size (E). Data are presented as the mean±standard error of the mean. Significance was assessed by using one-way analysis of variance with Tukey's multiple comparison test. The results were considered significant at *p<0.05, **p<0.01, ***p<0.005.

celecoxib, which is currently one of the leading drugs used for FAP. Administration of 75 mg/kg celecoxib to $Apc^{Min/+}$ mice for 16 weeks did not significantly alter the body weight of the mice (Fig. 4A). Celecoxib administration significantly reduced the number of polyps in the small bowel (Fig. 4B and C). The total number of polyps in celecoxib-treated mice was significantly reduced by 54.1% compared with that in control mice (p=0.0009) (Fig. 4D). In addition, administration of celecoxib resulted in a significant decrease only in medium-sized polyps (1–5 mm) compared with the effects in the control group (small: p=0.0772, medium: p<0.0001, large: p=0.8086) (Fig. 4E).

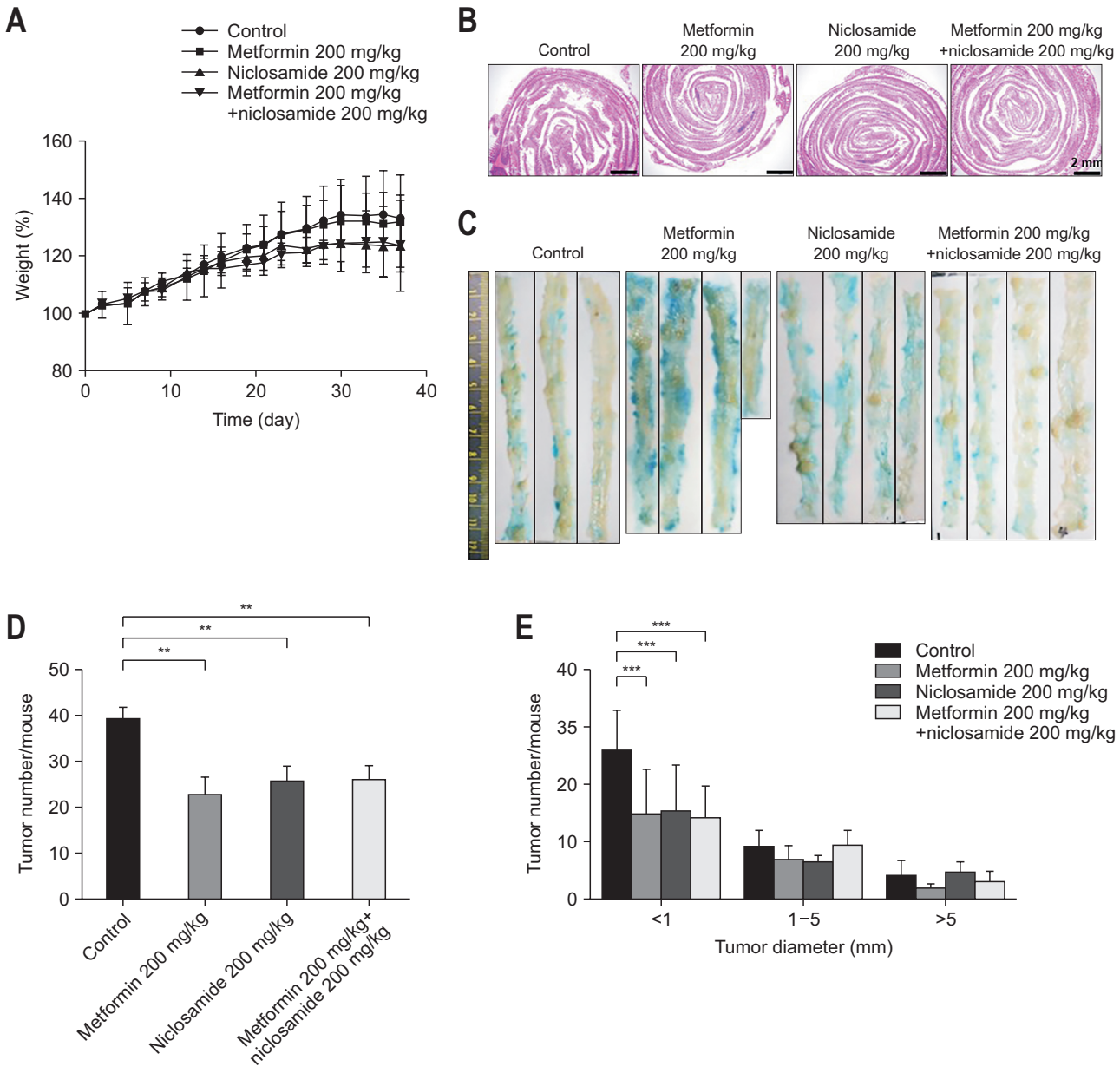


Fig. 3. Effect of the combination of high-dose niclosamide and metformin on intestinal polyps in $Apc^{Min/+}$ mice. Four groups of $Apc^{Min/+}$ mice were treated with a 200 mg/kg dose of metformin (n=7), a 200 mg/kg dose of niclosamide (n=7), a combination of metformin (200 mg/kg) and niclosamide (200 mg/kg) (n=7), or a phosphate-buffered saline vehicle solution (n=6) for 16 weeks. The mice were sacrificed, and the numbers and sizes of polyps were evaluated. (A) Changes in weight in the control and treatment groups. (B) Representative images of the mouse small intestine stained with hematoxylin and eosin (×12.5). (C) Representative images of the resected small intestine. Total number of polyps (D) and number of polyps by size (E). Data are presented as the mean±standard error of the mean. Significance was assessed by using one-way analysis of variance with Tukey's multiple comparison test. The results were considered significant at **p<0.01, ***p<0.005.

5. Dose-dependent drug interactions in Bliss independence model

To quantitatively characterize the interaction at each dose level, we applied the Bliss independence model to the total polyps count data (Table 1). The analysis revealed a clear dose-dependent shift in the interaction profile. At the 100 mg/kg dose, the synergy score was –0.059, indicating a nearly perfect additive interaction. In contrast, at the 200 mg/kg dose, the synergy score was –0.326, indicating a clear antagonistic interaction. A borderline additive or weakly synergistic trend was observed at the 50 mg/kg dose, with a synergy score of +0.079.

6. Prediction of PK interactions and safety margins using PBPK modeling

To investigate a potential PK basis for the observed

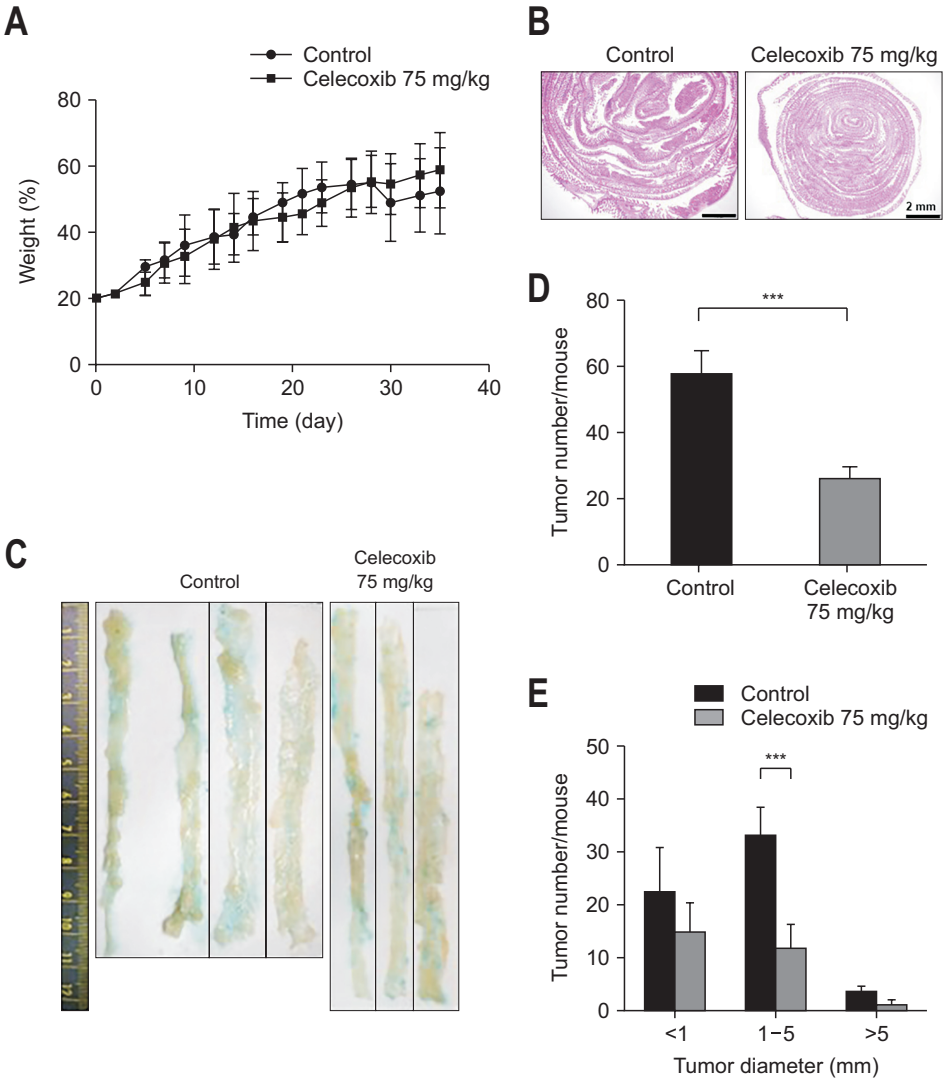


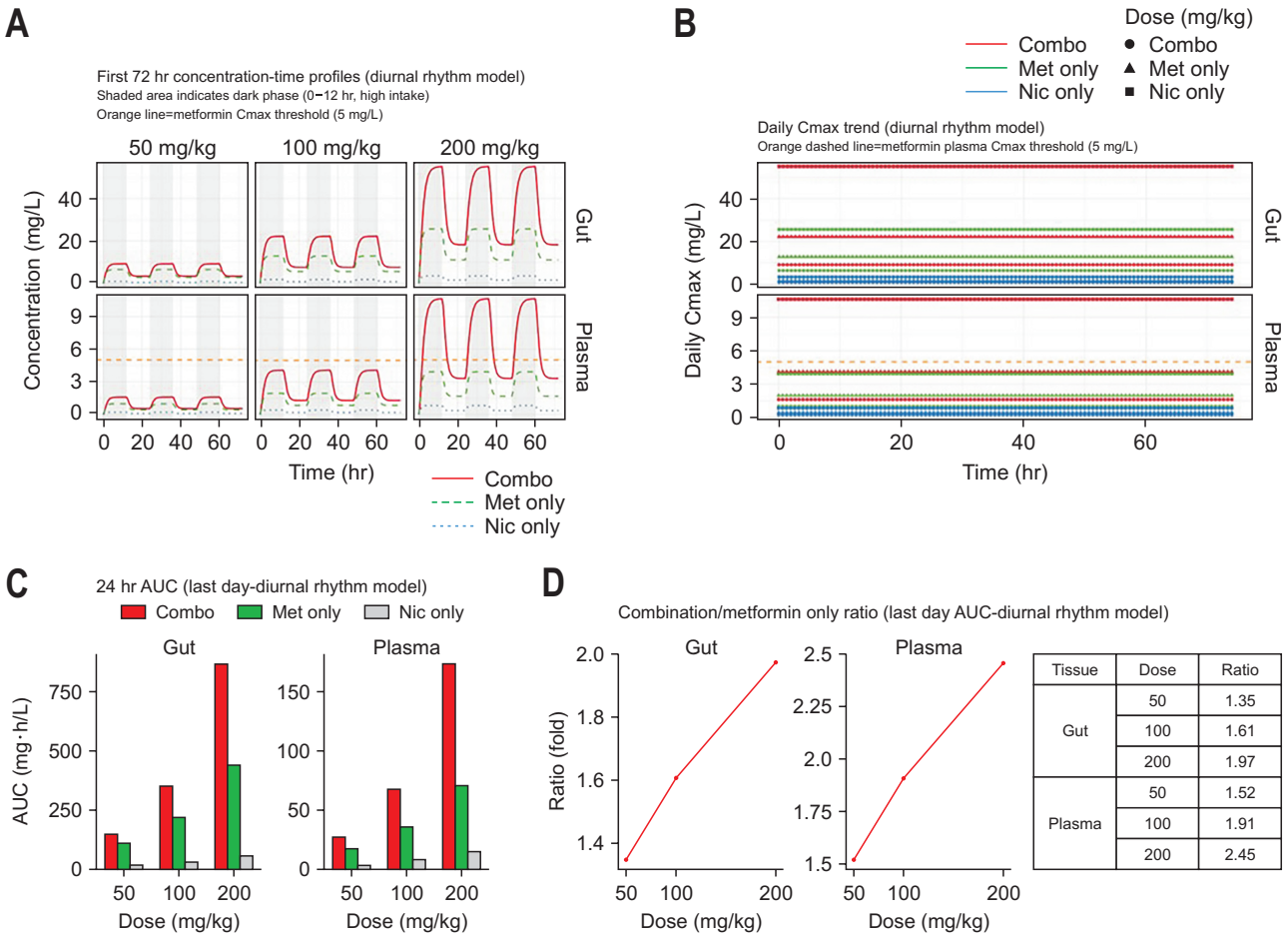
Fig. 4. Effect of celecoxib on intestinal polyps in $Apc^{Min/+}$ mice. Mice were treated with a dose of 75 mg/kg of celecoxib (n=6) or a phosphate-buffered saline vehicle solution (n=4) for 16 weeks. The mice were sacrificed, and the numbers and sizes of intestinal polyps were evaluated. (A) Weight changes in the control and treatment groups. (B) Representative images of the mouse small intestine stained with hematoxylin and eosin (×12.5). (C) Representative gross images of the resected small intestine. Total number of polyps (D) and number of polyps by size (E). Data are presented as the mean ± standard error of the mean. The significance of the findings was assessed by using one-way analysis of variance with Tukey's multiple comparison test. The results were considered significant at ***p<0.005.

Table 1. Bliss Independence Model Analysis of Combination Treatment of Niclosamide and Metformin

Dose	N_ctrl	N_met	N_nic	N_combo	E_met	E_nic	E_obs_combo	E_exp_bliss	Synergy score*	Interpretation
50 mg/kg	40	38	37	32	0.050	0.072	0.200	0.121	0.079	Additivity
100 mg/kg	32	20	21	15	0.375	0.344	0.531	0.590	–0.059	Additivity
200 mg/kg	40	23	26	28	0.425	0.350	0.300	0.626	–0.326	Antagonism

N_ctrl, mean total number of polyps per mouse in the control group; N_met, mean total number of polyps per mouse in the metformin-only group; N_nic, mean total number of polyps per mouse in the niclosamide-only group; N_combo, mean total number of polyps per mouse in the combination therapy group; E_met, fractional inhibition by metformin, calculated as (N_ctrl – N_met)/N_ctrl; E_nic, fractional inhibition by niclosamide, calculated as (N_ctrl – N_nic)/N_ctrl; E_obs_combo, observed fractional inhibition by the combination, calculated as (N_ctrl – N_combo)/N_ctrl; E_exp_bliss, expected fractional inhibition based on the Bliss model, calculated as E_met + E_nic – (E_met × E_nic); Synergy score, the difference between observed and expected inhibition, calculated as E_obs_combo – E_exp_bliss.
*A synergy score >0.1 was considered synergistic, <–0.1 as antagonistic, and between –0.1 and 0.1 as additive.

dose-dependent effects, we performed PBPK simulations reflecting the diurnal feeding patterns of the mice. The model predicted distinct daily fluctuations in drug concentrations, with higher levels during the active (dark) phase (Fig. 5A). Critically, the simulation predicted a significant DDI wherein niclosamide coadministration increased metformin exposure at all tested doses. At steady-state, the 24-hour area under the curve of metformin in the gut was predicted to increase by approximately 1.35-fold, 1.61-fold, and 1.97-fold at the 50, 100, and 200 mg/kg combination doses, respectively, compared to metformin alone (Fig. 5C and D, left panel). A similar, though less pronounced,



Tissue	Dose	Ratio
Gut	50	1.35
	100	1.61
	200	1.97
Plasma	50	1.52
	100	1.91
	200	2.45

Fig. 5. PBPK simulation of niclosamide and metformin pharmacokinetics under a medicated feed administration model. Drug concentrations were simulated by using a PBPK model that reflected the experimental conditions. Drug administration via medicated feed was modeled as a diurnal, two-phase, zero-order input rate (70% of the dose during a 12-hour dark phase and 30% during a 12-hour light phase) for 16 weeks in a mouse weighing 28 g. (A) Simulated concentration–time profiles during the first 72 hours of treatment. (B) Predicted daily maximum concentration (Cmax) trends over the 16-week treatment period. (C) Bar chart comparing the predicted 24-hour AUC on the final day of treatment. (D) (left) Line plot showing the ratio of the metformin AUC in the combination group versus the metformin-only group; (right) Table quantifying the metformin AUC ratio (combination vs metformin only) at each dose level. The orange dashed line in panels (A) and (B) represents the 5 mg/L metformin plasma concentration threshold associated with an increased risk of lactic acidosis. PBPK, physiologically based pharmacokinetic; AUC, area under the curve.

increase was predicted in the plasma (Fig. 5C and D, right panel), with the relative boost in exposure tending to increase with dose.

We then compared the predicted steady-state plasma Cmax for metformin against a literature-derived safety threshold of 5 mg/L, which is associated with an increased risk of lactic acidosis.[43] For the combination therapy, the predicted Cmax was well below this threshold at the 50 mg/kg (approximately 1.5 mg/L) and 100 mg/kg (approximately 2.8 mg/L) doses. However, the simulation predicted that the Cmax would exceed this threshold at the 200 mg/kg dose (approximately 6.5 mg/L), suggesting a potential for increased systemic side effects at this high dose (Fig. 5B).

DISCUSSION

Both metformin and niclosamide are well-established drugs that are inexpensive and have a favorable safety profile. Metformin has long been used as a standard antidiabetic drug with good safety, and niclosamide, an antihelminthic agent, shows very limited systemic absorption, supporting its safety.[44] Although the efficacy of metformin and niclosamide against cancer has been demonstrated, studies of their combined effects and effective doses are limited. Our previous study proposed a mechanism for the synergistic effect of the combination of these two drugs.[25] However, differences in the effects of different doses of each drug have not been evaluated. In this study, we evaluated the dose-dependent effects of the individual or combined drugs.

One of important observations in our study is the complex dose-response at the 50 mg/kg low-dose regimen, where a reduction in small polyps (<1 mm) was paradoxically accompanied by an increase in medium-sized polyps (1–5 mm). This seemingly counterintuitive result is not an isolated finding in $Apc^{Min/+}$ mouse studies. In fact, a similar pattern of decreased small polyps and increased medium polyps with no change in total polyp number was previously reported for niclosamide monotherapy. Remarkably, this same size-shift phenomenon—a decrease in small polyps with a concurrent increase in medium ones with minimal change in total polyp number—has also been observed with non-pharmacological interventions such as exercise.[25,39] This recurring pattern suggests that a suboptimal therapeutic dose can alter the dynamics of polyp progression, possibly by incompletely inhibiting early lesions which then survive and progress into a larger size category. Based on the mechanism of combination of metformin/niclosamide demonstrated in our previous report,[25] we propose a more specific molecular hypothesis for our novel metformin/niclosamide combination. We postulate that this effect stems from an imperfect balance between dual actions of the drug: at a low dose, partial Wnt inhibition by niclosamide may reduce new polyp initiation, but insufficient AMPK activation by metformin may fail to fully suppress the paradoxical, YAP-driven progression of some existing lesions. This imbalance appears to be resolved at the optimal 100 mg/kg dose, though direct molecular validation is needed.[25,39] In addition, to elucidate whether the observed size shifts represent true progression or suboptimal treatment effects, longitudinal assessments at multiple time points and/or serial endoscopic monitoring of polyp development would be necessary. This result is further supported by findings that a low dose of thymoquinone, another chemopreventive agent, failed to suppress the progression to large polyps and showed a tendency to increase medium and large polyps.[45] The complexity of these non-monotonic size-shifts has been noted with other drug combinations as well, and in some cases, certain agents have even increased overall polyp multiplicity despite reducing polyp size.[46,47] Therefore, our finding suggests that a net therapeutic benefit is only achieved through rigorous dose optimization, as a seemingly positive effect on one sub-population of polyps can be offset by a detrimental effect on another.

At higher doses (100 and 200 mg/kg), all drug regimens were significantly effective in reducing the overall number of polyps, particularly small polyps (<1 mm). The key finding was that the combination treatment with middle-doses of metformin (100 mg/kg) and niclosamide (100 mg/kg) showed a significant additive effect in reducing small polyps compared with the effects of either drug alone. This observation was quantitatively confirmed by our Bliss independence model analysis, indicating a nearly perfect additive interaction. In contrast, at the 200 mg/kg dose, the Bliss independence model revealed a strong antagonistic interaction, suggesting that a high dose (200 mg/kg) of metformin or niclosamide alone may have reached a saturated effect (maximum effect, Emax). This phenomenon is consistent with a U-shaped or biphasic dose-response, a well-documented principle in pharmacology where excessive drug concentrations can trigger negative feedback mechanisms that counteract the therapeutic benefits.[48] Furthermore, the pronounced antagonism, rather than a simple plateau, may suggest that excessive drug exposure triggers negative feedback mechanisms or off-target toxicities that actively counteract the therapeutic benefits.

To investigate a potential mechanism for this dose-specific different effect, we utilized a PBPK model reflecting the experimental conditions, including administration via medicated feed. The simulation predicted a significant DDI wherein niclosamide coadministration increased metformin exposure, suggesting that niclosamide boosts metformin's concentration at its site of action to a level sufficient to achieve a superior combined effect. Interestingly, our PBPK model predicted that the relative PK boost of metformin by niclosamide was maintained or even increased at the 200 mg/kg dose, but the synergistic or additive benefit of the combination disappeared and showed antagonistic effect in our *in vivo* results. This disconnect strongly supports our hypothesis that a high dose (200 mg/kg) of metformin or niclosamide alone may have reached a saturated effect (Emax). This phenomenon is consistent with other reports; in several studies,[49-52] metformin alone was effective at concentrations above 200 mg/kg, whereas combination therapy[50,53-55] was effective at or approximately 100 mg/kg. Furthermore, other studies have reported saturated effects of niclosamide at 100–200 mg/kg doses in other models[56,57] which aligns with our Emax saturation hypothesis. In addition, the PBPK simulation provided critical insights into the safety profile of this combination, showing the predicted Cmax (approximately 6.5 mg/L) exceeded the safety threshold at the 200 mg/kg dose. This reinforces that 100 mg/kg is the superior dose, as it achieves optimal efficacy while maintaining a favorable predicted safety margin. In addition, regarding the molecular mechanism,[25] our findings suggest that the 100 mg/kg dose at which the combination of niclosamide and metformin was most effective is the optimal dose at which metformin inhibited niclosamide-induced activation of YAP signaling by AMPK activation.[25]

To compare the relative effects of our combination to

those of previously established drugs, we confirmed the efficacy of celecoxib. The total number of polyps per mouse was significantly reduced, particularly that of medium-sized polyps (1–5 mm). Oshima *et al.*[58] reported that Cox2 expression is challenging to discern in tumors measuring <2 mm in APC-mutant mice, but its expression is more readily identified in tumors exceeding 2 mm in diameter. In addition, Cherukuri *et al.*[59] reported a decrease in the number of polyps measuring 2 to 4 mm in APC-mutant mice with ablated Cox2 expression compared with that in controls. Our findings showing the significant effect of celecoxib on medium-sized polyps are comparable to those of previous reports.

Compared with the effects of celecoxib, the combination of niclosamide and metformin more strongly suppressed small polyps (<1 mm), whereas celecoxib suppressed medium-sized polyps (1–5 mm), suggesting a potential difference in their primary targeted stage of tumorigenesis, with metformin/niclosamide possibly acting on earlier initiation events and celecoxib on later promotion stages. Thus, a combination of celecoxib and metformin/niclosamide may target both the initiation and promotion stages of carcinogenesis.

This study has several limitations that should be acknowledged. First, although this study used the $Apc^{Min/+}$ mouse, a representative animal model for FAP, the results may not perfectly reproduce the response in the human body. Therefore, further verification is needed before applying these findings to FAP patients. Second, while this study confirmed dose-dependent efficacy differences by macroscopic polyp count changes, it did not directly analyze the mechanistic changes at the molecular level for each dose. Third, the analytical models used in this study have inherent assumptions. The Bliss independence model, while useful for quantifying interactions from the mean responses, does not account for the variability within the experimental groups. Additionally, it is a critical limitation that the PBPK model used in this study is a theoretical simulation based on literature-derived parameters. It was not experimentally validated using plasma or tissue PK measurements from $Apc^{Min/+}$ mice. Therefore, the prediction of model should be interpreted as hypothesis-generating rather than definitive quantitative outcomes. Validating this prediction with direct plasma and tissue drug concentration measurements would be an essential next step before clinical translation. Fourth, our study did not investigate how nutritional factors modulate the efficacy of our chemopreventive combination. As example, Fini *et al.*[60] demonstrated a polyphenol extract had enhanced efficacy when combined with a balanced diet compared to a Western diet in $Apc^{Min/+}$ mice. Given that dietary composition can serve as a beneficial or aggravating factor,[61-64] we used a standardized diet to establish a baseline efficacy of the novel metformin and niclosamide combination. Future studies should explore interactions with varied diets for translational applications.

In conclusion, we demonstrated the combined effect of niclosamide and metformin in the $Apc^{Min/+}$ mice, based on the individual antitumor effects of each drug and their positive drug interactions. In addition, this study underscores that a comprehensive, multi-faceted approach is critical for defining the optimal dose in combination chemoprevention. By systematically integrating *in vivo* efficacy data, quantitative synergy analysis via the Bliss independence model, and mechanistic PBPK simulations, we successfully identified an optimal therapeutic window for the metformin and niclosamide combination. Ultimately, this work provides a robust, data-driven rationale for guiding the design of future clinical trials in FAP patients.

CONFLICTS OF INTEREST

J.H.C. is an editorial board member of the journal but was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article were reported.

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AUTHOR CONTRIBUTIONS

Study concept and design: S.J.P., T.I.K. Data acquisition: J.Y.K., D.K.K., Y.S. Data analysis and interpretation: J.Y.K., D.K.K., T.I.K. Drafting of the manuscript: D.K.K., J.Y. Critical revision of the manuscript for important intellectual content: D.K.K., S.J.P., T.I.K. Statistical analysis: J.Y.K., D.K.K., L.H.H. Obtained funding: S.J.P., T.I.K. Administrative, technical, or material support: T.I.K. Study supervision: J.W.Y., J.P., S.J.P., J.J.P., J.H.C., T.I.K. Approval of final manuscript: all authors.

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DATA AVAILABILITY STATEMENT

Data analyzed in this study are available from the corresponding author upon reasonable request.

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