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Discovery of ursolic acid prodrug (NX-201): Pharmacokinetics and *in vivo* antitumor effects in PANC-1 pancreatic cancer

Yeohong Yoon[a,c,*], Jee Woong Lim[a], Jiyoung Kim[a], Younggi Kim[b] and Keun Ho Chun[c,*]

[a]*Nexoligo Co., Ltd., 40 4F 3408, Simin-daero 365beongil, Dongan-gu, Anyang-si, Gyeonggi-do, 14057, Korea*
[b]*KNOTUS Co., Ltd, 189, Dongureung-ro, Guri-si, Gyeonggi-do, 02117, Korea*
[c]*Department of Chemistry, Soongsil University, 369, Sangdo-ro, Dongjak-gu, Seoul, 06978, Korea*

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ABSTRACT

The aim of our study was to develop ursolic acid (UA) prodrugs in order to overcome UA's weakness, which has an extremely low bioavailability. UA-medoxomil (NX-201), one of our UA prodrugs, showed an improved bioavailability about 200 times better than UA in rodent model. According to *in vivo* test performed with PANC-1 xenograft SCID mouse model, tumor growth rate decreased dose-dependently and 100 mg/kg dose of NX-201 had an anticancer effect comparable to gemcitabine. Most of all the combination of NX-201 (50 mg/kg, po, daily) and gemcitabine (40 mg/kg, iv, 2 times per week) even reduced tumor size after three weeks.

Natural products have been used for curing and preventing disease since long before, and medicinal chemists have contributed to the mankind by utilizing certain natural products as lead compounds in order to develop more effective and less toxic drug candidates. Among many natural products, pentacyclic triterpene series[1] have more diverse and interesting pharmacological activities, and various studies related to these activities have been frequently conducted. Recently ursolic acid (UA, 3β-hydroxy-urs-12-ene-28-oic acid, Figure 1.) has been proved to have many biological activities suggesting that it could be developed as a drug candidate for various intractable diseases.

Figure 1. Structure of ursolic acid (UA)

UA, which is a natural compound of ursane-type pentacyclic triterpenic acid, is present in many plants and can be supplied as a single compound. In view of new drug development, UA became known to have various pharmacological effects; hepatoprotective, immunomodulatory, anti-inflammatory, kidneyprotective, antidiabetic, anti-muscle atrophy, antibacterial, antiviral against HCV, HSV and HPV, anti-*trypanosoma cruzi* for Chagas disease, and anticancer.[2] Among them the anticancer effect has been drawing the most attention and consequently, the numerous studies related to mode of action and therapeutic method of UA as an anticancer drug have proceeded. UA has been known for STAT-3 inhibitor[3] which suppresses the proliferation of various cancer cell types. Until now several STAT-3 inhibitors[4] including UA have been developed but it is not yet potent enough to undergo clinical trials. However it has been reported that combination of UA and prescribed anticancer drugs such as cisplatin and paclitaxel, which is commercially available, would create a synergetic effects in reducing tumor growth rate.[5] Furthermore, there are also reports[6] that UA can act as a potent anticancer agent for some cancers which are resistant to currently available anticancer drug, for example gemcitabine-resistant pancreatic cancer.

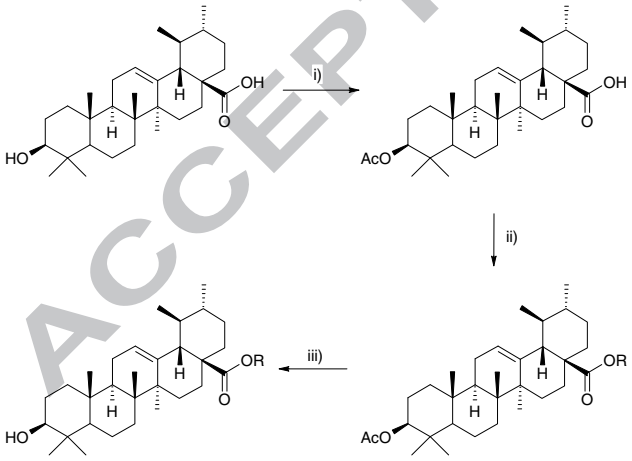
Pancreatic cancer is known as one of the most fatal cancers. Gemcitabine has been used as a standard chemotherapy, but 5-year survival rate is about 9% and attempts for the development of new drug have been much less compared to other intractable cancers such as lung cancer whose 5-year survival rate is now up to 22%. Therefore developing more efficient and less toxic pharmacotherapy for pancreatic cancer should be given priority in this field.

According to Lie's research, *in vivo* test was carried out with utilizing human pancreatic gemcitabine-resistant cancer cell lines

such as PANC-1, MIA and PaCA-2 on nude mouse xenograft model. The test was conducted for two weeks with UA 100 mg/kg and 200 mg/kg by ip injection. The result showed that tumor growth rate decreased strikingly dose-dependently. Unfortunately, UA has a serious physicochemical disadvantage resulting in an extremely low bioavailability, which obstructs oral administration of UA. For that reason injectable formulation or chemical modification of UA capable of showing more effectiveness have been regarded as alternatives in many ways. For example injectable UA liposome (UAL) was reported in terms of clinical phase 1 trial.[7] In spite of low toxicity and usefulness of UA, new derivatization of UA inevitably increases the risk of consumption of time and cost in the long process of confirmation of safety and efficacy.[8,9] Therefore, our research team put a great effort on developing an oral UA prodrug which results in better PK profile and can be expected to be safe comparable to UA itself.

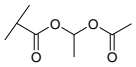
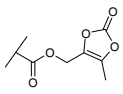
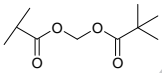
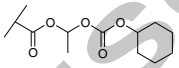
As shown in Figure 1, chemical structure of UA is very rigid, and has an alcohol group located on C-3, a double bond on C-12 and an unusual tertiary carboxylic acid on C-17. Since UA's pK_a is 5.29, UA might be absorbed through intestine after oral administration, however, we found that no absorption has occurred through PK test. Our team designed ester type prodrugs to enhance oral absorption rates by increasing lipophilicity of carboxylic acid on C-17. Several well-known promoieties of prodrugs which have been proved to be safe and easily biodegradable after orally taken, were introduced into UA.

Synthetic process of UA prodrugs shown in scheme 1 consisted of 3 steps. After protection of hydroxyl group on C-3 with acetyl group, carboxylic acid on C-17 was transformed to several esters of promoieties, and finally deprotection of hydroxyl group was performed to offer UA prodrugs. As for protection of C-3 alcohol, acetyl group was more suitable in the aspect of separation and purification of final products, compared to TBDMS, THP. Selective deacetylation of C-3 in final step was important to achieve high purity, so the acidic condition was favorable because of lability of C-17 ester under the basic condition. UA having 99% purity was used as a starting material, and all final products showed over 98% in HPLC purity.



Scheme 1. Synthesis of UA prodrugs. Reagent and conditions: (i) acetic anhydride, pyridine, DMAP cat., THF; (ii) K_2CO_3, RX (RX = 1-(acetoxyethyl)-bromide (axetil); 4-chloromethyl-5-methyl-2-oxo-1,2-dioxolane (medoxomil); 1-chloromethyl pivalate (pivoxetil); 1-chloroethyl cyclohexyl carbonate (hexetil)), KI, acetone; (iii) p-TsOH, CH_2Cl_2, MeOH.

Table 1. UA prodrugs: structure, purification method, yield

compound	Structure of promoiety (-CO_2R in Scheme 1)	Purification method	Total yield (%)
UA-axetil		Column chromatography	22
UA-medoxomil		Crystallization	47
UA-pivoxetil		Column chromatography	21
UA-hexetil		Column chromatography	25

Among the synthesized four derivatives in Table 1, UA-medoxomil (NX-201),[10] shown in Figure 2, was preferentially applied to *in vivo* PK and efficacy tests for the following two reasons. First, NX-201 has relatively good physicochemical properties, for example, crystallinity and stability, which allow easier purification in large scale. Second, medoxomil promoiety has been researched profoundly in terms of the bioconversion mechanism and toxicity in many cases including olmesartan medoxomil, even though all four derivatives would be considered as orally administrated prodrugs[11] that is rapidly and completely metabolized to UA in gastrointestinal mucosa, portal blood and liver before it comes into systemic circulation. Prodrug compounds other than NX-201 is currently followed up to evaluate their potential as a drug in parallel with NX-201 in our reseach team.

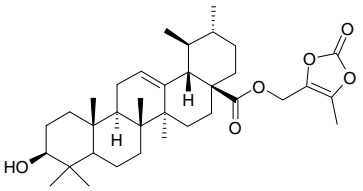


Figure 2. Structure of UA-medoxomil (NX-201)

As a result PK study shown in Table 2, bioavailability (F) of NX-201 was proved to be over 200 times better than that of UA and effective half-life was 15 times longer. UA's direct bioavailability data was unknown in previous PK studies, instead, result for oleanolic acid (OA), known for an isomer of UA, has been reported.[12] In that report OA's bioavailability was 0.7% with 25 mg/kg dose in rat. Although NX-201's bioavailability is less than 3% which is still very low level, it could be raised using an advanced formulation technology applied to poorly water-insoluble compounds.

Table 2. Pharmacokinetic data of UA and NX-201[13]

cpd	route	dose (mg/kg)	T_{max} (hr)	C_{max} (ng/ml)	AUC (h*ng/ml)	$T_{1/2}$ (hr)	F (%)
UA[a]	iv	0.5	0.08	378.0	114.3	1.55	-
UA[b]	po	25	0.25	1.9	0.6	0.3	< 0.01
NX-201[b]	po	25	1.46	30.3	132.2	4.72	2.3

[a]0.5 mg/ml, DMSO:PEG 300:water = 2:4:1

[b]0.5% CMC water suspension

Anticancer *in vivo* efficacy[14] of NX-201 was tried by using typical pancreatic cancer model, a SCID mouse bearing PANC-1 xenograft to provide evidence for pancreatic cancer drug. Figure 3 shows the comparison of test groups in the tumor size for four weeks.

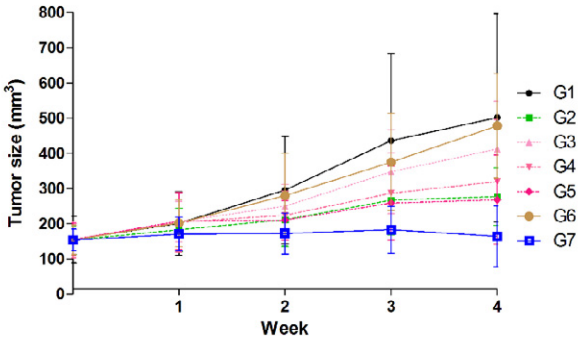


Figure 3. *In vivo* test in PANC-1 xenograft SCID mouse model

(Groups: G1 = negative control; G2 = positive control, gemcitabine, 40 mg/kg, iv, 2 times/week; G3 = NX-201, 25 mg/kg, po, daily; G4 = NX-201, 50 mg/kg, po, daily; G5 = NX-201, 100 mg/kg, po, daily; G6 = UA, 50 mg/kg, po, daily; G7 = combination of NX-201 (50 mg/kg, iv, daily) and gemcitabine (40 mg/kg, po, 2 times/week))

Repeated administration of single NX-201 (G3-G5) for 4 weeks resulted in the decrease of tumor growth rate dose-dependently. In case of UA (G6), no significant change in tumor size occurred as expected, that suggests the evidence of enhanced absorption of NX-201 by oral administration route. At 100 mg/kg dose of NX-201 (G5) tumor growth rate decreased on a similar level of gemcitabine, and this reflects the potent anticancer effect considering its low bioavailability. A noteworthy point is that the combination of NX-201 and gemcitabine (G7) even reduced tumor size after 3 weeks administration. In terms of body weight every test groups showed no significant change.

In conclusion, conversion of UA to ester-type prodrug, NX-201, ensured a high probability of new pancreatic cancer therapy. And furthermore, UA prodrugs including NX-201 can be utilized in the field of various cancer and other intractable diseases based on the previous reports about UA's pharmacological efficacies. Since UA has been well-known for very low-toxic natural product, it can be said that UA prodrugs have great potentials in many respects. In the view of anticancer drugs, combination of NX-201 and other currently prescribed drugs can be highly beneficial in the management of incurable cancer patients. In near future, our research team is planning to broaden the therapeutic applications in parallel with the development of anticancer drug.

Acknowledgments

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10. ^{1}H NMR (DMSO-d_6, 500MHz) δ 4.76~5.00 (2H, dd, J = 14Hz, medoxomil methylene), 5.16 (1H, t, J = 1.75Hz, C12), 4.27 (1H, d, J = 5Hz, hydroxyl), 2.96 ~ 3.01 (1H, m, C3), 2.15 (1H, d, C18), 2.11 (3H, s, medoxomil methyl), 1.04 (3H, s, C27), 0.92 (3H, d, J = 6Hz, C30), 0.89 (3H, s, C23), 0.84 (3H, s, C25), 0.81 (3H, d, J = 6.5Hz, C29), 0.67 (3H, s, C26), 0.56 (3H, s, C24); ^{13}C NMR ($CDCl_3$, 500MHz) δ 177.04 (C28), 152.25 (medoxomil carbonyl), 139.91 (medoxomil), 137.66 (C13), 133.84 (medoxomil), 126.05 (C12), 78.96 (C3), 55.20 (medoxomil methylene), 53.35 (C15), 52.87 (C18), 48.43 (C7), 47.45 (C9), 42.05 (C14), 39.50 (C8), 39.02 (C1), 38.82 (C4), 38.73 (C19), 38.60 (C20), 36.92 (C22), 36.61 (C10), 33.03 (C7), 30.54 (C21), 28.15 (C23), 27.82 (C2), 27.20 (C15), 24.12 (C16), 23.47 (C11), 23.24 (C27), 21.12 (C30), 18.28 (C6), 16.94 (C26), 16.63 (C24), 15.63 (C25), 15.40 (C26), 9.38 (medoxomil methyl); MALDI-TOF m/z [M+Na] calcd for $C_{35}H_{52}O_6Na$ 591.37, found 591.34; IR(cm^{-1}) 2918, 1882, 1686, 1720, 1455, 1383, 1219, 1186, 1044, 1029; mp 188~192℃.
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13. Sample collection and analysis condition about PK test of UA and NX-201(>99% purity on HPLC): (a) Blood samples (0.6 ml) were collected prior to serve as a control, and collection points are 5 min, 15 min, 30 min, 45 min, 1 hr, 2 hr, 4 hr, 8 hr and 12 hr after drug administration; (b) HPLC/MS/MS (Agilent 1200 series LC system) analysis condition: detector = AB API4000 LC/MS, column = Phenomenex Luna C18 column (50 mm * 2.0 mm, 3 μm), mobile phase = 10 mM ammonium acetate : methanole, 10 : 90 (v/v), flow rate = 0.3 ml/min, injection volumn = 5.0 μL, MRM = ursolic acid (m/z 455.4).
14. *In vivo* test: (a) This study was performed in accordance with the Animal Experimentation Policy of KNOTUS Co., Ltd. for animal experimentation ethics (b) Animal model = NOD, DB17 SCID

mouse xenograft model (n = 8 per group); (c) Cancer cell line = PANC-1 pancreas carcinoma; (d) Preparation of xenograft model: animals were subcutaneously inoculated with $2*10^6$ cells in 0.2 ml of medium by an administrator to prepare the xenograft model. About 22 days after inoculation, when tumor size reached 150~250 mm^3, animals were distributed referring to the rank of tumor sizes. Finally 8 animals per group were assigned for each group; (e) Statistical analysis: all analyses were performed with Prism 5.03 (GraphPad software Inc., San Diago, CA, USA) and significance levels were judged at $p < 0.05$. Group's significant differences compared to the G1 were G2 (4 week)'s $p < 0.05$, G5 (4 week)'s $p < 0.05$, G6 (4 week)'s $p < 0.05$, G7 (2 week)'s $p < 0.05$, G7 (3 week)'s $p < 0.01$, G7 (4 week)'s $p < 0.001$.

Graphical Abstract

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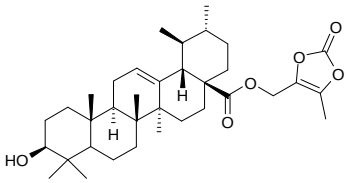
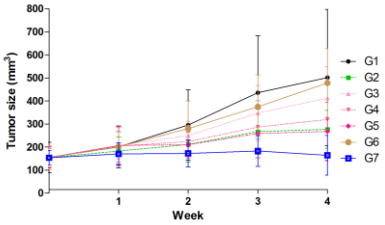


NX-201



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G5 = NX-201, 100 mg/kg, po, daily
G6 = UA, 50 mg/kg, po, daily
G7 = combination of NX-201 (50 mg/kg, iv, daily) and gemcitabine (40 mg/kg, po, 2 times/week)