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List of Abbreviations

ABBREVIATION	DEFINITION
AMPK	5' AMP-activated protein kinase
AUC	Area under the plasma concentration-time curve
BCG	Bacillus Calmette-Guerin
BET	Bromodomain and Extra-Terminal motif
bFGF	Basic fibroblast growth factor
BID	Twice a day
BIW	Twice a week
Cmax	Maximum plasma concentration
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
GI	Gastrointestinal
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Median inhibition concentration
Inh	Inhibition
ip or i.p.	Intraperitoneal
iv	Intravenous
LD_{50}	Median lethal dose
LDH	Lactate dehydrogenase
LFT	Liver function test
MetAP1	Methionine aminopeptidase I
MetAP2	Methionine aminopeptidase II
MS	Mass spectrometry

MTD	Maximum tolerated dose
mg	Milligram
mg/kg/day	Milligram per kilogram per day
MIC	Minimal inhibitory concentration
mTOR	mammalian target of rapamycin
ND	Not detected
ng	Nanogram
NMIBC	Non-Muscle Invasive Bladder Cancer
PK	Pharmacokinetics
RB1	retinoblastoma protein
SIRT1	Sirtuin 1
$t_{1/2}$	Terminal elimination half-life
TID	Three times a day
Tmax	Time of Cmax
TP53	Tumor protein 53
VEGF	Vascular endothelial growth factor
Vol.	Volume
w/	With
w/o	Without
wk	Week
Wt.	Weight

2.6.2.1 BRIEF SUMMARY

Nitroxoline has been discovered to have both anti-angiogenic and anti-tumor activities. Research results from the Johns Hopkins University demonstrated that nitroxoline inhibited tumor angiogenesis through a novel mechanism of synergistic dual inhibition of MetAP2 and SIRT1 enzymes (Shim, 2011). Further *in vitro* studies conducted by us and other labs confirmed nitroxoline's anti-proliferative activities in multiple human cancer cell lines (Jiang, 2011). In addition, the anti-angiogenesis and anti-tumor activities of nitroxoline have been demonstrated in several *in vivo* models. Most recently, nitroxoline has been shown to have synergistic effect when administered in combination with Bacillus Calmette–Guerin (BCG) in an orthotopic bladder cancer model. Results from these *in vitro* and *in vivo* studies are summarized below.

Table 2.6.2-1 List of *In Vitro* and *In Vivo* Studies Performed with Nitroxoline

Study type	Test System	Treatment	Read out
***In vitro* studies**			
Angiogenesis	HUVEC cells	Nitroxoline	Calcein-AM staining
Cell Proliferation	Various types of cancer cell lines	Nitroxoline	IC_{50}
Invasion	MCF-10A neoT cells	Nitroxoline	Inhibition of cell invasiveness
***In vivo* studies**			
Angiogenesis (Matrigel Plug Assay)	BALB/c, nu/nu-NCr mice	Nitroxoline	Masson trichrome staining
Tumor growth and angiogenesis	Human Breast Cancer HCC1954 Xenograft Model in BALB/c, nu/nu-NCr mice	Nitroxoline	Tumor volume and micro vessels staining
Tumor growth	Orthotopic Bladder Cancer KU7-luc Model in Hsd: Athymic Nude-*Foxn1*^{nu} mice	Nitroxoline	Tumor bioluminescence
Tumor growth	Orthotopic Bladder Cancer 5637 Model in Nu/Nu nude mice	Nitroxoline	Tumor weight
Tumor growth	Orthotopic Kidney Cancer KCC-853 Model in Nu/Nu nude mice	Nitroxoline	Tumor weight and volume
Tumor growth	Orthotopic Bladder Cancer Model MBT-2–luc in C3H/HeN mice, in combination with BCG	Nitroxoline, in combination with BCG	Tumor bioluminescence

PRIMARY PHARMACODYNAMICS

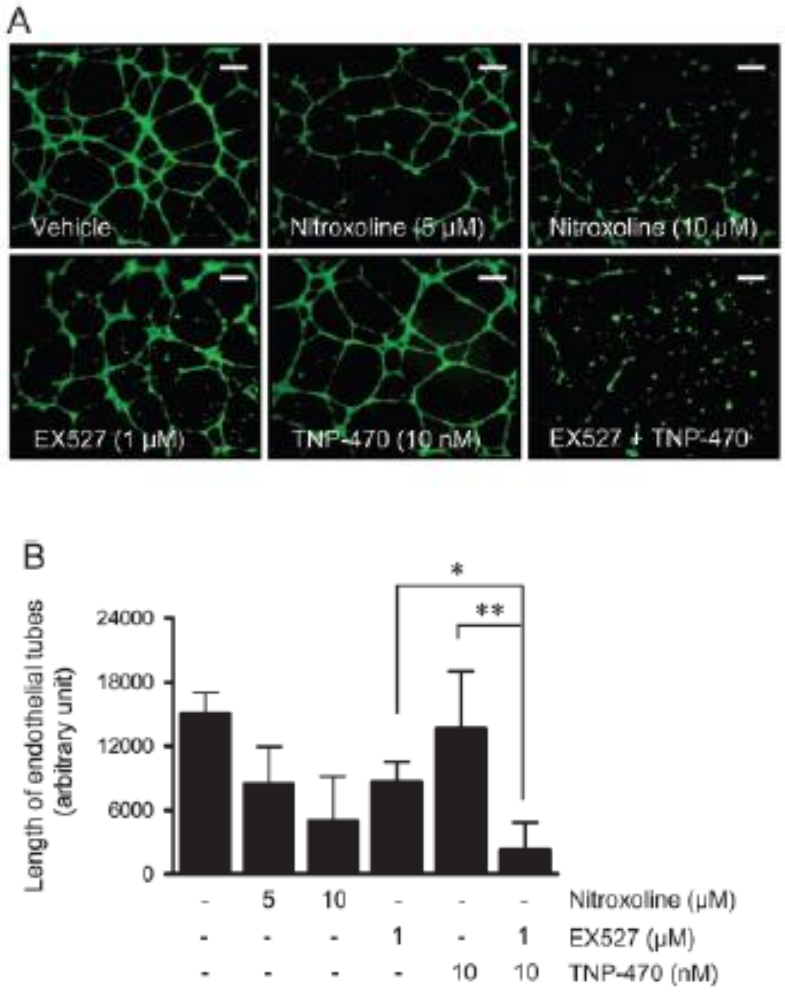
2.6.2.2.1 *In Vitro* Anti-Angiogenic Activities

Methionine aminopeptidases (MetAPs) are ubiquitously expressed Co^{2+} or Mn^{2+} dependent cytoplasmic enzymes and catalyze the removal of N-terminal methionine, a critical step in the maturation of many proteins and polypeptides (Sato, 2004). Eukaryotic organisms express two isoforms of MetAPs, MetAP1 and MetAP2. Inhibition of MetAP2 leads to the activation of tumor suppressor protein p53 (TP53) and stimulating the production of cyclin-dependent kinase inhibitor p21. Given the inhibition of endothelial cell proliferation by various human MetAP2 inhibitors, MetAP2 has served as a target for discovering and developing novel anti-angiogenic agents. TNP-470, a representative of a series of irreversible MetAP2 inhibitors, entered multiple phase I and II clinical trials in cancer patients, and had shown preliminary anti-cancer activities supported by documented tumor regression and anecdotal complete remission (Kruger, 2000).

The silent information regulator 2 (SIR2) family of nicotinamide adenine dinucleotide (NAD)–dependent deacetylases (also known as sirtuins [SIRTs]) has been shown to play important roles in gene silencing and DNA repair, among other cellular processes (Guarente, 1999). There are seven human sirtuins (SIRT1–7), and each isoform shows a distinct substrate specificity and subcellular localization (Finkel, 2009; Michishita, 2005). The SIRT1 protein is located in the nucleus and targets a variety of acetylated substrates including TP53 (Vaziri, 2001). There are accumulating evidences that SIRT1 plays an important role in cellular senescence and angiogenesis (Brooks, 2009). Inhibition of SIRT1 has been shown to result in promoting premature senescence of endothelial cells and consequently attenuates angiogenesis *in vivo* (Liu, 2009; Ota, 2007), which makes SIRT1 another promising target for discovering new inhibitors of angiogenesis.

Nitroxoline was discovered to have anti-angiogenic activities by the researchers at the Johns Hopkins University (Shim, 2011). Nitroxoline has been shown to have a novel dual mechanism in inhibiting tumor angiogenesis by modulating the activity of MetAP2 and SIRT1(Shim, 2011). The concurrent inhibition of MetAP2 and SIRT1 synergistically increases the level of acetylated TP53, leading to the induction of premature senescence in HUVEC. Nitroxoline was able to reproduce comparable effect by the combination of very potent SIRT1 inhibitor (EX527, IC_{50} = 38 nM (Solomon, 2006)) and MetAP2 inhibitor (TNP-470, IC_{50} = 50 pg/mL (Kruger, 2000)). As a result, the inhibitory effects of nitroxoline on endothelial cell proliferation was significantly enhanced (IC_{50} = 1.9 µM). The HUVEC tube formation was inhibited by nitroxoline in a dose-dependent manner (Figure 2.6.2-1).

Figure 2.6.2-1: *In Vitro* Effects of Nitroxoline on Angiogenesis



A) Effect of nitroxoline or SIRT1 inhibitor (EX527) and MetAP2 inhibitor (TNP-470) on endothelial tube formation. **Scale bar** = 200 µm. **B**) Quantitative analysis of mean tube lengths from three independent experiments is shown. **Error bars** = 95% confidence intervals. **P* = .001; ***P* = .0012.

2.6.2.2.2 Anti-Proliferative and Anti-Invasive Activities of Nitroxoline Against Various Cancer Cell Lines *In Vitro*

Nitroxoline showed anti-proliferative activities in a variety of cancer cell lines, with IC_{50} ranging from 0.44 µM to 14.2 µM (Table 2.6.2-2). The exact mechanism for nitroxoline's anti-proliferative activities is not well established. MetAP2 may play a critical role in tumorigenesis and cell growth, and inhibition of MetAP2 can induce cytostasis in tumor cells (Wang, 2003; Tucker, 2008; Chang, 2015; Jiang, 2017). Jiang H. (2011) reported that the cytotoxicity of nitroxoline was associated with an increase of reactive oxygen species (Jiang, 2011). It was also suggested that nitroxoline induces anticancer activity through AMPK-dependent inhibition of mTOR-p70S6K signaling pathway and cyclin

D1-Rb-Cdc25A axis, leading to G1 arrest of cell cycle and apoptosis (Chang, 2015). Recently nitroxoline was identified as a selective BET inhibitor and effectively inhibited the proliferation of MLL leukemia cells by inducing cell cycle arrest and apoptosis (Jiang, 2017).

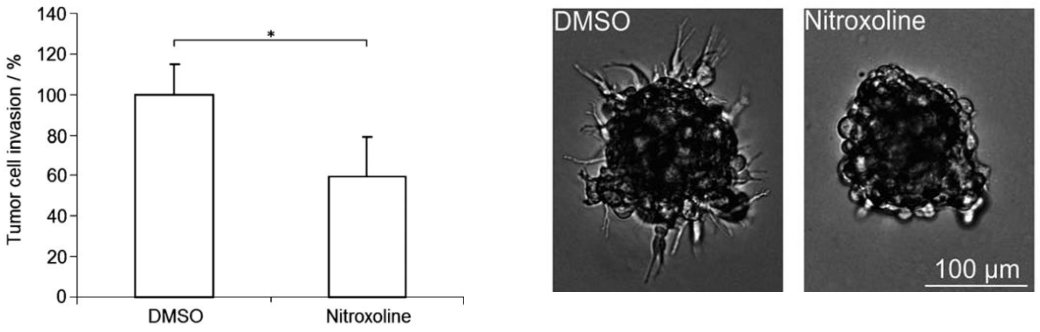
Table 2.6.2-2: *In Vitro* Activity of Nitroxoline on Cancer Cell Proliferation

Tumor Types	Cell Lines	IC_{50} (µM)	Data Sources
Bladder Cancer	5637	2.7	Our internal study
	T24	3.0	
Kidney Cancer	KCC853	3.7	
Liver Cancer	HepG2	14.2	
Gastric Cancer	SGC-7901	4.4	
Lymphoma	Raji	0.44	Jiang, 2011
	DHL-4	2.7	
Leukemia	HL-60	12.9	
Pancreatic Cancer	Panc-1	5.9	
Ovarian Cancer	A2780	13.0	
Prostate cancer	PC-3	4.6	Chang, 2015
	DU-145	5.5	
	LNCaP	4.2	
MLL leukemia	MV4.11	4.87	Jiang, 2017
	Thp-1	8.04	

As shown in Figure 2.6.2-2, nitroxoline has been shown to inhibit the invasion of the transformed human breast epithelial cell line, MCF-10A neoT (Mirković, 2011). In this report, nitroxoline was found to inhibit cathepsin B with inhibition constants K_i of 39.5 ± 2.8 µM (with the enzyme-substrate complex) and 154.4 ± 26.7 µM (with the free enzyme). Cathepsin B plays a role in the degradation of extracellular matrix proteins in tumor tissues, enabling tumor cells to migrate, invade and metastasize.

Figure 2.6.2-2: *In Vitro* Effects of Nitroxoline on Tumor Cell Invasion

A) **B)**

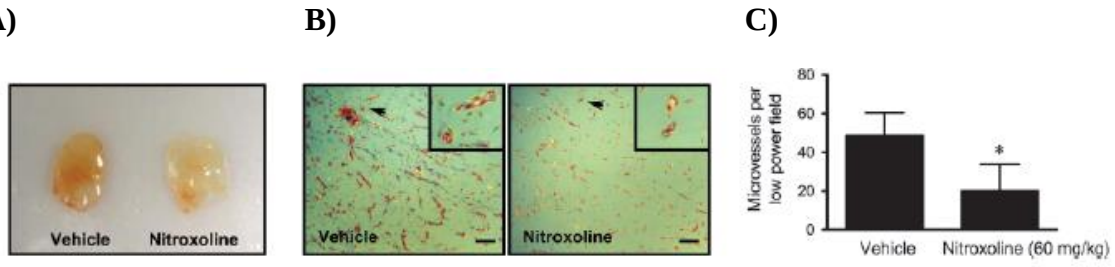


A) Effect of 10 µM nitroxoline on tumor cell invasion as assessed with a two-dimensional *in vitro* invasion assay. Data are presented as the percentage of invading cells in the presence of DMSO or 10 µM nitroxoline (mean+/-SD, n=3). B) Effect of 10 µM nitroxoline on tumor cell invasion as evaluated with a three-dimensional *in vitro* tumor model based on cell aggregates implanted in Matrigel.

2.6.2.2.3 *In Vivo* Anti-Angiogenic Pharmacological Studies

Nitroxoline was reported to have an *in vivo* effect on angiogenesis in a Matrigel plug assay in nude mice (Shim, 2011) (Figure 2.6.2-3). Mice were injected with either vehicle or 60 mg/kg nitroxoline (n = 5 in each group) intraperitoneally, once daily for 3 days, and Matrigel plugs containing VEGF and bFGF were implanted subcutaneously into mice. Treatment with vehicle or nitroxoline continued, once daily, for additional 7 days. Ten days after initial treatment, the Matrigel plugs were removed and photographed (Figure 2.6.2-3, A), and the newly invaded blood vessels were assessed microscopically (Figure 2.6.2-3, B). Compared with vehicle, nitroxoline at 60 mg/kg reduced the number of new micro vessels by 63% (vehicle vs. nitroxoline, mean = 48.6 vs. 20 micro vessels, *P* = 0.002) (Figure 2.6.2-3, B and C).

Figure 2.6.2-3: *In Vivo* Effects of Nitroxoline on Angiogenesis



A) Analysis of *in vivo* angiogenesis using Matrigel plug assay. B) Representative images of Masson trichrome staining of Matrigel plugs. **Arrowheads** indicate erythrocyte-filled micro vessels (insets show the magnified view). Magnification ×100. Scale bar= 150 μm. C) Quantitative analysis of micro vessels in the Matrigel plugs shown in (B) by counting five randomly selected fields in low-power (magnification ×100) fields.

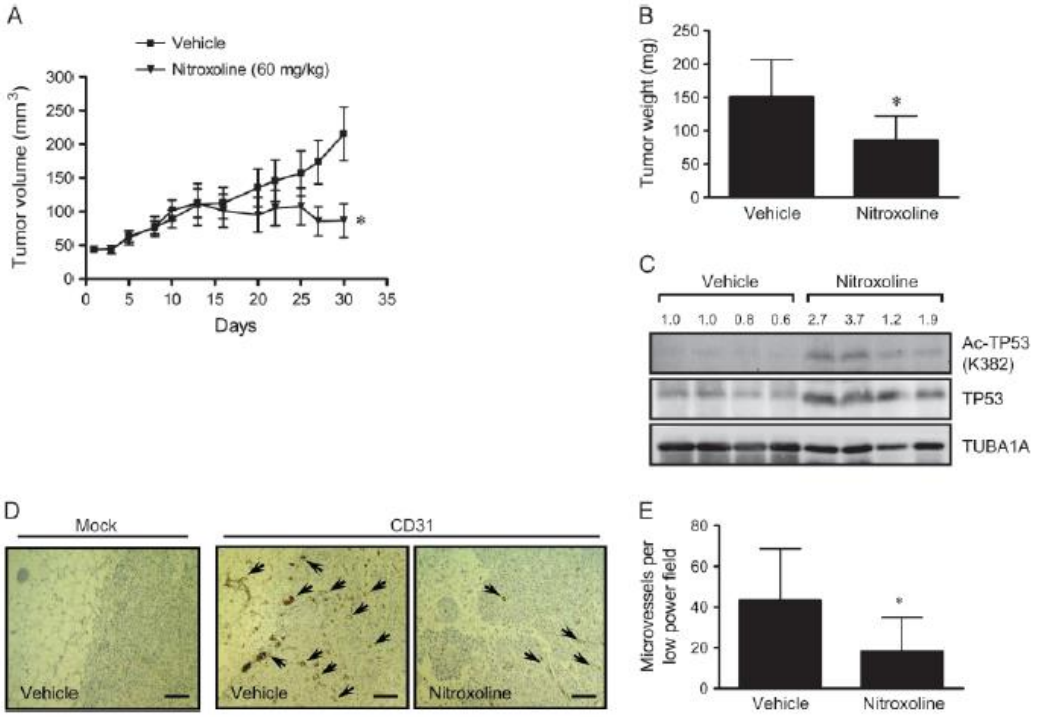
2.6.2.2.4 *In Vivo* Anti-Cancer Pharmacological Studies

Effect of Nitroxoline in a Human Breast Tumor Xenograft Model

Nitroxoline was reported to have an effect on the growth of breast tumors in a mouse xenograft model (Shim, 2011). HCC1954 cells were subcutaneously inoculated into female athymic nude mice, and the mice were injected either with vehicle (n = 5) or 60 mg/kg nitroxoline (n = 5) once every other day intraperitoneally for 30 days. Mice treated with nitroxoline showed statistically significantly reduced growth of HCC1954 xenografts (Figure 2.6.2-4). A 60 percent inhibition of tumor volume (vehicle vs. nitroxoline, mean = 215.4 vs. 86.5 mm^3, *P* =0.012) and a 43% inhibition of tumor weight (vehicle vs. nitroxoline, mean = 150.9 mg vs. 86.1 mg, *P* =0.036) were achieved on day 30 (Figure 2.6.2-4, A and B). To determine whether nitroxoline affected SIRT1 activity *in vivo*, an immunoblot analysis of the protein levels of TP53 and acetylated TP53 (K382) was performed in the tumor tissues (four tumors per group) from day 30. As shown in Figure 2.6.2-4, C, representative tumors from mice treated with nitroxoline

showed a substantial increase in the levels of TP53 and acetylated TP53 (K382). Tumors from mice treated with nitroxoline showed a 58% inhibition of angiogenesis (CD31-positive vessels, control vs. nitroxoline, mean = 43.2 vs. 18.2, P = 0.04) (Figure 2.6.2-4, D, E).

Figure 2.6.2-4: *In Vivo* Effects of Nitroxoline on the Growth and Angiogenesis of Human Breast Cancer



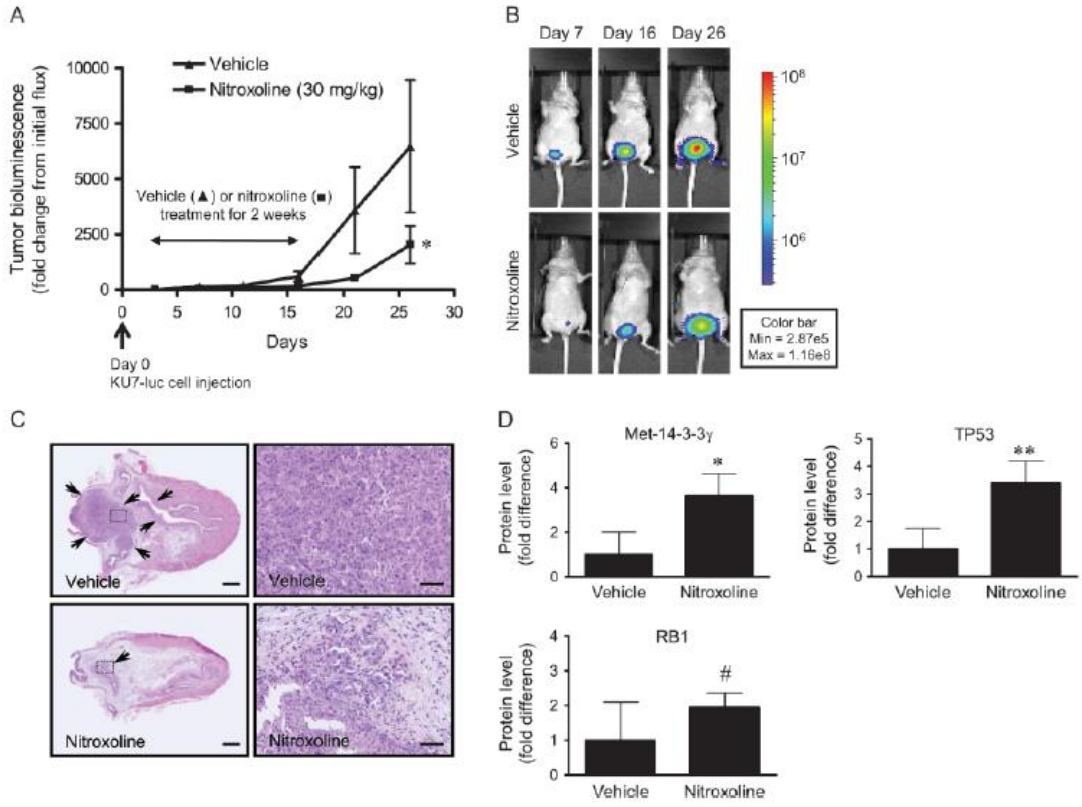
A) Analysis of tumor volume. B) Analysis of tumor weight. C) Immunoblot analysis of the levels of total TP53 and Ac-TP53 (acetyl-K382) in tumor samples. TUBA1A was used as the loading control. The Ac-TP53 (acetyl-K382) levels were normalized to total TP53 protein levels and their relative differences with the control are shown. D) Analysis of tumor micro vessels by immunohistochemistry. Magnification ×100. Scale bar = 200 μm. E) Quantitative analysis of micro vessels in the tumor tissues shown in (D) by counting four randomly selected fields in low-power (magnification ×100) fields.

Effect of Nitroxoline in an Orthotopic Superficial Bladder Cancer Model (KU7-luc)

Nitroxoline was also reported to have an effect on the growth of superficial bladder tumors *in vivo* (Shim, 2011). Experimentally, KU7-luc cells were implanted orthotopically onto the wall of mouse bladders in 11 female athymic nude mice (Hsd: Athymic Nude-*Foxn1nu*). Three days after tumor cell injection, mice were given nitroxoline (30 mg/kg/day) (n = 6) or vehicle (n = 5) orally for 2 weeks, and tumor growth was measured on days 7, 11, 16, 21, and 26 using bioluminescence imaging (Figure 2.6.2-5, A and B). Mice treated with nitroxoline showed a statistically significant inhibition of tumor growth compared with the vehicle-treated mice (Figure 2.6.2-5, A, *P*

= 0.045). Histopathologic analysis of xenograft tumors showed that tumors occupied a large area of bladders in the vehicle group, but there was a substantial reduction in tumor area in the bladders of mice treated with nitroxoline (Figure 2.6.2-5, C). To assess the inhibitory effect of nitroxoline on SIRT1 and MetAP2 proteins *in vivo*, bladder tissues from mice treated with vehicle or nitroxoline were subjected to immunoblot analysis. Tissues from mice treated with nitroxoline showed an increased level of Met-14-3-3γ (a substrate of MetAP2), TP53, and RB1 proteins (Figure 2.6.2-5, D), indicating that nitroxoline inhibited the activities of MetAP2 and SIRT1 in the tumors.

Figure 2.6.2-5: Effects of Nitroxoline on Tumor Growth in a Mouse Model Orthotopically Xenografted with Human Bladder Cancer Cell KU7-luc



A) Bioluminescence analysis. B) Bioluminescence measurement of bladder cancer growth in vehicle- and nitroxoline-treated mice on day 7, 16, and 26. Representative images are shown. C) Hematoxylin and eosin staining of representative bladder sections. Arrows indicate tumor areas in the bladder that stained more intensively compared with normal bladder cells. Whole bladder images are shown in the left panels (magnification ×10), and representative tumor areas (open boxes) are magnified (magnification ×150) in the right panels. Scale bar = 1 mm (left panels) and 100 μm (right panels). D) Quantitative immunoblot analysis of methionine on 14-3-3γ (Met-14-3-3γ), TP53 and retinoblastoma 1 (RB1) proteins from whole bladder tissue extracts. *P = 0.001; **P < .001, #P = 0.025.

Effect of Nitroxoline in an Orthotopic Superficial Bladder Cancer Model (5637)

The effect of nitroxoline on tumor growth was further characterized by us using a second superficial bladder cancer model of orthotopically xenografted human bladder cancer cells 5637. Under anesthetization, the inside walls of the bladders of Nu/Nu nude mice were slightly scratched with needle tips placed through the animal urethra. Bladder cancer cells 5637 in saline suspension were intravesically instilled into the animal bladders for two hours. Three weeks after the tumor cell instillation, animals were randomly divided into 5 groups, which received the treatment of vehicle, cisplatin, and three nitroxoline dosage groups (60, 120, 240 mg/kg/day), respectively. The body weights of animals were measured twice a week. At necropsy after four weeks of drug dosing, animal bladders including tumors were excised for weight measurement and photography. Tumor tissues were separated from the normal bladder tissues and were weighted to calculate the inhibition rates. The results are summarized in Table 2.6.2-3. After 28 days of treatment, nitroxoline showed statistically significant inhibitions of tumor growth in all three dose groups. It is worthwhile to note that 30 and 60 mg/Kg, BID, in mice are equivalent to 300 mg and 600 mg total daily doses in human, respectively.

Table 2.6.2-3: Effects of Nitroxoline in a Superficial Bladder Cancer Model Orthotopically Xenografted with Human Bladder Cancer Cell 5637

Groups	Admin Route	Animal No.		Body Weight (g)		Tumor Weight	Inh. Rates (%)
		Start	End	Start	End	(g)	
Vehicle	po.	8	7	22.41±1.35	20.02±2.52	0.191±0.050	—
Cisplatin, 5 mg/Kg, BIW	iv.	8	7	22.99±1.23	19.86±1.57	0.103±0.036**	46.3
Nitroxoline, 30 mg/kg, BID	po.	8	6	22.70±1.39	20.40±2.79	0.080±0.015***	58.0
Nitroxoline, 60 mg/kg, BID	po.	8	6	22.98±2.42	21.85±2.87	0.056±0.023***	70.7
Nitroxoline, 120 mg/kg, BID	po.	8	5	22.70±1.82	18.66±2.28	0.043±0.019***	77.3

$p<0.05$: *; $p<0.01$: **; $p<0.001$: ***.

Effect of Nitroxoline in an Orthotopic Kidney Cancer Model (KCC-853)

We have further evaluated the effect of nitroxoline on tumor growth by using an orthotopic kidney cancer model. Under anesthetization, human kidney cancer KCC-853 tumor tissues in size of 1.0x1.0 mm^2 were placed into one kidney capsule of Nu/Nu nude mice. After two weeks, animals were randomly divided into vehicle, cisplatin, and three nitroxoline dose groups (30, 60, 120 mg/kg/day) and dosed accordingly (Table 2.6.2-4). The body weights of animals were measured twice a week. At necropsy after four weeks of drug dosing, animal kidneys on both sides were excised for weight and photography. Tumors were separated from normal tissues and measured in weights and volumes. The results are summarized in Table 2.6.2-4. After 28 days of treatment, nitroxoline showed statistically significant inhibitions of tumor growth in all three dose groups.

Table 2.6.2-4: Effects of Nitroxoline in a Cancer Model Orthotopically Xenografted with Human Kidney Cancer Cell KCC-853

Groups	Admin Route	Animal No.		Tumor Wt. (W)	Tumor Vol. (V)	Inh. Rates (%)	
		Start	End	(g)	(mm^3)	W	V
Vehicle	po.	9	6	0.1954±0.0539	717.28±263.36	—	—
Cisplatin, 5 mg/kg, BIW	iv.	9	6	0.0798±0.0430**	209.21±170.24**	59.2	70.8
Nitroxoline, 30 mg/kg, BID	po.	9	6	0.0830±0.0429**	389.67±129.08*	57.5	45.7
Nitroxoline, 60 mg/kg, BID	po.	9	6	0.0805±0.0152***	301.48±295.79*	58.8	58.0
Nitroxoline, 120 mg/kg, BID	po.	9	6	0.0412±0.0182***	124.08±77.99***	78.9	82.7

p<0.05: *; p<0.01: **; p<0.001: ***

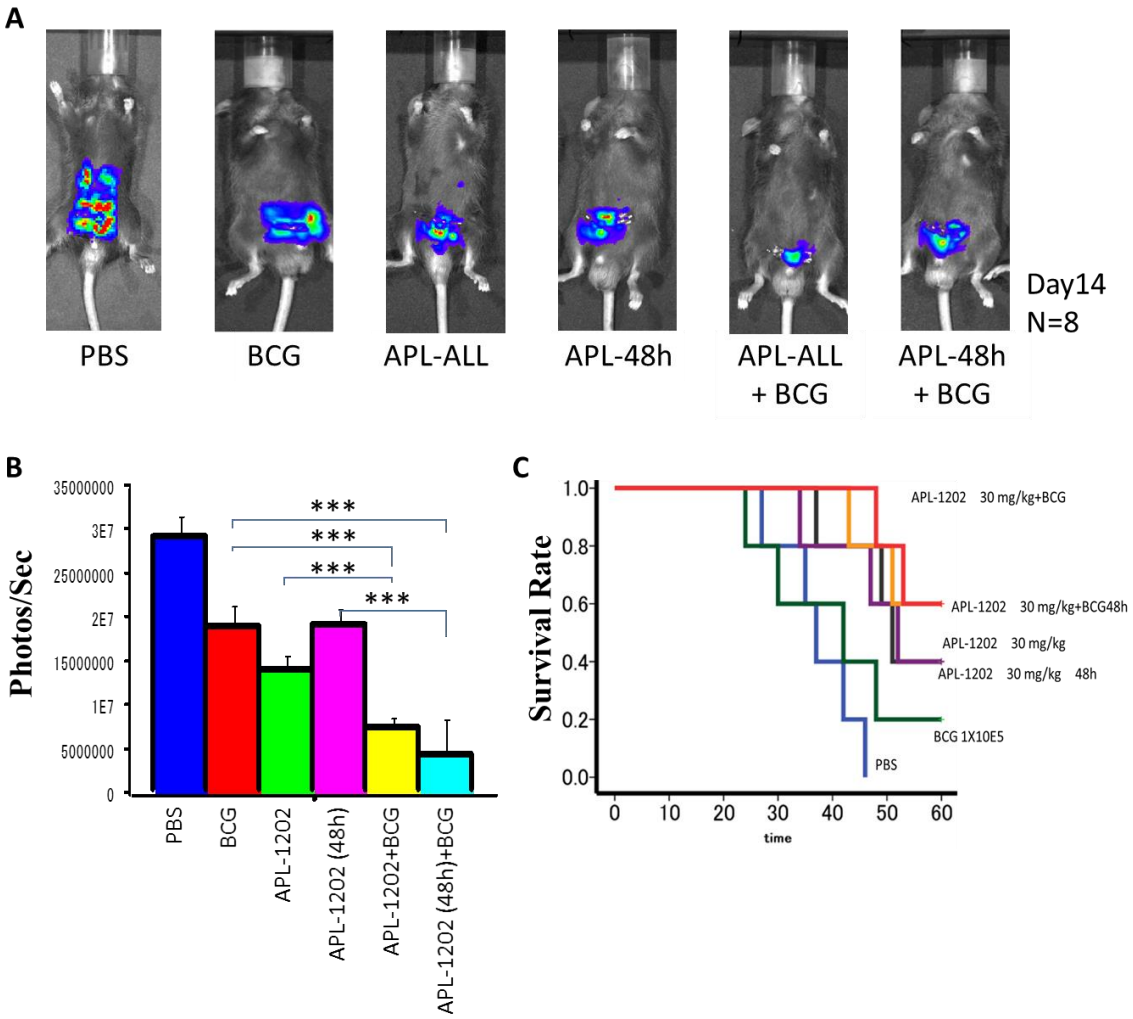
Effect of Nitroxoline in Combination with Bacillus Calmette–Guerin (BCG) in an Orthotopic Bladder Cancer Model (MBT-2–luc)

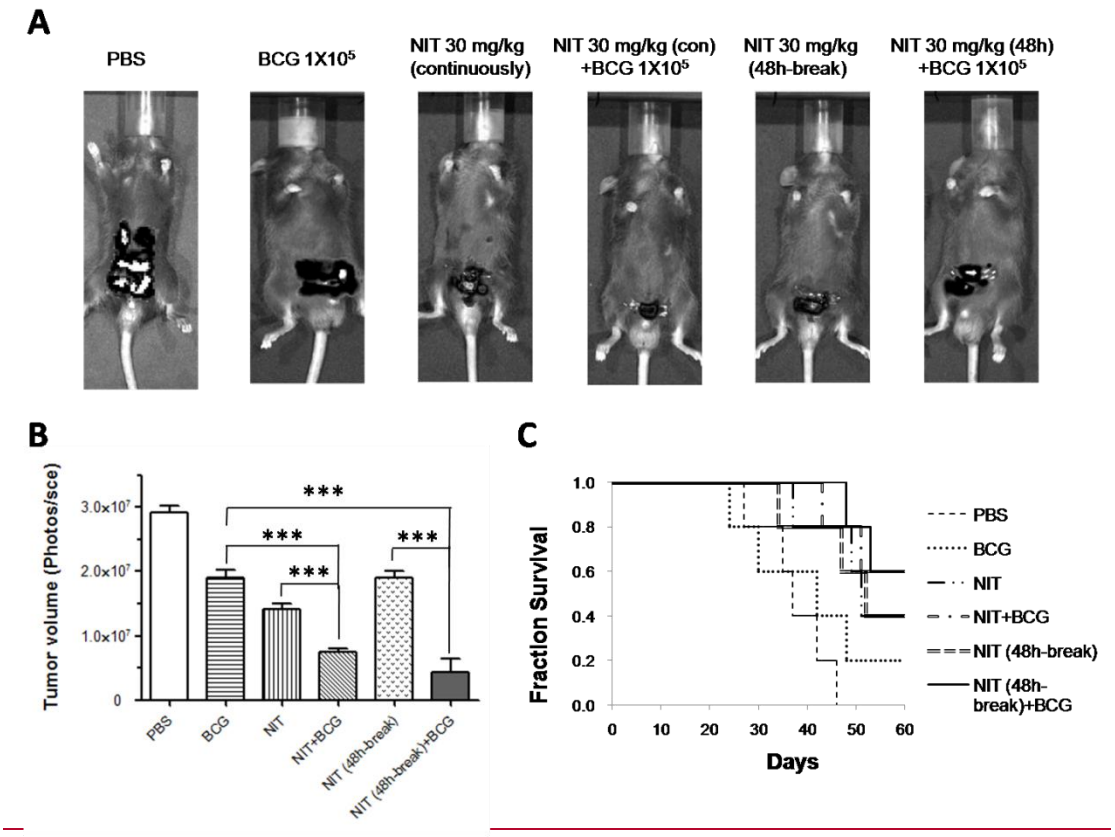
Given that a significant fraction of nitroxoline is excreted through urine (see Section 2.6.4.5 METABOLISM) and BCG is the first-line therapy for intermediate- and high-risk non–muscle invasive bladder cancer (NMIBC), the effect on tumor growth of nitroxoline in combination with BCG was evaluated in a mouse orthotopic bladder cancer model.

Orthotopic bladder cancer tumors were established with MBT-2-Luc cells in six-week-old female C3H/HeN mice as described by Huang (2015). ~~Five~~ Seven (7) days after tumor cell implantation, the mice carrying orthotopic bladder cancer tumors were randomly divided into six groups and respectively treated with: 1) vehicle, oral daily (~~Vehicle~~ PBS group); 2) BCG, intravesically, ~~once weekly for 3 weeks~~Day 2 and Day 9 (BCG group); 3) ~~NIT~~APL-1202, oral daily, 30 mg/kg/day as BID for 2~~3~~ weeks (~~NIT~~ APL-ALL group); 4) ~~NIT~~APL-1202, oral daily, 30 mg/kg/day as BID for 2~~3~~ weeks, except for a 24-hour break before and after ~~the weekly scheduled BCG~~ vehicle instillation at Day 2 and Day 9 (~~NIT~~ APL-48h-break group); 5) combination of ~~NIT~~ APL-1202 and BCG (~~NIT~~APL-ALL+BCG group) ; and 6) combination of ~~NIT~~ APL-1202 with 48-hr break and BCG instillation at Day 2 and Day 9 (~~NIT~~ APL-48h-break+BCG group). Tumor size was evaluated weekly using a bioluminescence imaging system (Figure 2.6.2-6A).

As shown in Figure 2.6.2-6B, oral administration of nitroxoline inhibited the tumor growth, both consecutively and with a 48-hour break, and the magnitude of inhibition was slightly higher or similar to that of intravesical BCG, respectively. When oral nitroxoline (consecutively or with a 48-hour break) was dosed in combination with intravesical BCG, the magnitude of inhibition was significantly higher compared to either oral nitroxoline or intravesical BCG alone (p<0.0001 for all comparisons). In addition, similar effects on the survival of the treated animals were observed (Figure. 2.6.2-6C).

Figure 2.6.2-6. Combination of Orally Dosed Nitroxoline and Intravesically Instilled BCG Revealed Synergistic Antitumor Effects in MBT-2–luc Orthotopic Mouse Bladder Cancer Model





A) The tumor volume was analyzed by the Xenogen IVIS200 system, and a typical IVIS image for each treatment group of indicated were shown. B) The tumor volume of each group was determined by region-of-interest analysis of total photons per second. Eight mice were analyzed in each group (***, $p<0.0001$). C) The Kaplan-Meier analysis was performed to evaluate mice survival for all treatments groups.

The results of the Bliss independence model calculations of additivity for tumor growth inhibition and survival proportions are summarized in Table 2.6.2-5. When oral NIT was administered in combination with intravesical BCG, either consecutively or with a 48-hour break, the magnitude of observed tumor inhibition was significantly increased compared to either agent alone. The actual observed values of the NIT and BCG combination are much greater than their Expected Bliss Additive Value, suggesting a robust synergy in tumor inhibition by the NIT and BCG combination.

Table 2.6.2-5: Bliss Independence Model Calculation of Additivity for Oral Nitroxoline with Intravesical BCG in MBT-2–luc Orthotopic Mouse Bladder Cancer Model

NIT Dosing	Single Agent Effect	NIT +BCG Combination effect

	NIT	BCG	Expected Bliss Additive Value	Actual Observed Value	Interaction
Consecutively	0.52	0.35	0.69	0.75	Synergy
48-h break	0.35	0.35	0.58	0.85	Synergy

Additivity was determined using the Bliss Independence model: $E_{xy} = E_x + E_y - (E_xE_y)$, where E_{XY} is the additive effect of the 2 compounds *x* and *y* as calculated by the product of the individual effect of the 2 compounds, E_x and E_y. Synergy was established when the actual observed tumor inhibition value was greater than the expected tumor inhibition value determined by the Bliss Independence model (Yan, 2010).

2.6.2.3 SECONDARY PHARMACODYNAMICS

No secondary pharmacodynamic studies have been performed.

2.6.2.4 SAFETY PHARMACOLOGY

No safety pharmacology studies have been performed.

2.6.2.5 PHARMACODYNAMIC DRUG INTERACTIONS

In our preliminary study, nitroxoline revealed antibacterial activity against BCG *in vitro*. The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined as 11 µM under the experimental conditions, in which BCG were continuously exposed to nitroxoline for 13 days.

2.6.2.6 DISCUSSION AND CONCLUSIONS

Nitroxoline has been marketed as an antimicrobial drug since 1960s, and recently re-discovered to have both anti-angiogenic and anti-tumor activities. Compared to its antimicrobial MICs ranging from 7.9-28.9 µM (Kuss, 1962), nitroxoline is several folds more potent in inhibiting the growth of HUVEC (IC_{50}= 1.9 µM) and several bladder and kidney cancer cell lines (IC_{50}= 2.7-3.7 µM). *In vivo* effects of nitroxoline in inhibiting the growth of bladder cancer and kidney cancer have been demonstrated in multiple orthotopic models in nude mice. The effective doses of 30-60 mg/kg/day, equivalent to human doses at 150-300 mg/day, were equal to or lower than the clinical doses (300-800 mg/day) used for treating urinary tract infections. Moreover, nitroxoline (30 mg/kg/day) showed synergistic effects when administered together with intravesical BCG in an orthotopic bladder cancer model.

Preliminary drug interaction study showed nitroxoline has moderate inhibitory effects on BCG when co-incubated *in vitro*. However, the clinical significance of this finding is not clear. In our clinical trial phase II, we confirmed that the plasma clearance rate of nitroxoline in human (T1/2) is < 2 hours. In addition, we observed that nitroxoline concentration in patients' urine reached > 10 µM in some of the patients, if not all. However, amongst the urine samples in which nitroxoline was detected, the concentration of nitroxoline was decreased below 10 µM in 82.8% of patients treated with over 150 mg

by 8 hours following the nitroxoline treatment. In clinical applications, BCG instillation for bladder cancer patients is 2-3 hours and then BCG is urinated. Thus, the duration of drug-drug interaction between nitroxoline and BCG in patient bladder is short. In our animal study comparing the antitumor efficacies of two treatment regimen, namely mice were treated with BCG in combination either with nitroxoline daily treatment or with a 24-hour break before and another 24-hour break after each BCG treatment. As shown in Figure 2.6.2-6 and Table 2.6.2-5, no significant difference in antitumor efficacy as well as overall survivals between the two treatment regimens, suggesting that no drug-drug interaction between nitroxoline and BCG in this study. These data collectively suggest that the potential of a drug-drug interaction of BCG and nitroxoline in the clinical would be minimal.

In summary, these non-clinical pharmacology studies support the clinical development of nitroxoline as a cancer treatment agent, particularly in NMIBC patients. Furthermore, the synergistic anti-tumor effect of nitroxoline and BCG combination supports the clinical evaluation of the effect of oral nitroxoline in combination with intravesical BCG in NMIBC patients.