ISSN 1881-7831 Online ISSN 1881-784X

DD&T

Drug Discoveries & Therapeutics

Volume 8, Number 1
February, 2014

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www.ddtjournal.com

DD&T
Drug Discoveries & Therapeutics

ISSN: 1881-7831
Online ISSN: 1881-784X
CODEN: DDTRBX
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.

Drug Discoveries & Therapeutics is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group and is published bimonthly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA and Shandong University China-Japan Cooperation Center for Drug Discovery & Screening (SDU-DDSC).

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CONTENTS

Volume 8, Number 1, 2014

Review

Brief Report

Original Articles

CONTENTS (*Continued*)

Review

DOI: 10.5582/ddt.8.1

Pharmacological effects and clinical applications of ultra low molecular weight heparins

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Summary **Heparin, one of the common anticoagulants, is clinically used to prevent and treat venous thromboembolism (VTE). Though it has been the drug of choice for many advanced medical and surgical procedures with a long history, the adverse events, such as bleeding, heparin-induced thrombocytopenia (HIT), allergic reactions, follow. Therefore, low molecular weight heparins (LMWHs) and ultra low molecular weight heparins (ULMWHs), with lower molecular weights, higher anti-FXa activity, longer half-life times and lower incidence of adverse events than unfractionated heparin (UFH), were researched and developed. Fondaparinux, a chemically synthesized ULMWH of pentasaccharide, has the same antithrombin III (AT-III)-binding sequence as found in UFH and LMWH. In addition, AVE5026 and RO-14, another two ULMWHs, are obtained by selective chemical depolymerization. In this paper, we review the preparation process, pharmacological effects and clinical applications of fondaparinux, AVE5026 and RO-14.**

Keywords: Pharmacological effects, clinical applications, ultra low molecular weight heparin, fondaparinux, AVE5026, RO-14

1. Introduction

Heparin was discovered in 1916. Because it plays an essential role in many medical and surgical procedures, heparin has been widely used clinically since 1934 (*1*). Currently, heparin is the drug of choice to prevent and treat venous thromboembolism (VTE). Pharmaceutical heparin is usually obtained from porcine intestines or bovine lungs (*2-5*). The chemical parameters, such as purity, molecular mass distribution and degree of sulfation, must be strictly controlled to ensure appropriate biological activities. There are three forms of heparin drugs: UFH (average molecular weight (MWavg) ~15,000), LMWHs (MWavg 3,500 to 6,000) and ULMWHs (MWavg < 3000).

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Heparin, a linear sulfated polysaccharide, consists of repeating disaccharide subunits of α-1,4 linked uronic acid and D-glucosamine (panel A of Figure 1) (*6*). The uronic acid residue of heparin may be either α-L-iduronic acid (IdoA) or β-D-glucuronic acid (GlcA) and can be unsubstituted or sulfonated at the 2-*O* position. The glucosamine residue may be unmodified (GlcN), *N*-sulfonated (GlcNS) or *N*-acetylated (GlcNA), and can contain variable patterns of *O*-sulfonation at the 3-*O* and/or 6-*O* positions. The major disaccharide sequence of heparin is the trisulfonated L-IdoA(2S)-D-GlcNS(6S). It has been demonstrated that the locations of the sulfo groups, IdoA, and GlcA lead to the anticoagulant activity of heparin.

Due to the ability to bind to antithrombin III (AT-III), heparin has anticoagulant activity. AT-III has a conformational change when heparin interacts with it, exposing the reactive center. Then, this reactive center within AT-III can interact with coagulant enzymes, such as thrombin and factor Xa (FXa). A unique pentasaccharide domain, the residue A to E in panel A of Figure 1, is critical in inducing the conformational change of AT-III. Therefore, both the pentasaccharide domain and the thrombin-binding domain (Figure 2) are acquired

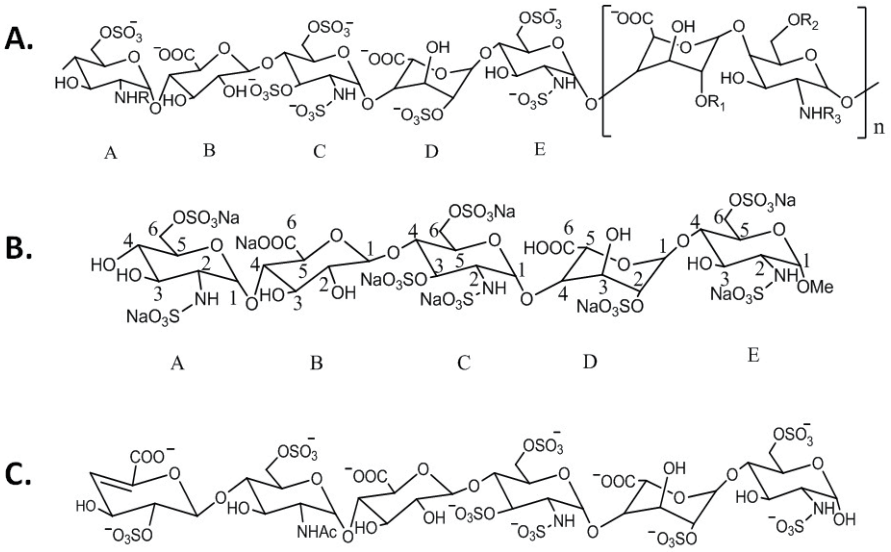

Figure 1. The structure of UFH, fondaparinux and ΔIIa-IIs-Is. A: The unique AT-III-binding pentasaccharide sequence and repeating disaccharide units of heparin. (R_1 = H, SO_3^-; R_2 = H, SO_3^-; R_3 = H, acetyl, SO_3^-). **B:** The structure of fondaparinux. C: The structure of the ΔIIa-IIs-Is.

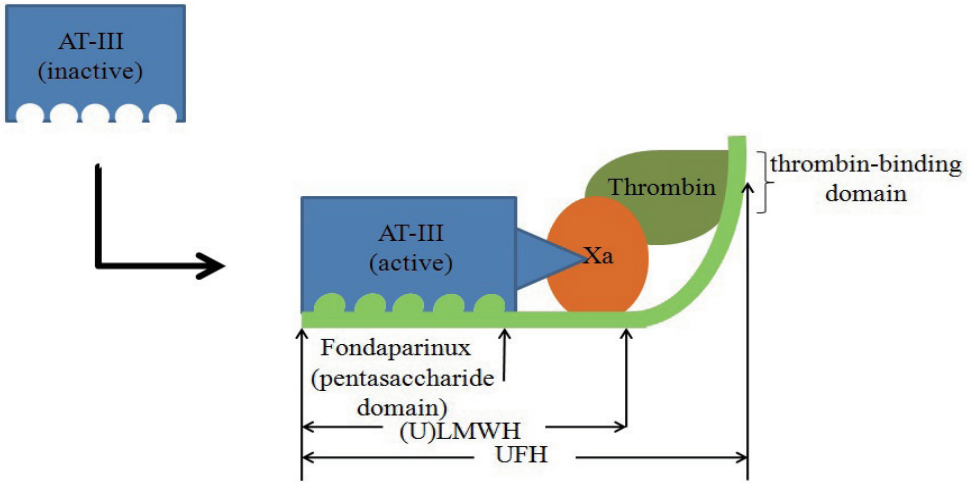

Figure 2. Schematic illustrating the heparin inactivation of FXa and thrombin by antithrombin.

for heparin's antithrombin activity, which means the minimum size of fragment with antithrombin activity is 18 monosaccharides in heparin-based drugs. The schematic of the inactivation of FXa and thrombin by heparin and AT-III is shown in Figure 2.

Though UFH works well as an anticoagulant drug, an adverse bleeding event occurs sometimes when UFH is used for antithrombotic therapy, because all heparin polysaccharides obtained from different preparation processes include saccharide-fragments with high antithrombin activity (Table 1) (*7*). In addition, UFH has other adverse events, such as heparin-induced thrombocytopenia (HIT), allergic reactions, and so on. It is reported that UFH results in a 1%-6% incidence of HIT, a life-threatening complication (*8*). LMWHs, depolymerized products of heparin polysaccharide, have a longer half-life than UFH and can be subcutaneously administered. Because of these advantages, LMWHs have emerged as the most widely prescribed heparins in the US (*9*). However, although the frequency of adverse bleeding events has declined, the antithrombin activity of LMWHs cannot be ignored. For example, the anti-FIIa activity of enoxaparin is 27 IU/mg (Table 1) and this can lead to bleeding in a few patients. Among the three forms of heparin, ULMWHs have the highest anti-FXa activity and the lowest anti-FIIa activity compared to heparin and LMWHs, resulting in the lowest anticoagulant activity and incidence of bleeding (Table 1). In Table 1, we make a comparison of AVE5026, RO-14, fondaparinux, enoxaparin, and UFH.

Fondaparinux, AVE5026 and RO-14 are the three most common ULMWHs. Fondaparinux, a chemically synthesized pentasaccharide, has the same AT-III-binding sequence as the natural heparin polysaccharide. One pentasaccharide domain contained in both UFH (panel A of Figure 1) and fondaparinux (panel B of

Table 1. Comparison of AVE5026, RO-14, fondaparinux, enoxaparin, and UFH

	MeanMW (Da)	Anti-FXa (U mg^{-1})	Anti-FIIa (U mg^{-1})	$t_{1/2}$ (h)	Route	Clearance (primary)	Antidote
UFH	15,000	193	193	0.5-2.5	IV/SC	Cellular metabolism and renal clearance	Protamine
Enoxaparin	4,500	105	27	4.0-4.7	SC	Renal clearance	Protamine may have partial neutralizing effects
AVE5026	2,400	150-200	< 5	16-20	SC	Renal clearance	Protamine may have partial neutralizing effects
RO-14	2,200	80-140	≤ 10	8.1	SC	Renal clearance	Protamine may have partial neutralizing effects
Fondaparinux	1,728	850 ± 27	< 0.1	17	SC	Renal clearance	Recombinant factor VIIa may be effective

Figure 1) is shown from residue A to residue E in Figure 1, and this pentasaccharide domain is critical in inducing the conformational change of AT-III which interacts with it. Therefore, it has good anti-FXa activity. However, because this pentasaccharide is too short to bridge AT-III to thrombin, fondaparinux scarcely has anti-FIIa activity. Fondaparinux has been indicated for primary prevention of VTE in patients undergoing orthopedic or abdominal surgery and for the treatment of VTE (*10*). AVE5026 is a compound from a chemoselective depolymerization of the heparin macromolecule. It was studied for use in prophylaxis of VTE in patients with cancer. Meanwhile, RO-14 exhibited dose-proportional pharmacokinetics and a favorable safety profile. Data from clinical studies of RO-14 have not been published. In addition, bemiparin is approved for use in the prophylaxis of VTE in medical patients and patients undergoing general or orthopedic surgery and for secondary prophylaxis in patients with deep vein thrombosis (DVT) in the US since 1998. Only few articles classify bemiparin as an ULMWH and many others consider it to be the second generation LMWHs. The development of deligoparin was terminated because of a study evaluating its use as an anti-inflammatory treatment in patients with ulcerative colitis failed to meet its end points (*11*). Therefore, we reviewed the preparation process, pharmacological effects, and clinical applications of fondaparinux, AVE5026, and RO-14.

2. Fondaparinux sodium

2.1. *Structure and structure-activity relationships*

Fondaparinux sodium (Arixtra), a chemically-synthesized pentasaccharide, is a specific inhibitor of FXa (*12*) and it went on the market in the USA and Europe in 2002. It is synthesized through a block synthesis in about 55 steps from naturally occurring carbohydrates. When the pentasaccharide skeleton was obtained, *O*-sulfation-hydrogenation-*N*-sulfation became the critical process for synthesis of fondaparinux sodium. Recently, Manikowski and his colleagues reported an alternative way for fondaparinux sodium synthesis based on an efficient and facile one-step *O*- and *N*-sulfation of the appropriate pentasaccharide (*13*). The advantage of this updated approach is minimizing byproduct formation, simplifying the fondaparinux sodium synthesis in comparison to the contemporary methods.

Except for the residue at the reducing end (residue E in panel B of Figure 1), fondaparinux sodium has the same structure as the pentasaccharide sequence contained in all anticoagulant heparin sulfate (HS) isolated from natural sources. The hydroxyl group on position 1 of the reducing end residue in fondaparinux is methylated. The structure and the structure-activity relationships are recounted in detail in Petitou's review (*14*). Briefly, the sulfate groups or carboxylate groups on position 6 of residue A in panel B of Figure 1 (C_A6), C_B5, C_C2, C_C3, C_D5, and C_E2 are essential for the activation of AT-III, whereas the sulfate groups on C_A2, C_D2, and C_E6 only help to increase the biological activity. Meanwhile, though significant anti-FXa activity remains if some carbohydrate units (*e.g.* residues A and D) are replaced with more flexible mimetics, like "open" pyranose analogues and other "open" saccharide analogues, but for others the rigidity is essential. For example, if GlcA (residue E in panel B of Figure 1) in the pentasaccharide is replaced with a flexible "open" pyranose analogue, only 2% of the anti-FXa activity remains. In addition, the extra 3-*O*-sulfate in this GlcA can interact with Arg 46 and Arg 47 in the AT-III, which enhances the interaction between the saccharide and AT-III.

2.2. *Pharmacological effects and clinical applications*

2.2.1. *Prophylaxis and treatment of acute coronary syndromes*

Fondaparinux is currently one of the drugs of choice to prevent and treat VTE and acute coronary syndromes (ACS) (*15*,*16*). Its efficiency in the treatment of patients with non-ST-segment elevation ACS (NSTE-ACS) was also proven (*12*). Compared with LMWH, fondaparinux resulted in a 17% decrease in mortality at 30 days and was associated with a 50% reduction in major bleeding

for the treatment of NSTE-ACS (*17*). In addition, in the Fifth Organization to Assess Strategies in Acute Ischemic Syndromes (OASIS-5) study, it was also demonstrated that fondaparinux was similarly efficient compared to enoxaparin in preventing ischaemic events but reduced major bleeding, mortality and morbidity in NSTE-ACS patients regardless of their risk of persistent ischemia (*17*,*18*). Based on OASIS-5 population data, it was shown that fondaparinux was a more cost-effective antithrombotic agent than enoxaparin during both short and long term NSTE-ACS treatment (*19*). Therefore, use of fondaparinux for treatment of patients with NSTE-ACS is superior to that of enoxaparin in terms of prevention of further cardiovascular events and at a lower cost (*20*). Michel and his colleagues also found that using fondaparinux instead of enoxaparin in patients with NSTE-ACS could yield substantial savings at the local as well as the national level in Switzerland (*21*). The use of fondaparinux in the post-coronary artery bypass grafting (CABG) population appears to be safe and is not associated with an increase in bleeding, transfusion or re-operation for bleeding (*22*). However, the risk of catheter thrombosis was higher with fondaparinux than with LMWH or heparin in ACS patients who underwent percutaneous coronary intervention (PCI) (*23*), and because of this problem, fondaparinux was of no benefit in patients undergoing urgent PCI (*24*).

2.2.2. *Prophylaxis and treatment of DVT*

The soluble fibrin (SF) and D-dimer tests might be affected after administration of fondaparinux, complicating the diagnosis of DVT. The D-dimer test on postoperative day 7 is useful for DVT screening in patients treated with fondaparinux. The SF on postoperative day 4, 7, and 14 and D-dimer levels on postoperative day 14 and 21 in patients treated with fondaparinux without DVT were lower than the ones in patients without fondaparinux treatment, while the D-dimer levels on postoperative day 14 and 21 in patients treated with fondaparinux with DVT were higher than that in patients without fondaparinux treatment (*25*). In the fondaparinux treated group, the frequency of DVT and the hemoglobin level were significantly lower than those in the group without fondaparinux treatment, indicating that fondaparinux is useful for the prophylaxis of DVT, but may increase bleeding (*26*).

2.2.3. *Applications in patients with a history of HIT*

Because fondaparinux did not cross-react with HIT-associated antibodies, it had a decreased risk of causing HIT syndrome as compared to enoxaparin (*27*). Savi's group designed a prospective and blinded study in which 39 sera from patients with clinically and serologically confirmed HIT and 15 control sera were collected followed by a particular HIT assay by 3 different specialized laboratories (*28*). The serotonin release assay, heparin-induced platelet agglutination assay, and platelet aggregation assay were performed in these labs independently. The results showed that fondaparinux was nonreactive to HIT sera, suggesting the possibility that this pentasaccharide could be applied to prophylaxis and treatment of thrombosis in patients with a history of HIT.

2.2.4. *Applications in orthopedic surgery patients*

In vitro studies had shown that fondaparinux did not have a negative effect on human osteoblast proliferation by comparing the effects of LMWHs (enoxaparin and dalteparin) and fondaparinux on bone metabolism (*29*,*30*). Papathanasopoulos *et al.* reported that fondaparinux had no adverse effects on either mesenchymal stem cell (MSC) proliferation or osteogenic as well as chondrogenic cell differentiation *in vitro* (*31*). Based on these characteristics, it would be more safe and efficient when fondaparinux was applied in orthopedic surgery patients.

2.2.5. *Applications in heparin allergy patients*

The anti-inflammatory effect of fondaparinux has been demonstrated in intestinal ischemia and reperfusion injury models (*32*). It seems that fondaparinux is characterized by an anti-inflammatory effect manifested by reduction of plasma monocyte chemotactic protein-1 (MCP-1) (*33*), one receptor of fondaparinux (*7*). Palmo-plantal pruritus after application of heparins was an early sign of the immediate type hypersensitivity reaction (*34*). Fondaparinux was found to be a safe alternative for immediate heparin allergy (*34*).

2.2.6. *Applications in renal dysfunction patients*

Fondaparinux is primarily metabolized through the kidney, and thus, is contraindicated in renal-impaired patients (creatinine clearance < 30 mL/min) (*27*). According to post hoc analysis (*17*), the rate of major bleeding during fondaparinux administration was the highest in patients with moderate renal impairment, aged 75 years or more, and body weight less than 50 kg (*35*). Yukizawa's group (*36*) enrolled 85 patients who received subcutaneous fondaparinux 2.5 mg after total hip arthroplasty (THA). Then, the anti-FXa activity was measured on postoperative days 1, 3, 7, and 14. The data obtained in their study indicated that anti-FXa activity levels were significantly higher in patients with renal dysfunction and a poor correlation was observed between the plasma levels of anti-FXa activity and age or body weight. Besides, the patients with normal renal function also showed an increase in anti-FXa activity with repeated administration of fondaparinux.

2.2.7. *Applications in pregnancy*

Different from LMWHs, fondaparinux sodium could cross the placenta though the mechanism of crossing is not clear (*37*). Data on the use of fondaparinux in pregnancy are limited to animal models and a few case reports. In a study of Knoll's group, it was found that fondaparinux did not cause hypersensitive skin reactions and was not associated with bleeding or other complications in the mother and child (*38*). The other several separately reported cases also did not show adverse events to the mother or child (*39-43*). The limited data show that fondaparinux appears efficacious in pregnancy, but bleeding risk is not absent, so care is required when used as a second-line therapy (*44*).

2.3. *The advantages and disadvantages of fondaparinux*

One of the main advantages of fondaparinux is that its dosage does not need to be adjusted based on age or weight, because of its pharmacokinetics, namely, its specific binding to anti-thrombin and near 100% bioavailability (*27*). Fondaparinux with subcutaneous administration has a critically high bioactivity and is mainly excreted by the kidneys with a half-life of 17-21 h (*45*). The recommended dose of fondaparinux sodium in the US and European Union (EU) is 2.5 mg once daily (QD) as a subcutaneous injection, administered postoperatively. Although fondaparinux has been safely administered in many patients, it still has some disadvantages. First, unlike UFH, fondaparinux has no antidote. This is a limitation for patients who are a risk for bleeding. Second, fondaparinux has a long half-life and accumulates in patients with renal dysfunction and even in normal renal function patients (*36*), and its subcutaneous absorption may be unpredictable in a hemodynamically unstable patient. Last, some immune mediators also affect the absorption and metabolism or the activity of fondaparinux, so the dose of fondaparinux should be adjusted when these immune mediators are pre-administered. For example, interleukin-10 (IL-10) decreases the elimination rate of fondaparinux, suggesting that pre-treatment with IL-10 may allow reducing the fondaparinux dose (*46*).

2.4. *Adverse events*

The higher prophylactic efficacy of fondaparinux is associated with a higher risk of bleeding complications (*47,48*), although fondaparinux is supposed to only minimally enhance bleeding and not affect platelet functions (*49*). Bleeding was more prominent in the fondaparinux group compared to the enoxaparin group at an equipotent dose of anti-FXa activity (*50*).

In 2013, Orostegui *et al.* reported a case of liver toxicity likely due to fondaparinux administered to a child (*51*). The mechanism of fondaparinux hepatotoxicity may not be related to drug metabolites since fondaparinux does not undergo hepatic metabolism and is recovered in the urine as the unchanged compound, and it also does not interact with other drugs administered concomitantly, under physiological conditions (*52*). It is hypothesized that inflammatory mediators may have contributed to sensitizing hepatocytes to injurious effects of an unknown compound.

3. AVE5026 (Semuloparin)

AVE5026, a novel and hemi-synthetic ULMWH, is in clinical development for the prevention of VTE (*56*). It possesses a higher anti-FXa activity with a residual anti-FIIa activity (< 5 IU/mg), and has a MWavg of 2.4 kDa (Table 1) and a unique AT-III-binding oligosaccharide ΔIIa-IIs-Is (panel C of Figure 1). It has the AT-III-binding sequence and the 4,5-unsaturated uronic acid-2-*O*-sulfated ester residue. The anti-FXa activity of this characteristic hexasaccharide was found to be 740 IU/mg and it is the major constituent of the AT-III-binding hexasaccharide fraction. AVE5026 is the saccharide fragments of a chemoselective depolymerization of the heparin macromolecules by 2-tert-butylimino-2-diethylamino-1,3-dimethyl-perhydro-1,2,3-diaza-phosphorine (BEMP). The reaction principle is a *β*-eliminative reaction. The hemi-synthetic pathway has six steps (Figure 3) (*53*).

3.1 *The dose-range study of AVE5026*

A dose-range study of AVE5026 for the prevention of VTE in patients after total knee replacement surgery was completed in 2009 (*54*). In this parallel-group, double-blind and double-dummy study, 690 patients were enrolled randomly, and 678 of them were treated with once-daily doses of AVE5026 (5, 10, 20, 40, or 60 mg) or enoxaparin 40 mg. The primary efficacy end point was VTE until post-operative day 11, defined as DVT detected by bilateral venography, symptomatic DVT, non-fatal pulmonary embolism (PE) and VTE-related death. The primary safety outcome was the incidence of major bleeding. The primary efficacy outcome was assessed in 464 patients. There was a significant dose-response across the AVE5026 groups for VTE prevention ($p < 0.0001$) and for proximal DVT ($p = 0.0002$). The incidence of VTE ranged from 5.3% to 44.1% compared to 35.8% in the enoxaparin group. Also, a significant dose-response for AVE5026 was seen for major bleeding ($p = 0.0231$) and any bleeding ($p = 0.0003$). Six patients experienced major bleeding in all the groups treated with the AVE5026 and four of them belonged to the 60 mg group. Meanwhile, none experienced major bleeding in the enoxaparin group. In addition, the risk of VTE was reduced by 58% [95% confidence interval (CI), 26-76], 61% (95%

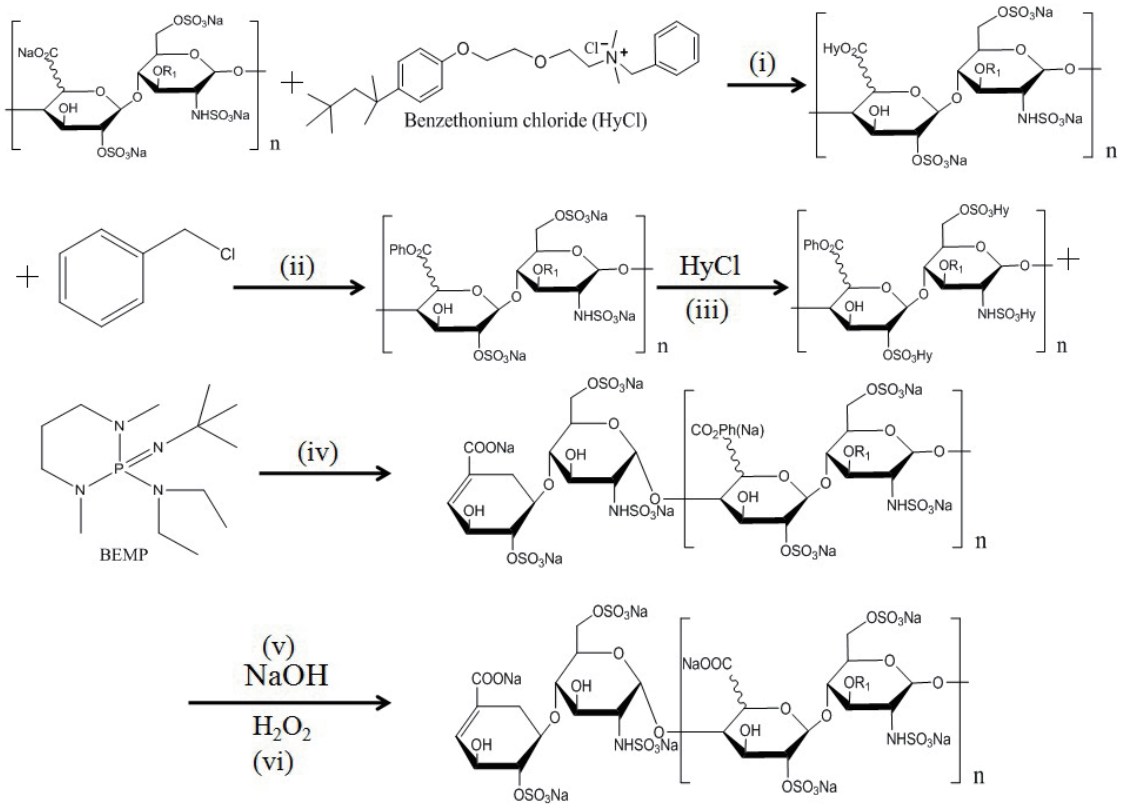

Figure 3.The hemi-synthetic pathway of AVE5026. The hemi-synthetic pathway has 6 steps: (*i*) transalification of heparin sodium with benzethonium salts; (*ii*) esterification of heparin benzethonium salts by benzyl chloride; (*iii*) transalification of the heparin benzyl ester by benzethonium salts; (*iv*) depolymerization of the heparin benzyl ester, benzethonium salts by BEMP; (*v*) saponification of benzyl esters; (*vi*) purification to obtain AVE5026.

CI, 30-79) and 85% (95% CI, 60-94) in the AVE5026 20 mg, 40 mg, and 60 mg groups, respectively. It was demonstrated that AVE5026 at high doses (≥ 20mg) was significantly more effective at reducing confirmed VTE compared to enoxaparin. The safety and efficacy results of this study suggested that a dose range of AVE5026 between 20 mg and 40 mg presented an adequate benefit-to-risk ratio.

3.2 *The phase III trials of AVE5026*

There have been 6 phase III trials during the development of AVE5026 for VTE prophylaxis. In addition, another trial, the seventh one, was initiated but terminated early in acutely ill medical patients. According to the sponsor's reports about these clinical studies, AVE5026 was successful against placebo (evaluation of AVE5026 in the prevention of VTE in cancer patients undergoing chemotherapy (SAVE-ONCO); evaluation of AVE5026 as compared to placebo for the extended prophylaxis of VTE in patients undergoing hip fracture surgery (SAVE-HIP3)). AVE5026 did not meet the primary efficacy endpoint, any VTE or any death caused by either VTE or other reasons (all-causes of death), in 3 of the 4 completed enoxaparin-controlled trials, including both superiority and non-inferiority study designs. These were conducted in patients undergoing orthopedic surgery, including the study comparing the efficacy and safety of AVE5026 with enoxaparin for the prevention of VTE in patients undergoing elective knee replacement surgery (SAVE-KNEE) and the study comparing the efficacy and safety of AVE5026 with enoxaparin for the prevention of VTE in patients undergoing hip fracture surgery (SAVE-HIP2), and the study comparing the efficacy and safety of AVE5026 with enoxaparin for the prevention of VTE in patients undergoing major abdominal surgery (SAVE-ABDO). One of the 4 enoxaparin-controlled trials (the study comparing the efficacy and safety of AVE5026 with enoxaparin for the prevention of VTE in patients undergoing elective hip replacement surgery (SAVE-HIP1)) met the primary efficacy endpoint (any VTE or all-causes of death), but did not meet the secondary efficacy endpoint (major VTE or all-causes of death). The proportion of patients was larger in the enoxaparin group (10.6%) than in the AVE5026 group (4.5%) for the safety population in this study.

3.2.1 *The study of SAVE-ONCO*

The efficacy and safety of AVE5026 for prevention of VTE in patients receiving chemotherapy for cancer was being evaluated in the study of SAVE-ONCO, a double-blind, multicenter trial (*55*). In this study, 3,212 patients who were undergoing chemotherapy for locally advanced or metastatic cancers of lung,

pancreas, stomach, colon/rectum, bladder, or ovary were enrolled. Patients were randomized 1:1 to receive either AVE5026 20 mg QD subcutaneously (SC) or placebo for a minimum of 3 months while receiving chemotherapy. In the results, the median treatment duration was 3.5 months. VTE occurred in 20 of 1,608 patients (1.2%) receiving AVE5026, as compared to 55 of 1,604 (3.4%) receiving placebo (hazard ratio (the ratio of 1.2% to 3.4%), 0.36; 95% CI, 0.21 to 0.60; $p < 0.001$), with consistent efficacy among subgroups defined according to the origin and stage of cancer and the baseline risk of VTE. The incidence of clinically relevant bleeding was 2.8% and 2.0% in the AVE5026 and placebo groups, respectively (hazard ratio, 1.40; 95% CI, 0.89 to 2.21). Major bleeding occurred in 19 of 1589 patients (1.2%) receiving AVE5026 and 18 of 1583 (1.1%) receiving placebo (hazard ratio, 1.05; 95% CI, 0.55 to 1.99). Incidences of all other adverse events were similar in the two study groups. In conclusion, this study showed that AVE5026, as compared to placebo, reduced the incidence of VTE in patients with locally advanced or metastatic cancer, with no apparent increase in major bleeding.

3.2.2 *The study of SAVE-ABDO*

Regarding other trial with AVE5026 for VTE prophylaxis in patients with cancer, in the study of SAVE-ABDO, 81% (2451) of the primary efficacy population were patients with cancer undergoing oncologic surgery. SAVE-ABDO was a randomized active-controlled trial in patients undergoing major abdominal surgery. A total of 4,413 patients were randomized 1:1 to receive either AVE5026 20 mg QD SC or enoxaparin 40 mg QD SC for a duration of 7-10 days after surgery. A US Food and Drug Administration (FDA) exploratory analysis in the subgroup of patients with cancer showed a numerically higher proportion of subjects with VTE events in the AVE5026 group compared to the enoxaparin group (7.1% *vs.* 5.9%, respectively; Odds Ratio 1.23 (0.89, 1.69)). This trial failed to meet its primary efficacy endpoint of any VTE or all-causes of death in a noninferiority comparison of AVE5026 versus enoxaparin (Odds Ratio 1.16, with the upper bound of the 95% CI (1.59) failing to meet the pre-specified noninferiority margin of 1.25).

A total of 7,616 patients have been exposed to AVE5026 across 21 clinical trials, including one phase II clinical dose-finding study, seven phase III clinical efficacy/safety studies of AVE5026 for VTE prophylaxis (6,826 patients, except for the patients in a phase III clinical study which was initiated but terminated early) and thirteen phase I clinical pharmacology studies (354 exposed to AVE5026, including 255 healthy subjects). The totality of safety data from these studies suggested that the safety profile of AVE5026, including bleeding adverse events, was similar to that of enoxaparin.

4. RO-14

RO-14, a derivative of bemiparin (*57*), developed by Laboratorios Farmaceúticos Rovi, S.A. of Spain, is obtained by selective chemical depolymerization of UFH in a non-aqueous medium. The reaction mechanism is β-elimination, as well. The anti-FXa activity of RO-14 is between 80 and 140 IU/mg, and the anti-FIIa activity is lower than or equal to 10 IU/mg (Table 1). The ratio of anti-FXa to anti-FIIa is higher than 20 (*58*). Its molecular weight is between 1.8 kDa and 3.0 kDa and the MWavg is about 2.2 kDa.

So far, only two articles were published about the research of RO-14 (except reviews and conference reports), one is about RO-14 reducing the endothelial angiogenic features elicited by leukemia, lung cancer or breast cancer cells (*59*), and the other is a phase I clinical study about RO-14 (*58*). Vignoli and his colleagues have evaluated whether RO-14 may retain the antiangiogenic properties observed with LMWH. In this study, they investigated the capacity of RO-14 to inhibit the angiogenic features of the endothelium stimulated by leukemic, breast cancer, and small cell lung cancer cells, or by standard proangiogenic factors in an *in vitro* system of interaction of cancer cells with microvascular endothelial cells. They found that RO-14 had an antiangiogenic activity, suggesting RO-14 can be applied in cancer treatment as an adjuvant drug.

The phase I clinical study was a two-stage, single-center, open-label, randomized study. Eighteen volunteers were enrolled in this study. Thirteen of the volunteers participated in one stage which assessed 6 ascending dose levels of RO-14 (1,750, 2,450, 3,500, 4,550, 5,600, 6,650 IU anti-FXa), and 12 of the volunteers participated in the other stage which assessed 6 additional strengths of RO-14 (7,700, 10,150, 12,600, 15,050, 17,500, and 19,950 IU anti-FXa). Blood samples were collected in tubes containing citrate sodium 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, 18, and 24 h after drug administration for pharmacodynamic analysis. Safety was assessed by spontaneous/elicited adverse events, medical examination and laboratory tests. In this study, all doses were well tolerated and there were no bleeding events. The anti-FXa activity at the lowest and the highest dose levels were 0.16 (± 0.02) IU/mL and 1.67 (± 0.15) IU/mL, respectively. At the highest dose levels, the $t_{1/2}$ was 8.05 h. The mean T_{max} was 2.86 (± 0.39) h at all dose levels. RO-14 showed proportional and linear pharmacodynamics. There were no clinically significant changes in the platelet count, activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), fibrinogen, and antithrombin. This phase I clinical study demonstrates that RO-14 has a high anti-FXa activity for prophylaxis or treatment of VTE and a good safety profile, linear pharmacodynamics and a long elimination half-life.

5. Conclusion

Here, we reviewed the preparation processes, pharmacological effects and clinical applications of fondaparinux, AVE5026 and RO-14. Though fondaparinux is a chemically synthesized pentasaccharide and both AVE5026 and RO-14 are obtained by degradation of UFH through a β-elimination reaction, all of them have lower molecular weight, higher anti-FXa activity and a longer half-life time than enoxaparin (Table 1). The US FDA has recently approved the generic forms of LMWHs and ULMWHs, which underscores the rapid growth in the development of heparin-based drugs. Among fondaparinux, AVE5026, and RO-14, fondaparinux was the only one that has gone on the market, and has been wildly applied in prophylaxis and treatment of DVT. Fondaparinux does not have the same contamination risks associated with animal-sourced UFH and LMWH, is subcutaneously bioavailable and has reduced risks of HIT and osteoporosis. However, fondaparinux is contraindicated in renal-impaired patients and lacks an antidote. Therefore, better ULMWHs are being developed by many groups and companies. AVE5026 and RO-14 have a high anti-FXa activity for prophylaxis or treatment of VTE according to limited data. However, Sanofi-Aventis, the development company of AVE5026, revoked the listing application of AVE5026 for prophylaxis of VTE in patients receiving chemotherapy for locally advanced or metastatic pancreatic or lung cancers or for locally advanced or metastatic solid tumors with a VTE risk score ≥ 3. The FDA advisory committee denied the application, because the absolute efficiency is low although the relatively efficiency is statistically significant. Despite this, we believe that research steps for ULMWHs will still be going on and new ULMWHs with high anti-FXa activity and low incidence of adverse events will be developed in the future.

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(Received January 10, 2014; Revised January 24, 2014; Accepted January 25, 2014)

Brief Report

DOI: 10.5582/ddt.8.11

Design, synthesis and biological evaluation of naphthalimide-based fluorescent probes for α_1-adrenergic receptors

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Summary **α_1-Adrenergic receptors (α_1-ARs), as one of the most important members of G protein-coupled receptors (GPCRs), can mediate lots of physiological responses of the sympathetic nervous system. Until now, α_1-ARs have been divided into at least three subtypes, α_{1A}, α_{1B}, and α_{1D}, which distribute in various tissues and organs. In this research, we designed and synthesized several napthalimide-based small-molecule fluorescent probes for α_1-ARs, which mainly contained two parts: the pharmacophore (quinazoline and phenylpiperazine) that binds to α_1-ARs and the fluorophore (naphthalimide) that labels the receptors with fluorescent properties. Moreover, some of these compounds demonstrated potent affinity to α_1-ARs and cell imaging potential.**

Keywords: α1-Adrenergic receptors, fluorescent probes, high affinity, cell imaging

1. Introduction

The α_1-adrenergic receptors (α_1-ARs) are one of the most important members of G protein-coupled receptors (GPCRs), distribute in varieties of organs, tissues and cells, which mediate many important physiological effects in the human body. Until now α_1-ARs have been divided into at least three subtypes, α_{1A}, α_{1B} and α_{1D}, according to the differences on their gene structure, tissue distribution and pharmacological action (*1-3*).

Studies have confirmed that α_1-ARs are closely related to various diseases, such as hypertension, benign prostatic hyperplasia, prostate cancer, and so on (*4-6*). Therefore, it is extremely useful to develop drugs for diseases' examination and clinical diagnosis. So far, researchers have designed and synthesized several α_1-ARs antagonists, including quinazoline and phenylpiperazine-based derivatives, to prevent and treat diseases related to α_1-ARs abnormally expressed (*7*). However, in the absence of their three-dimensional crystal structures, it is difficult to study the biological and pharmacological characteristics of α_1-ARs with conventional research approaches.

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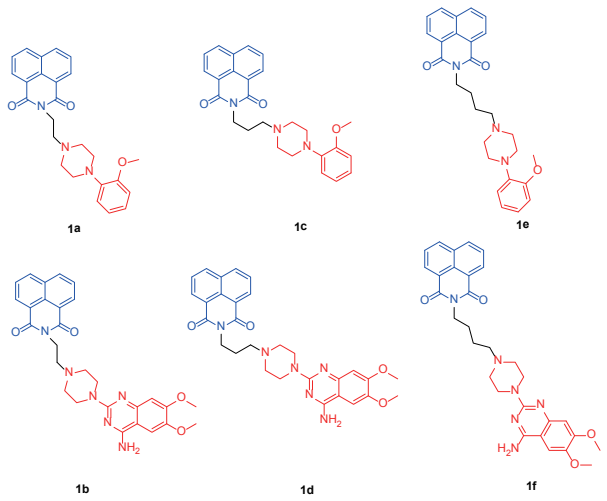
Nowadays, fluorescence analysis technology has developed rapidly in various areas and small-molecule fluorescent probes have demonstrated high sensitivity and selectivity in the detection of biomacromolecules, such as proteins, enzymes, *etc*. (*8-10*). Small-molecule fluorescent probes usually consist of two parts: the pharmacophore that could be bind to the targets, and the fluorophore that labels the targets with fluorescent properties.

Based on our previous work (*11-14*), we chose quinazoline and phenylpiperazine moiety as the pharmacophore with high affinity to α_1-ARs, and naphthalimide as the fluorophore to provide fluorescent properties. Biological evaluation confirmed that our probes demonstrated high affinity to α_1-ARs and reasonable cell fluorescence imaging potential. These interesting results indicated that these fluorescent probes could be employed as fluorescent competitive substrates in α_1-ARs ligand activity screening.

2. Materials and Methods

2.1. *Chemicals*

In summary, a series of naphthalimide derivatives were well designed and synthesized as fluorescent probes for α_1-ARs (Scheme 1). In this case, quinazolines and phenylpiperazines acted as pharmacophores, and naphthalimide as fluorophores.



Scheme 1. The fluorescent probes for α1-ARs

2.2 *Optical property*

The ideal optical property is of importance for a reasonable fluorescent probe. We firstly measured the optical properties of the synthesized fluorescent probes. The optical properties were performed on a Thermo-Fisher Varioskan microplate reader by dissolving the probes in 50 mM PBS, pH 7.4.

2.3 *Affinity to α_1-ARs*

Besides the optical properties, the affinity to the targets is also a key characteristic for fluorescent probes. Therefore, the affinity activities to three different adrenergic receptor subtypes (α_{1A}-, α_{1B}- and α_{1D}-AR) of these probes were evaluated by radio-ligand binding test, in which the phentolamine was taken as a positive control.

2.4 *Fluorescence imaging*

HEK293A cells were transfected with α_{1A}-AR (HEK293A-α_{1A}-AR cells). The cell lines were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum (Gibco) in an atmosphere of 5% CO_2 at 37°C.

The cell lines were cultured in 35 mm glass bottom culture dishes (Mat Tek) at 37 °C for 24 h. Then cells were washed with DMEM medium (without fetal bovine serum) and incubated in DMEM medium (without fetal bovine serum) containing the probes for 10 min at 37 °C. Fluorescence imaging was performed on a Zeiss Axio Observer A1.

3. Results and Discussion

The optical results demonstrated that most of the probes had reasonable optical properties (Table 1). After being excited, the probes had a large stokes shift. And as we can see in Table 2, all probes had high affinity to α_1-ARs at the nM level or even at pM level. Further more,

Table 1. The optical properties of the fluorescent probes

Compound	UV λ_{max} (nm)	λ_{ex} (nm)	λ_{em} (nm)
1a	332	333	380
1b	333	333	380
1c	332	333	380
1d	333	333	385
1e	333	333	380
1f	333	333	380

Table 2. The affinity of probes to α_1-ARs

	Ki (nM)			IC_{50} (nM)		
Compound	α_{1A}	α_{1B}	α_{1D}	α_{1A}	α_{1B}	α_{1D}
Phentolamine	0.8	7.7	11.1	1.4	19.2	18.0
1a	0.07	0.2	0.1	0.1	0.5	0.2
1b	19.4	2.3	5.1	41.5	13.5	11.6
1c	0.3	0.5	0.8	0.6	1.1	1.2
1d	5.0	5.6	6.8	10.6	33.6	15.2
1e	0.2	0.5	0.02	0.3	1.1	0.03
1f	35.8	12.3	18.7	76.6	73.9	42.1

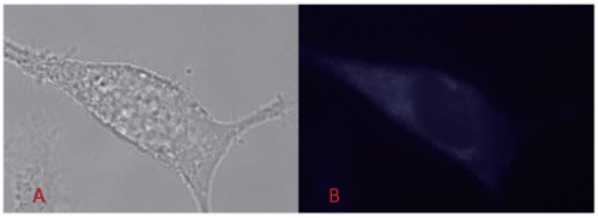


Figure 1. The fluorescence imaging results of 1d. The probe 1d (20 nM) was incubated at 37°C for 10 min. **A:** the bright field of HEK293A-α_{1A}-AR cells; **B:** the fluorescence image of 1d in HEK293A-$\alpha 1_A$-AR cells.

the phenylpiperazine-based probes had about 20-fold higher affinity to α_1-ARs than phentolamine (1a, 1c, 1e), which were more sensitive and can be a direction for developing other probes or antagonists for α_1-ARs. The fluorescent image of 1d (Figure 1B) showed that the probes could target the α_1-ARs expressed in the live cells with fluorescence properties at the nanomolar level (20 nM), which would be a direction for developing longer wavelength fluorescent probes for α_1-ARs. Moreover, these probes could be employed as competitive fluorescent substrates in α_1-ARs ligand activity screening.

4. Conclusion

The current paper describes several naphthalimide-based small-molecule fluorescent probes with high affinity for α_1-ARs. These results can provide further direction for structure-based fluorescent probes or ligands for α_1-ARs. Moreover, these fluorescent probes can help us to understand how α_1-ARs bind with their ligands and these probes can be expected to be fluorescent competitive substrates for α_1-ARs activity screening.

Acknowledgements

The present work was supported by grants from the Fok Ying Tong Education Foundation (No. 122036), the Program of New Century Excellent Talents in University (No. NCET-11-0306), the Shandong Natural Science Foundation (No. JQ201019) and the Independent Innovation Foundation of Shandong University, IIFSDU (No. 2010JQ005 and 2012JC002).

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(Received January 3, 2014; Revised January 15, 2014; Accepted January 25, 2014)

Appendix

1. Chemistry: general procedures

All materials were purchased from commercial companies (Aladdin and J&K Scientific) and used without further purification. Twice-distilled water was used throughout all experiments. Mass spectra were performed by the analytical and the mass spectrometry facilities in Drug Analysis Center at Shandong University on Agilent Technologies 1100 infinity HPLC, Applied Biosystems API4000. ^{1}H-NMR and ^{13}C-NMR were recorded on a Bruker 300 MHz NMR spectrometer.

The synthetic routes of these probes were in two different ways in Scheme 2 and Scheme 3. We got the key intermediate **c3** and **d3** through the protection of 3-bromopropan-1-amine and then got the probe **1c** and **1d** (Scheme 2). The key intermediate of probe **1a**, **1b**, **1e**, and **1f** were obtained through the Gabriel reaction (Scheme 3).

1.1. *Benzyl (3-bromopropyl)carbamate (**1**)*

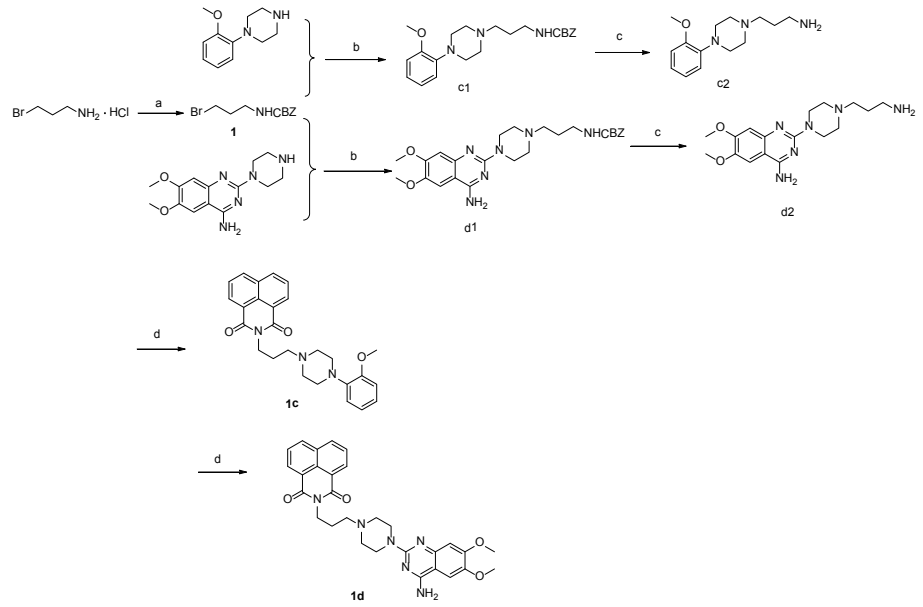
To a solution of 3-bromopropan-1-amine hydrobromide (5 g, 23 mmol) in 3N NaOH (77 mL) and $CHCl_3$ (77 mL) was added benzyl carbonochloridate (Cbz-Cl) at 0°C. Then stirred at room temperature overnight. The $CHCl_3$ layer was separated and washed with H_2O and brine, then dried with $MgSO_4$. The solvent was evaporated in vacuo and separated by column chromatography by using 10% ethyl acetate and 90% petroleum ether to get colorless liquid. ESI-MS calcd for $C_{11}H_{15}BrNO_2$ ($M + H^+$): 272.0; found: 272.3.

1.2. *Benzyl (3-(4-(2-methoxyphenyl)piperazin-1-yl) propyl)carbamate (**c1**)*

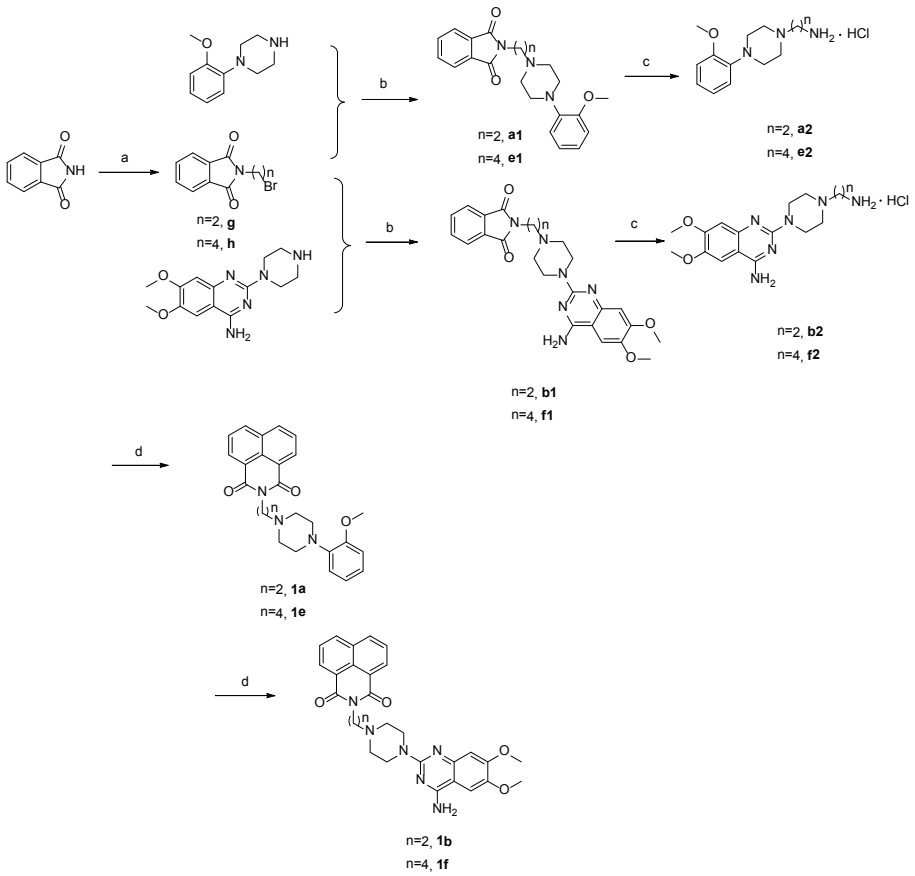
The 1-(2-methoxyphenyl)piperazine (0.83 g, 4.3 mmol), the **1** (1.43 g, 5.3 mmol) (1.2 equiv) and K_2CO_3 (1.25 g, 9 mmol) (2.1 equiv.) were heated to reflux in 25 mL CH_3CN solvent for 5 h under nitrogen atmosphere. Then the mixtures cooled down to room temperature. Then evaporated the solvent in vacuo and the residue dissolved in H_2O and extracted with ethyl acetate. The organic layer was washed by brine and dried by $MgSO_4$. Filtered and solvent evaporated in vacuo, separated by column chromatography (50% petroleum ether and 50% ethyl acetate) to give the product as yellow oil (1.52 g, 92%). ESI-MS calcd for $C_{22}H_{30}N_3O_3$ ($M + H^+$): 384.2; found: 384.4.

1.3. *Benzyl (3-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)propyl)carbamate (**d1**)*

Using the general synthetic procedure of **c1** above to give the product as a yellow solid. ESI-MS calcd for $C_{25}H_{33}N_6O_4$ ($M + H^+$): 481.3; found: 481.4.



Scheme 2. Reagents and conditions. (a) Cbz-Cl, 3 mol/L NaOH, $CHCl_3$, overnight; **(b)** K_2CO_3, CH_3CN, 80°C, 5 h; **(c)** H_2, Pd/C,30°C, overnight; **(d)** 1,8-naphthalic anhydride, CH_3CH_2OH, 85°C, 3 h.



Scheme 3. Reagents and conditions. (a) K_2CO_3, DMF, 30°C, overnight; **(b)** triethylamine, CH_3CN, 85°C, 6 h; **(c)** (*i*) hydrazine hydrate, EtOH, 85°C, 3 h, (*ii*) HCl/EtOH; **(d)** 1,8-naphathalic anhydride, EtOH, 85°C, 3 h.

1.4. *3-(4-(2-Methoxyphenyl)piperazin-1-yl)propan-1-amine (**c2**)*

The mixture of compound **c1** (0.58 g, 1.5 mmol) and Pd/C (36 mg) in 10 mL CH_3OH stirred overnight under hydrogen atmosphere. Filtered and solvent evaporated *in vacuo* to give the product as a brown oil (0.35 g, 95%). ESI-MS calcd for $C_{14}H_{24}N_3O$ ($M + H^+$): 250.2; found: 250.4.

1.5. *2-(4-(3-Aminopropyl)piperazin-1-yl)-6,7-dimethoxyquinazolin-4-amine (**d2**)*

Using the general synthetic procedure of **c2** above to give the product **d2**. ESI-MS calcd for $C_{17}H_{27}N_6O_2$ (M + H^+): 347.2; found: 347.4.

1.6. *2-(3-(4-(2-Methoxyphenyl)piperazin-1-yl)propyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1c**)*

The naphthalimide (150 mg, 0.76 mmol) and **c2** were heated to reflux in 30 mL EtOH for 3 h, and then cooled to room temperature. Solvent evaporated *in vacuo* and separated by column chromatography (40% petroleum ether and 60% ethyl acetate) to give the product as yellow solid (294 mg, 90%). m.p. 151-153°C. ESI-HRMS calcd for $C_{26}H_{28}N_3O_3$ (M + H^+): 430.2125; found: 430.2127. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.52 (d, 2H, *J* = 6.9 Hz), 8.46 (d, 2H, *J* = 7.5 Hz), 7.89 (t, 2H, *J* = 7.8Hz), 6.94-6.86 (m, 2H), 6.85 (td, 1H, 7.8 Hz, 2.4 Hz), 6.67 (d, 1H, *J* = 6.9 Hz), 4.17 (t, 2H, *J* = 7.2 Hz), 3.73 (s, 3H), 2.72 (s, 4H), 2.51-2.43 (m, 6H), 1.89-1.8 (m, 2H, *J* = 6.9 Hz). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 163.5(2C), 151.9, 141.1, 134.2(2C), 131.3, 130.6(2C), 127.4, 127.2(2C), 122.3, 122.2(2C), 120.7, 117.7, 111.8, 55.7, 55.2(2C), 52.8(2C), 49.8(2C), 24.1.

1.7. *2-(3-(4-(4-Amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)propyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1d**)*

Using the general synthetic procedure of **1c** above to give the product as yellow solid (153mg, 73%). ESI-HRMS calcd for $C_{29}H_{31}N_6O_4$ (M + H^+): 527.2401; found: 527.2400. ^{1}H-NMR (300MHz, DMSO-d6) δ: 8.53 (dd, 2H, *J* = 7.5 Hz, 0.6 Hz), 8.46 (d, 2H, *J* = 8.4 Hz), 7.9 (t, 2H, *J* = 7.5 Hz), 7.38 (s, 1H), 7.06 (s, 2H), 6.69 (s, 1H), 4.18 (t, 2H, *J* = 6.6 Hz), 3.81 (s, 3H), 3.77 (s, 3H), 3.5 (s, 4H), 2.45 (t, 2H, *J* = 6.6 Hz), 2.34 (s, 4H), 1.89 (t, 2H, *J* = 6.9 Hz). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 163.5(2C), 161.0, 158.3, 154.1, 148.8, 144.8, 134.2(2C), 131.3, 130.6(2C), 127.4, 127.2(2C), 122.2(2C), 105.1, 103.6, 102.7, 55.9, 55.8(2C), 55.3, 52.8(2C), 43.3(2C), 24.3.

1.8. *2-(2-Bromoethyl)isoindoline-1,3-dione (**g**)*

Potassium phthalimide (0.93 g, 5 mmol) was added to a solution of 1,2-dibromoethane (1.3 mL, 15 mmol) in DMF (8 mL). The mixture was stirred at room temperature overnight and evaporated the solvent in vacuo, the residue dissolved in H_2O and extracted with ethyl acetate. The organic layer was washed by brine and dried by $MgSO_4$. Filtered and solvent evaporated in vacuo, recrystallized from ethyl acetate to give white solid (566 mg, 45 %).

1.9. *2-(4-Bromobutyl)isoindoline-1,3-dione (**h**)*

Using the general synthetic procedure of **g** above to give the product **h** as a white solid (4.1 g, 85 %).

1.10. *2-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl) isoindoline-1,3-dione (**a1**)*

The compound **g** (0.36 g, 1.42 mmol), 1-(2-meth oxyphenyl)piperazine (0.35 g, 1.84 mmol) and triethylamine (990 μL, 7.1 mmol) were heated in 20 mL CH_3CN at 85°C for 6 h. The mixture was cooled to room temperature and solvent evaporated, separated by column chromatography (50% petroleum ether and 50% ethyl acetate) to give the product as yellow solid (0.35 g, 67%). ESI-MS calcd for $C_{21}H_{23}N_3O_3$ (M + H^+): 366.2; found: 366.3. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.92-7.83 (m, 4H), 6.96-6.91 (m, 2H), 6.87-6.83 (m, 2H), 3.76-3.72 (m, 5H), 2.88 (s, 4H), 2.61-2.57 (m, 6H).

1.11. *2-(4-(4-(2-Methoxyphenyl)piperazin-1-yl)butyl) isoindoline-1,3-dione (**e1**)*

Using the general synthetic procedure of **a1** above to give the product **e1** as yellow solid (1.34 g, 85 %). ESI-MS calcd for $C_{23}H_{28}N_3O_3$ (M + H^+): 394.2; found: 394.4. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.89-7.82 (m, 4H), 6.96-6.92 (m, 2H), 6.86-6.84 (m, 2H), 3.76 (s, 3H), 3.62 (t, 2H, *J* = 6.9 Hz), 2.92 (s, 4H), 2.51-2.46 (m, 4H), 2.35 (t, 2H, *J* = 6.9 Hz), 1.68-1.58 (m, 2H), 1.50-1.41 (m, 2H).

1.12. *2-(2-(4-(4-Amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)ethyl)isoindoline-1,3-dione (**b1**)*

Using the general synthetic procedure of **a1** above to give the product **b1** as yellow solid (553 mg, 60%). ESI-MS calcd for $C_{24}H_{27}N_6O_4$ (M + H^+): 463.2; found: 463.4. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.91-7.83 (m, 4H), 7.40 (s, 1H), 7.08 (s, 2H), 6.71 (s, 1H), 3.82 (s, 3H), 3.77 (s, 3H), 3.77 (t, 2H, *J* = 6.3 Hz), 3.62 (s, 4H), 2.59 (t, 2H, *J* = 6.3 Hz), 2.52-2.46 (m, 4H).

1.13. *2-(4-(4-(4-Amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)butyl)isoindoline-1,3-dione (**f1**)*

Using the general synthetic procedure of **a1** above to give the product **f1** as yellow solid (1.3 g, 88 %). ESI-MS calcd for $C_{26}H_{31}N_6O_4$ (M + H^+): 491.2; found: 491.5. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.90-7.82 (m, 4H), 7.40 (s, 1H), 7.08 (s, 2H), 6.71 (s, 1H), 3.82 (s, 3H), 3.78 (s, 3H), 3.66 (s, 4H), 3.63 (t, 2H, *J* = 6.9 Hz), 2.35-2.28 (m, 6H), 1.68-1.59 (m, 2H), 1.52-1.45 (m, 2H).

1.14. *2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethanamine hydrochloride (**a2**)*

Hydrazine hydrate (200uL, 3.3 mmol) was added to a solution of **a1** (0.3 g, 0.8 mmol) in EtOH (20 mL). The reaction mixture was stirred at 85°C for 3h. Then cooled to room temperature, filtered the precipitate

and add HCl/EtOH to the filtrate, filtered and got the crude product (161 mg, 72 %), which was used without further purification.

1.15. *4-(4-(2-Methoxyphenyl)piperazin-1-yl)butan-1-amine hydrochloride (**e2**)*

Using the general synthetic procedure of **a2** above to give the product **e2** as gray solid, which was used without further purification.

1.16. *2-(4-(2-Aminoethyl)piperazin-1-yl)-6,7-dimeth oxyquinazolin-4-amine hydrochloride (**b2**)*

Using the general synthetic procedure of **a2** above to give the product **b2** as gray solid, which was used without further purification.

1.17. *2-(4-(4-Aminobutyl)piperazin-1-yl)-6,7-dimeth oxyquinazolin-4-amine hydrochloride (**f2**)*

Using the general synthetic procedure of **a2** above to give the product **f2** as gray solid, which was used without further purification.

1.18. *2-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1a**)*

Using the general synthetic procedure of **1c** above to give the product **1a** as faint yellow solid (112 mg, 67%). ESI-HRMS calcd for $C_{25}H_{26}N_3O_3$ ($M + H^+$): 416.1969; found: 416.1972. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.54-8.47 (m, 4H), 7.92 (t, 2H, *J* = 7.5 Hz), 6.96-6.85 (m, 4H), 4.26 (t, 2H, *J* = 7.2 Hz), 3.77 (s, 3H), 2.93 (s, 3H), 2.64 (s, 6H).

1.19. *2-(4-(4-(2-Methoxyphenyl)piperazin-1-yl)butyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1e**)*

Using the general synthetic procedure of **1c** above to give the product **1e** as faint yellow solid (55mg, 16%). ESI-HRMS calcd for $C_{27}H_{30}N_3O_3$ ($M + H^+$): 444.2282; found: 444.2282. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.52-8.45 (m, 4H), 7.88 (t, 2H, *J* = 4.5 Hz), 6.96-6.84 (m, 4H), 4.1 (t, 2H, *J* = 6.9 Hz), 3.76 (s, 3H), 2.92 (s, 4H), 2.51-2.5 (m, 4H), 2.37 (s, 2H), 1.73-1.63 (m, 2H), 1.57-1.48 (m, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 163.4(2C), 151.9, 141.2, 134.3(2C), 131.3, 130.7(2C), 127.4, 127.2(2C), 122.3, 122.1(2C), 120.8, 117.8, 111.9, 57.6, 55.3(2C), 53.0(2C), 50.0(2C), 25.6, 23.9.

1.20. *2-(2-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1b**)*

Using the general synthetic procedure of **1c** above to give the product **1b** as yellow solid (54 mg, 41%). ESI-HRMS calcd for $C_{28}H_{29}N_6O_4$ ($M + H^+$): 513.2245; found: 513.2244. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 8.53-8.46 (m, 4H), 7.91 (t, 2H, *J* = 7.8 Hz), 7.4 (s, 1H), 7.09 (s, 2H), 6.72 (s, 1H), 4.26-4.2 (m, 2H), 3.83 (s, 3H), 3.78 (s, 3H), 3.67 (s, 4H), 3.37 (s, 4H), 2.64 (t, 2H, *J* = 7.2 Hz). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 163.4(2C), 161.0, 158.5, 154.1, 148.8, 144.8, 134.3(2C), 131.3, 130.7(2C), 127.4, 127.2(2C), 122.0(2C), 105.2, 103.6, 102.8, 55.7, 55.3(2C), 55.2, 53.0(2C), 43.7(2C).

1.21. *2-(4-(4-(4-Amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)butyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1f**)*

Using the general synthetic procedure of **1c** above to give the product **1f** as yellow solid (230mg, 42%). ESI-HRMS calcd for $C_{30}H_{33}N_6O_4$ ($M + H^+$): 541.2558; found: 541.2558. ^{1}H-NMR (300MHz, DMSO-d_6) δ: 8.51-8.45 (m, 4H), 7.9-7.84 (m, 2H), 7.61 (s, 3H), 4.11 (t, 2H, *J* = 6.6 Hz), 3.85 (s, 3H), 3.82 (s, 3H), 3.36 (s, 6H), 3.07-3.02 (m, 4H), 1.92-1.7 (m, 4H).

2. Affinity test

2.1. *Materials and reagents*

(1) [^{3}H] Prazosin: 1 mCi/mL, 85.3 Ci/mmol, Cat. No.# NET823025UC; Perkin-Elmer;
(2) Phentolamine: Cat. No.# P7547; Sigma-Aldrich;
(3) Human ADRA1A Receptor Membrane Preparation; Cat. No. # M00354; GenScript USA Inc.;
(4) Human ADRA1B Receptor Membrane Preparation; Cat. No. # M00355; GenScript USA Inc.;
(5) Human ADRA1D Receptor Membrane Preparation; Cat. No. # M00405; GenScript USA Inc.;
(6) DMSO: Cat. No.# 0231; Amresco;
(7) UniFilter-96 GF/C filter plates; Cat. No.#6005177; PerkinElmer;
(8) Binding buffer: 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4; filtered and stored at 4°C; Wash buffer: 50 mM Tris-HCl, pH 7.4; filtered and stored at 4°C.

2.2. *Procedures*

(1) Each well of Uni-filter 96 GF/C microplate was incubated with 100 µL 0.5% PEI (Polyethyleneimine, Sigma-Aldrich, Cat. No.# 408727, dissolved in milli-Q water) at 4°C for 30-60 min;
(2) PEI was discarded by filtration with Millipore vacuum manifold (8-15 mmHg) and plates were washed with 2 mL/well wash buffer (4-8°C);
(3) The reaction mixtures including membrane, labeled and unlabeled ligand were prepared in 24-well plates and incubated at 25°C for 2 h with a shaking speed of 500 RPM;
(4) The reaction system was transferred into the filter

plates and filtered with Millipore vacuum manifold (8-15 mmHg);
(5) The wells was washed with 3 mL/well cold wash buffer and dried at RT for 60 min;
(6) The bottom of the plates was sealed with Bottom seal™ (opaque) (Perkin-Elmer);
(7) 50 µL MicroScint 20™ (Perkin-Elmer) was added to each well;
(8) The plates were sealed with Topseal A (Perkin-Elmer) and counted on TopCount NXT for 1 min/well.

Original Article

DOI: 10.5582/ddt.8.18

Antioxidant activity of *Rafflesia kerrii* flower extract

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Summary ***Rafflesia kerrii* has been used in Thai traditional remedies for treatment of several diseases. However, scientific data particularly on biological activities of this plant is very rare. The present study explores an antioxidant activity of *R. kerrii* flower (RKF). Extracting solvent and extraction procedure were found to play an important role on the activity of RKF extract. The extract obtained from water-ethanol system showed higher antioxidant activity than that from water-propylene glycol system. Fractionated extraction using different solvents revealed that methanol fractionated extract (RM) possessed the highest antioxidant activity with Trolox equivalent antioxidant capacity (TEAC) and inhibitory concentration of 50% inhibition (IC_{50}) values of approximately 39 mM/mg and 3 µg/mL, respectively. Phytochemical assays demonstrated that RM contained extremely high quantity of phenolic content with gallic antioxidant equivalent (GAE) and quercetin equivalent (QE) values of approximately 312 mg/g and 16 mg/g, respectively. Ultraviolet-visible spectroscopy (UV-VIS) and high-pressure liquid chromatography (HPLC) indicated that gallic acid was a major component. RM which was stored at 40°C, 75% RH for 4 months showed slightly significant change ($p < 0.05$) in phytochemical content and antioxidant activity with zero order degradation. The results of this study could be concluded that *R. kerrii* flower was a promising natural source of strong antioxidant compounds.**

Keywords: *Rafflesia kerrii*, antioxidant, hydroalcoholic extract, fractionated extract, stability

1. Introduction

Rafflesia kerrii is a unique plant in family Rafflesiaceae which is the family of holoparasitic flowering plants most famous for being the world's largest single flower. As for all holoparasitic plants, this family relies upon their host plant for both water and nutrients (*1*). The plants in genus *Rafflesia* grow in a tropical rainforest area. Most species like *R. arnoldii*, *R. kerrii, R. azlanii*, and *R. hasseltii* are found in Malasia whereas *R. kerrii* is found in Thailand with a local name of "Bua Phut". *R. kerrii* is a second largest species after *R. arnoldii* (*2*). It lives as a holoparasite plant depends entirely on a host plant known as Liana due to the lack of root, stem and leaf. *R. kerrii* flower has a diameter of about 70 cm.

Phytochemical and biological properties of *R. hasseltii* which is similar to *R. kerrii* have been reported to contain alkaloids and phenolic compounds (*3*) which exhibited broad spectrum of antimicrobial activity (*4*) and accelerating the wound healing process in rats (*5*). For *R. kerrii*, the report on phytochemical and biological aspects is still rare. Kanchanopoom *et al*. (*6*) reported that *R. kerrii* was rich of tannin and phenylpropanoid glycoside. The flower of *R. kerrii* has been used by local people to restore the uterus of post-natal's women, cure infectious disease, reduce fever, and even as sexual stimulant without scientific support. Recently, some authors demonstrated the anticancer activity of *R. kerrii* extract on skin cancer cell lines (*7*). It is realized that cancer incidence is due to the oxidative stress that is resulted from an imbalance between formation and neutralization of prooxidants. Oxidative stress is initiated by free radicals like hydroxyl, peroxyl and superoxide radicals, which become stable through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. The damage caused by oxidative stress has been implicated as a potential contributor to a severe disease like cancer (*8*). Substances which

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possess antioxidant activity can prevent damage to cells caused by these free radicals and can be used for cancer prevention. In the present study, antioxidant activity of *R. kerrii* flower is explored for the first time. The effect of extracting solvent as well as extraction procedure on antioxidant activity and phytochemical content of the extracts was evaluated. The stability of the extract which possessed the highest antioxidant activity and kept in the stress condition of a high temperature and humidity was also investigated.

2. Materials and Methods

2.1. *Chemicals*

Trolox, potassium persulfate, 2,'-azinobis-(3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS), 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, quercetin and sodium carbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Organic solvents (AR grade) were from Merck (Darmstadt, Germany). Water was deionized and purified by Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were of the highest grade available.

2.2. *Plant materials*

All plant samples (Table 1) were collected as a fresh condition from local area of Southern Thailand. The samples were washed with clean water and dried under the controlled temperature of 50°C. The dried samples were then pulverized and kept in the vacuum desiccators until used.

2.3. *Preparation of hydroalcoholic extracts*

The dried plant samples were separately macerated in water-ethanol (5:95) or water-propylene glycol (PG) (1:1) solvent system for 3 days. After that, the mixtures were filtered through Whatman filter paper No. 1. The filtrates were kept at 4°C until used.

2.4. *Preparation of fractionated extracts*

The dried powder of *R. kerrii* flower (RKF) was sequentially macerated at room temperature in different polar solvents. First, it was macerated with n-hexane (48 h × 3). The residue after the third filtration was dried at room temperature for 24 h in order to remove n-hexane. After that, the dried residue was further macerated with ethyl acetate, n-butanol and methanol, respectively, in the same procedure as n-hexane. The filtrates of each solvent were collected and dried using a rotary evaporator under vacuum. The fractionated extracts of each solvent were kept at 4°C until used.

Table 1. Plant names and part used for extraction

Latin name	Common name	Part used	Abbreviation
Rafflesia kerrii	Bua Phut	Flower	RKF
Rosa damascene	Damask rose	Flower	RDF
Etlingera elatior	Torch ginger	Flower	EEF
Terminalia chebula	Cheburic myrobalan	Fruit	TCF
Terminalia bellirica	Beleric myrobalan	Fruit	TBF

2.5. *Determination of antioxidant activity*

2.5.1. *ABTS assay*

The ABTS assay was carried out according to a procedure described by Saeio *et al*. (*9*) with minor modification. Briefly, the free radical ABTS was firstly generated by reacting ABTS solution with potassium persulfate. The mixture was allowed to stand in the dark at room temperature for 12 h and diluted with absolute ethanol to obtain the absorbance of approximately 0.7 units at 750 nm. The extracts were diluted appropriately concentration with absolute ethanol. The ethanolic solution of each sample was added to ABTS free radical solution. The mixture was left to stand for 5 min at room temperature then the absorbance at 750 nm was recorded using a microtiter plate reader. Trolox was used as the calibration curve. The result was expressed as Trolox equivalent antioxidant capacity (TEAC) in millimolar concentration of Trolox which antioxidant capacity was equivalent to 1 mg of the test extract.

2.5.2. *DPPH assay*

The DPPH assay was determined using a procedure described by Okonogi *et al*. (*10*) with minor modification. Briefly, the solution of DPPH free radicals was firstly prepared by dissolving the free radicals in absolute ethanol to a concentration of 100 µM. The extracts were diluted with absolute ethanol of different concentrations. The ethanolic solution of each sample was added to DPPH solution. The mixture was left to stand for 20 min in the dark at room temperature. The amount of DPPH remaining in each period of stand was determined at 540 nm using a microtitre plate reader. Antioxidant activity was calculated as % inhibition of DPPH free radicals. The concentration of the extract that showed 50% inhibition (IC_{50}) value was recorded.

2.6. *Determination of total phenolic content*

Total phenolic content of RKF extracts was determined using Folin-Ciocalteu assay described by Sato *et al*. (*11*) with some modification. Briefly, an exact amount of the test extract was dissolved in absolute ethanol and mixed with Folin-Ciocalteu reagent. After 2 min of mixing, sodium carbonate was added. The mixture was allowed to stand for 2 h at room temperature before the absorbance was measured at 750 nm. Gallic acid was

used as the standard for the calibration curve. Total phenolic content was expressed as gallic acid equivalent (GAE) in mg of gallic acid to 1 g of the test extract.

2.7. *Determination of total flavonoid content*

Total flavonoid content of RKF extracts was determined by aluminium chloride colorimetric assay (*12*). The 100 µL sample solution was mixed with 20 µL 10% $AlCl_3$, 20 µL 1 M sodium acetate and 860 µL DI water. The mixture was allowed to stand for 30 min at room temperature before the absorbance at 415 nm was measured. Quercetin was used as the standard calibration curve. Total flavonoid content was expressed as quercetin equivalent (QE) in mg of quercetin to 1 g of the test extract.

2.8. *Ultraviolet-visible spectroscopy (UV-VIS) and high-pressure liquid chromatography (HPLC) analysis*

The most effective fractionated extract of RKF was examined under visible and UV light for proximate analysis. A solution of the test extract was prepared and filled in a cuvette with a solvent kept as blank in UV-VIS spectrophotometer (UV-2450, Shimadzu, Japan) and the maximum absorption (λ_{max}) was recorded.
The HPLC analysis was performed using an HP1100 system with a thermostatically controlled column oven and a UV detector (Hewlett-Packard, Palo Alto, CA, USA). A reversed phase column Zorbax SB-C18 (250 × 4.6 mm *i.d*., 5µm, Algilent, CA, USA) was connected with a Zorbax SB-C18 guard column (125 × 4.6 mm *i.d*., 5 µm, Algilent, CA, USA). Isocratic condition of a 4:1 volume ratio mixture of acetronotrile and 0.3% v/v o-phosphoric acid in DI water was used as a mobile phase at ambient temperature with a flow rate of 1 mL/min. An exact quantity of 20 µL of sample was injected. Samples and mobile phases were filtrated through a 0.45 µm Millipore filter, type GV (Millipore, Bedford, MA, USA) prior to HPLC injection. Gallic acid was used as a standard for identification of phenolic compounds in the extract.

2.9. *Stability test*

The stability of RKF extract which possessed the highest antioxidant activity was investigated by keeping the extract in a stress condition of 40°C and 75% relative humidity for 4 months. The physical characteristic of the extract was observed. The antioxidant activity and the total phenolic content were determined during the storage time.

2.10. *Statistical analysis*

All experiments were carried out in triplicate. The results were expressed as mean values ± S.D. To determine statistical difference between means ($p < 0.05$), ANOVA and Duncan's test were calculated using SPSS statistical software package v.10.

3. Results and Discussion

3.1. *General*

The antioxidant activity of the plant extracts is according to the constituents existing in them. The amount of such compounds existing in each plant is usually different. In this study, five different medicinal plants including *R. kerrii* were firstly investigated for their antioxidant activity. The hydroalcoholic (water-PG) extracts of these samples were used for comparison of their antioxidant power. Several methods could be used for determination of antioxidant activity. Each method is usually responded for the different mechanism of antioxidant action (*13,14*). Free radicals are a major cause of the propagation stage of oxidation process. The higher potential of free radical scavenging causes the stronger inhibition of the spreading of oxidation. Therefore, ABTS and DPPH assays were used for antioxidant activity testing in the present study because these two methods can determine the free radical scavenging activity of the test samples directly.

3.2. *Antioxidant activity of water-PG extract*

Water-PG extract of RKF in comparison with that of other four medicinal plants demonstrated that RKF extract possessed the highest antioxidant activity with the maximum TEAC value of 3 mM/mg and the minimum IC_{50} values of 0.06 mg/mL as shown in Table 2. Phytochemical study demonstrated that RKF possessed the highest total phenolic content with GAE value of 23 mg/g while the highest total flavonoid content belonged to TCF with QE value of 6 mg/g. The antioxidant power of the four other plants was previously reported (*15-21*). However, antioxidant activity of *R. kerrii* has not yet been reported elsewhere. The present work is the first study that demonstrates the antioxidant activity of *R. kerrii*. The highest antioxidant activity of RKF among the other high-antioxidant plants found in this study suggested that RKF is the potential natural source of antioxidant compounds and suitable for further investigation.

Table 2. Antioxidant activity and phytochemical content of water-PG extracts of five medicinal plants

Plant extracts	Antioxidant activity		Phytochemical content	
	TEAC (mM/mg)	IC_{50} (ug/mL)	GAE (mg/g)	QE (mg/g)
RKF	3.3 ± 0.2	57.6 ± 2.2	23.2 ± 0.6	6.1 ± 0.3
RDF	0.9 ± 0.0	330.4 ± 30.7	5.3 ± 0.1	2.3 ± 0.3
EEF	0.2 ± 0.0	1,563.8 ± 83.3	1.6 ± 0.5	1.3 ± 0.2
TCF	2.4 ± 0.2	87.3 ± 2.5	18.5 ± 1.1	5.6 ± 1.7
TBF	2.4 ± 0.2	80.6 ± 1.2	17.3 ± 0.9	6.1 ± 0.5

3.3. *Effect of extracting solvent*

Extraction of active compounds from plants is generally carried out using various types of solvents such as hexane, ethyl acetate, butanol, acetone, ethanol, methanol and water. As an alternative to the extraction processes, water-cosolvent systems have been investigated for use in the extraction of various plant compounds (*22*). Water-ethanol and water-methanol systems have been investigated for extraction of several phytochemicals from medicinal plants (*23,24*) but it is still less data for water-PG which might be due to the high viscosity of PG that could affect the diffusion of a substance. However, PG has a better benefit than ethanol when the solution form of extract has been used according to its non volatile property. In the present study, two extracting water-cosolvent systems, water-ethanol (5:95) and water-PG (1:1) systems were compared. The results found that the physical status of the RKF extracts was different. The water-ethanol extract was semi-solid while the water-PG extract was liquid state due to not feasibly complete evaporation of PG from the extract. The water-ethanol extract showed extremely higher TEAC and lower IC_{50} values than the water-PG extract as shown in Table 3, indicating its higher antioxidant activity. Phytochemical study demonstrated that the total phenolic content and the total flavonoid content of the water-ethanol extract were obviously higher than the water-PG extract. The results suggested that the phenolic and flavonoid compounds existing in the extracts played an important role on antioxidant activity of RKF. The lower antioxidant property of RKF water-PG extract was considered to be due to the poorer ability of this solvent system to dissolve the antioxidant compounds from RKF sample in addition with the dilution effect of the solvent system.

3.4. *Effect of extraction procedure and extracting solvent*

Numerous extraction methods have been investigating in order to extract the active components optimally from various plant samples. The techniques range from a simple solvent extraction to the modern methods such as supercritical fluid extraction (*25*), pressurized liquid extraction (*26*), microwave-assisted extraction (*27*), solid phase micro-extraction (*28*) and ultrasound-assisted extraction (*29*). Among these, two common and mostly used procedures are a simple extraction using only one solvent (usually 95% ethanol) and a fractionated extraction using many solvents sequenced by their polarity. The extract of the former procedure is usually called "crude extract" where that of a fractionation is called "fractionated crude extract" or "fractionated extract". As the suitable polarity to many compounds, the crude extract always contains more kinds of compounds than the fractionated extract. In fractionated extraction, only compounds of similar polarity and high dissolution to the respective extracting solvent are extracted. Therefore, the fractionated extracts are considered to be less kind of compounds than the crude extracts. Many reports found that the fractionated extracts exhibited stronger activity than the crude extracts (*30,31*). However, several authors demonstrated the synergistic effect among the major active compounds and some minor components in the extract (*32*). As extraction procedure gives different kinds of compounds in the extracts, therefore, the effect of extraction procedure on antioxidant activity of RKF was undertaken in this study. It was found that the yield and antioxidant activity of the crude extract of RKF, namely RE and the fractionated extracts, namely RH, RA, RB and RM for hexane, ethyl acetate, butanol and methanol, respectively, were different. From this result RE, RA and RB were classified as a moderated activity group whereas RH was the lowest activity extract. It was found that RM fractionated extract possessed the strongest antioxidant with the highest TEAC value of 39 mM/mg and IC_{50} value of 3 µg/mg as shown in Table 4. This result suggested that the fractionated extraction was the suitable extraction procedure for RKF. This procedure could effectively separate the potential compounds with high antioxidant activity from RKF. The results also revealed that RKF contained phenolic and flavonoid compounds. The highest quantity of total phenolic content and total flavonoid content were obtained from RM with GAE value of 312 mg/g and QE value of 16 mg/g, as shown in Table 5. The results suggested that the phenolic compounds existing in the extract played an important role on antioxidant activity of RKF.

3.5. *UV-visible spectroscopy and HPLC analysis*

RM was selected to analyze in this experiment as this extract showed the highest antioxidant activity of RKF.

Table 3. Antioxidant activity and phytochemical content of RKF extracted from different extracting solvent system

Extracting solvent system	Antioxidant activity		phytochemical content	
	TEAC (mM/mg)	IC_{50} (ug/mL)	GAE (mg/g)	QE (mg/g)
Water -ethanol	22.1 ± 0.3	5.4 ± 0.3	176.5 ± 5.2	5.3 ± 1.0
Water-PG	3.3 ± 0.2	57.6 ± 2.2	23.2 ± 0.6	6.1 ± 0.3

Table 4. Comparative antioxidant activity of RKF extracted from different extraction procedure

Extraction procedure	Yield (%)	TEAC (mM/mg)	IC_{50} (µg/mL)
Single extraction			
95% Ethanol	42.0	22.1 ± 0.3	5.4 ± 0.3
Fractionated extraction			
Hexane	0.2	2.3 ± 0.0	51.8 ± 0.2
Ethyl acetate	0.5	22.5 ± 0.9	6.3 ± 0.2
Butanol	3.9	16.4 ± 0.4	10.0 ± 0.3
Methanol	32.3	38.8 ± 0.4	3.2 ± 0.6

Table 5. Comparative phytochemical content of RKF extracted from different extraction procedure

Extraction procedure	Extracts obtained	GAE (mg/g)	QE (mg/g)
Single extraction			
95% Ethanol	RE	176.5 ± 5.2	5.3 ± 1.0
Fractionated extraction			
Hexane	RH	4.9 ± 0.7	15.8 ± 0.3
Ethyl acetate	RA	182.8 ± 2.6	8.5 ± 0.2
Butanol	RB	146.5 ± 4.9	5.7 ± 0.8
Methanol	RM	311.9 ± 12.5	16.0 ± 0.0

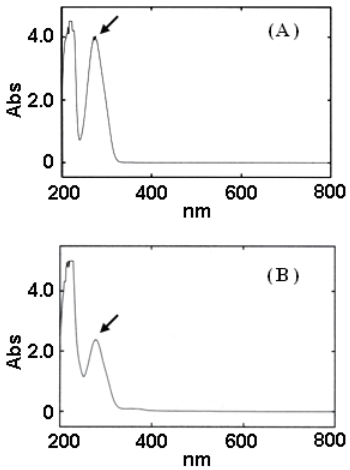

Figure 1. UV-VIS spectrum of RM (A) and gallic acid (B).

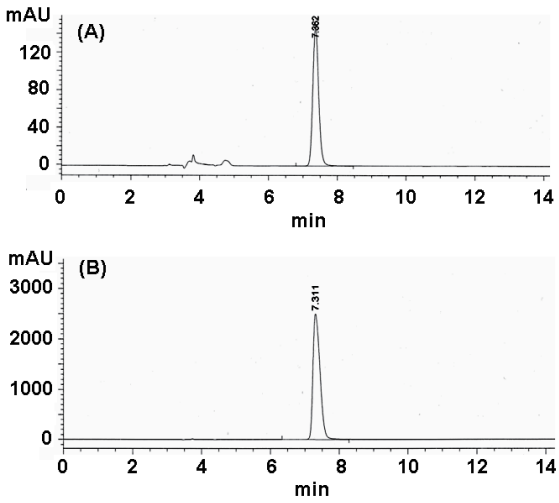

Figure 2. HPLC chromatogram of RM (A) and gallic acid (B).

The qualitative UV-VIS spectrum profile of RM was done at wavelength from 200 to 800 nm due to sharpness of the peaks and proper baseline was obtained. The UV-VIS profile of the extract showed the maximum absorption peaks at 273.2 ± 0.5, same as a standard gallic acid (274.0 ± 0.3 nm) as shown in Figure 1. This wavelength was use for UV detection in HPLC analysis. The HPLC chromatogram of RM displayed many peaks at different retention time. In comparison with a standard solution of gallic acid, the extract showed a major peak at the same retention time of gallic acid as shown in Figure 2. This result confirmed that gallic acid was one of the major active polyphenolic compounds existed in RM. Gallic acid was reported to be one of the major flavonoids possessed an antioxidant activity in grape seeds and skins (*33*). In the present study, this compound therefore was considered to play an important role on the antioxidant activity of RKF.

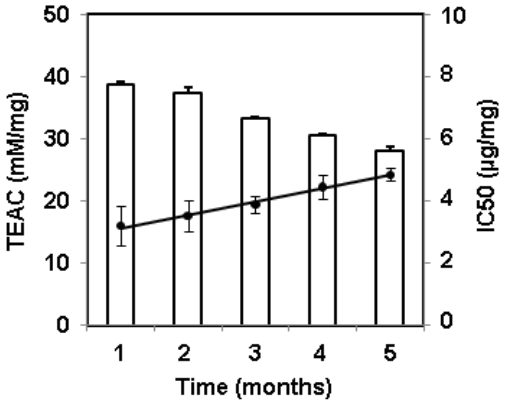

Figure 3. TEAC values (column) and IC50 values (●) of antioxidant activity of RM kept at 40°C and 75% relative humidity at various time.

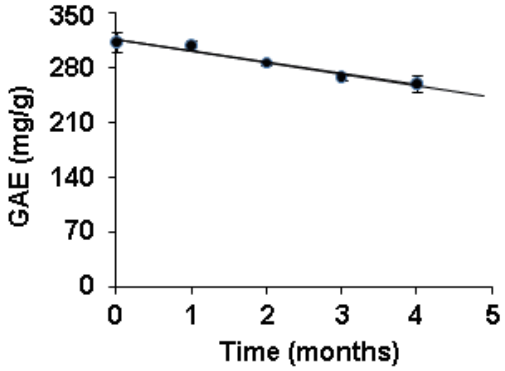

Figure 4. Total phenolic content of RM kept at 40°C and 75% relative humidity at various time.

3.6. *Stability of RM under stress condition*

Previous works observed the significant decrease in biological activities when plant extracts were subjected to a high temperature condition (*34*). In the present study, RM, the most effective antioxidant extract of RKF was subjected to a stress condition of 40°C and 75% RH for 4 months. Color change from reddish brown to slightly intense color was observed after the extract was stored for 3 months where the state of matter of the extract was still unchanged, still being as a solid mass. Antioxidant activity of RM during storage exhibited a decrease of TEAC values and an increase of IC_{50} values as shown in Figure 3, confirming the decrease of its activity. Total phenolic content of the extract depicted a decrease along the time of storage as shown in Figure 4. The decrease of these compounds was found to be a linear relationship with the antioxidant activity as shown

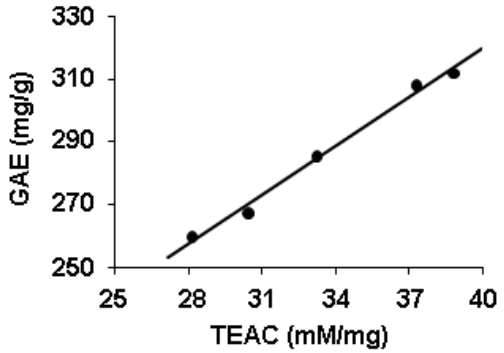

Figure 5. Relationship of total phenolic content (GAE value) and antioxidant activity (TEAC value) of RM.

in Figure 5. Therefore, the decrease of antioxidant activity of RM was considered to be due to the depletion of these active phytochemical content existing in the extract. Zero order kinetic equation (*d[A] = kdt*) was found to fit to the degradation of the existing total phenolic content in RM with the correlation coefficient (R^2) of 0.9582. According to this kinetic model, the rate constant of these phenolic compounds calculated from GAE values was 14.5 mg/month or approximately 50 µg/h. In comparison with the single antioxidant compound such as quercetin (*35*), catechins (*36*) and anthocyanin (*37*), RM seemed to exhibit higher stability than those compounds. This was considered to be due to some minor components existing in RM that might act as the desirable stabilizer for the antioxidant compounds.

Acknowledgements

The authors are grateful the TRF-MAG Grant from the Thailand Research Fund (TRF) for the financial support and the Graduate School, Chiang Mai University for their support.

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(Received December 25, 2013; Revised January 28, 2014; Accepted February 1, 2014)

Original Article

DOI: 10.5582/ddt.8.25

The comparative study of acetyl-11-keto-beta-boswellic acid (AKBA) and aspirin in the prevention of intestinal adenomatous polyposis in *APC*$^{Min/+}$ mice

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Summary **Acetyl-11-keto-beta-BA (AKBA), a component of the gum resin of *Boswellia serrata*, has been recognized as a promising agent for the prevention of intestinal tumorigenesis. Aspirin, a non-steroidal anti-inflammatory drug (NSAID), has also been considered to have the activity against intestinal tumorigenesis. However, the prevention of colonic cancer is insufficient and no definitive recommendation has been made for clinic use. Herein, we compared the efficacy of AKBA with that of aspirin in an adenomatous polyposis coli intestinal neoplasia (*APC*$^{Min/+}$) mouse model. *APC*$^{Min/+}$ mice were administered AKBA or aspirin orally for 7 consecutive weeks. Mice were sacrificed by anesthetizing. The whole intestine was removed from each mouse. The number, size and histopathology of intestinal adenomatous polyps were examined under microscopy. The adenomatous polyps were removed for further analysis by the assays of western blotting and immunohistochemical staining. AKBA significantly prevented the formation of intestinal adenomatous polyps without toxicity to mice. Statistical analysis indicated that AKBA's activity both in the prevention of small intestinal and colonic polyps was more potently than aspirin. Histopathologic examination revealed that AKBA's effect, that is the reduction of polyp size and degree of dysplasia, was more prominent in larger sized polyps, especially those originating in colon. These effects of AKBA were associated with its role in the induction of apoptosis in carcinomas. The assays of western blotting and immunohistochemistry staining indicated that the efficacy of AKBA might arise from its activity in the modulation of the Wnt/β-catenin pathway and NF-κB/COX-2 pathway in adenomatous polyps. Conclusion, AKBA by oral application prevented intestinal tumorigenesis more potential than aspirin.**

Keywords: Acetyl-11-keto-beta-boswellic acid, aspirin, *APC*$^{Min/+}$ mouse model, intestinal adenomatous polyps, Wnt/β-catenin pathway, apoptosis

1. Introduction

Colorectal cancer (CRC) is the most common malignancy worldwide. CRC has been considered to develop through a gradual series of histological changes of adenomatous polyps, called 'adenoma-carcinoma' sequence. Intestinal adenomatous polyps are thus believed to be major precursors of CRC. Epidemiologic studies indicate that almost half of the population will develop at least one benign adenomatous colonic polyp during life, with 3% of those cases going on to develop colorectal cancers (*1*). Further studies indicated that the mutations of adenomatous polyposis coli (*APC*) gene are responsible for the development of colorectal cancer in all familial adenomatous polyposis (FAP) cases and in 59% of sporadic colorectal cancer cases (*2*). Mutation of *APC* gene will cause the dysregulation of the Wnt/β-catenin

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signaling pathway in which accumulate high levels of β-catenin in nucleus and then lead to the transcription of many cell proliferation-related genes (*3*). Currently, a combination of surgery and chemotherapy is used to treat colon cancers. The 5-year survival expectation for CRC patients ranges from 93% for early stages to 8% in fully advanced stages. Unfortunately, only about 37% of patients are diagnosed at early stage (*4*). Thus, the chemoprevention regimen seems to be an attractive option for early stage of CRC.

Several drugs have been studied in this field. There is considerable evidence that nonsteroidal antiinflammatory drugs (NSAIDs), particularly aspirin, could reduce the risk of intestinal adenomatous polyps (*5*). The epidemiologic reports determined the anti-carcinogenic effects of aspirin in high-risk population of CRC (*6*). Clinical trials in patients with familial adenomatous polyposis showed that aspirin might reduce the risk of CRC through regression of intestinal adenomatous polyps (*7*). These studies suggested that aspirin might prevent adenocarcinoma growth through the mechanism of anti-inflammatory activity, e.g. the activities of COX-2 and 5-LOX (*8*). However, long-term use of aspirin is not free from toxicities. Some studies indicated that the risk-benefit profiles of aspirin for prevention of CRC are insufficient and no definitive recommendations can be made (*9*).

Boswellic acids (BAs) were a mixture of pentacyclic triterpene acids which isolated from the gum resin of *Boswellia serrata* (*10*). BAs have been used as anti-inflammation agents for the treatment of many diseases, such as chronic colitis, ulcerative colitis and osteoarthritis (*11*). Acetyl-11-keto-beta-BA (AKBA) is one of these BAs which have been considered to be most effective among these BAs. In our previous study, we found that AKBA by p.o. gavage prevented the formation of intestinal adenomatous polyposis in $APC^{Min/+}$ mice. AKBA might exert its chemopreventive action through multiple mechanisms including anti-proliferation, apoptosis induction, anti-inflammation and anti-angiogenesis (*12*). In this study, we compared these effects of AKBA with that of aspirin in the prevention of intestinal adenomatous polyposis in $APC^{Min/+}$ mice.

2. Materials and Methods

2.1. *Drugs*

AKBA was purchased from Sigma-Aldrich (A9855). The purity of AKBA was 99.5%. Aspirin (A5376) was also purchased from Sigma-Aldrich with ≥ 99.0% purity. AKBA and aspirin were both suspended in 0.5% sodium carboxymethyl cellulose (CMC) in sterile water before application.

2.2. $APC^{Min/+}$ *mouse model and drug application*

$APC^{Min/+}$ male mice were obtained from Jackson Laboratories, USA. In our group, $APC^{Min/+}$ male mice were crossed with wild type C57BL/6 female mice to generate $APC^{Min/+}$ mice (*12*). A total of 18 male mice (age, 4 weeks) were randomly divided into three groups. After one week of acclimation, the mice were administrated with the vehicle control (0.5% CMC, w/v), AKBA 80 mg/kg and aspirin 80 mg/kg. Drugs and vehicle were given by *p.o*. gavage daily (0.2 mL/10 g body weight) for 7 consecutive weeks. Mice were weighed weekly and observed daily for any signs of toxicity. This research protocol was approved by the Animal Care and Use Committee at Shandong University.

2.3. *Quantification of macroscopic and microscopic intestinal adenomatous polyp*

Mice were sacrificed by anesthetizing. After sacrifice, the whole intestine including small intestine and colon were removed from each mouse. The intestines were spread onto filter paper, opened longitudinally with fine scissors, and washed with saline. Adenomatous polyps on the intestines were counted, and their sizes were measured under a dissecting microscope. After counting, the intestines were fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin for further examination.

2.4. *Immunohistochemistry staining assay*

Immunohistochemistry staining assay was performed to analyze the expressions of β-catenin, cyclin D1, cyclooxygenase-2 (COX-2), and 5-LOX in the adenomatous polyps. Paraffin-embedded sections (4 μm thick) were cut from formalin-fixed intestinal adenomatous polyps. After deparaffinization, the immunohistochemistry staining assay was carried out as described previously (*12,13*). The primary antibodies included β-catenin (9562), COX-2 (4842), cyclin D1 (2922, Cell Signaling), 5-LOX (bs-0526R, Bioss, China). Second antibodies included anti-mouse IgG and anti-rabbit IgG (Santa Cruz).

The effects of AKBA or aspirin on the growth of adenomatous polyps were estimated by determining the levels of these proteins in adenomatous polyps. Positive staining with cyclin D1 was defined as brown staining in nuclei of adenomatous cells. The percentage of positive cells was calculated as described previously (*14*). Positive staining with COX-2 and 5-LOX was determined based on brown intensity in membrane, cytoplasm and nucleus of adenomatous cells (*15*). β-Catenin is a dynamic protein enriched in the membrane of normal cells and enters the nucleus and cytoplasm of adenomatous cells (*16*). We determined the brown intensity of β-catenin in the cytoplasm and nucleus of adenomatous cells. The levels of these

proteins were quantified by scoring the intensity as 0 (no staining), +1 (very weak), +2 (weak), +3 (moderate), and +4 (strong) in five randomly selected fields at 400× magnification in each sample (*12*).

2.5. *TUNEL staining assay*

The apoptotic cells in intestinal adenomatous polyps were determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using an *in situ* cell death detection kit (Roche, Germany). Serial 4-μm sections of intestinal adenomatous polyps were prepared and the staining was performed according to manufacturer's instructions. Adenomatous cells with brown-stained nuclei were recognized as TUNEL positive (**12**). The proportion of apoptotic cells in 6 mice was scored in randomly chosen fields under a microscope.

2.6. *Western blotting assay*

Western blotting assay was performed to determine the expressions of proteins-related to tumorigenesis in intestinal adenomatous polyps. Adenomatous polyps were incubated with 50 μL RIPA lysis buffer at 4°C for 30 min and then the lysates (30 μg of protein per lane) were fractionated by SDS-PAGE. The proteins were electro-transferred onto PVDF membranes and detected using the primary antibodies as described previously (*17*). The primary antibodies included those specific for NF-kB (sc-8008), p-NF-kB Ser^{536} (sc-33020), TOPO IIβ (sc-25330, Santa Cruz), caspase-3 (9662), caspase-9 (9502), β-catenin (9562), COX-2 (4842), cyclin D1 (2922), p-c-Jun (3270), survivin (2808), cleaved PARP (9541), PCNA (2586), β-actin (5779-1, Cell Signaling), Wnt-2 (3169-1, Epitomics) and c-Myc (ab32072, Abcam). The PVDF membranes were washed in 0.05% Tween-20/TBS and then incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were visualized using an enhanced chemiluminescence reagent (Millipore) and quantified by densitometry using ChemiDoc XRS+ image analyzer (Bio-Rad). Densitometric analyses of bands were adjusted with β-actin as loading control.

2.7. *Statistical analysis*

Data were described as mean ± S.D. Statistical analysis was done with SPSS/Win13.0 software (SPSS, Inc., Chicago, Illinois). Comparisons between $APC^{Min/+}$ control and AKBA-treated mice were conducted by two-tailed Student's *t* test. Comparison multiple groups were analyzed by one-way ANOVA and multiple between-group comparisons were performed using the S-N-K method. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. *General observation*

During the course of study, AKBA and aspirin were well tolerated by mice. There was no significant difference in body weight, peripheral blood element counts and other biological signs between AKBA or aspirin -treated and the vehicle control mice (data not shown)..

3.2. *Prevention of intestinal adenomatous polyp formation by AKBA or aspirin*

At age of 12 weeks, the number of adenomatous polyps in small intestine and colon of control mice were 29.0 ± 2.9 and 5.8 ± 1.0, respectively. Both AKBA and aspirin were found to have the activity against adenomatous polyp formation, whereas, AKBA's activity was more potently than aspirin. As shown in Figures 1A and 1B, treatment with AKBA resulted in the prevention of intestinal polyps by 45.7% and 78.7% ($p < 0.01$ *vs*. the vehicle control), respectively, in small intestines and colons. Aspirin prevented intestinal polyps by 27.6% ($p < 0.05$ *vs*. the vehicle control) in small intestines and 20.4% ($p < 0.05$ *vs*. the vehicle control) in colons. Statistical analysis indicated a significant difference between AKBA and aspirin ($p < 0.05$, Figures 1A and 1B).

The different efficacy of AKBA and aspirin was also observed by measuring the size of these adenomatous polyps. We divided the intestinal adenomatous polyps into four groups by small (< 1 mm), medium (1-2 mm), large (2-3 mm), and super large polyps (> 3 mm). In

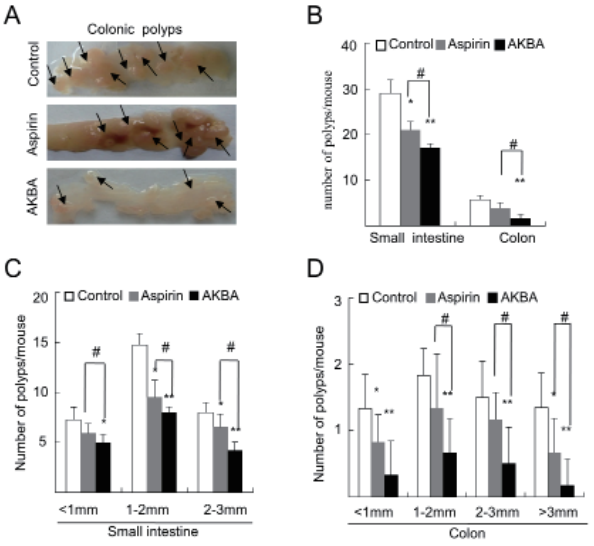

Figure 1. AKBA and aspirin prevented spontaneous intestinal polyposis in $APC^{Min/+}$ mice. (A): representative images of colon polyps; **(B)**: the number of polyps per mouse in small intestine and colon; **(C)**: the size distribution in small intestine. **(D)**: the size distribution in small colon; *, $p < 0.05$; **, $p < 0.01$ versus the vehicle control; #, $p < 0.05$; ##, $p < 0.01$ versus aspirin. The bars represent mean ± S.D. of six mice.

this study, we did not find super large polyps in small intestines. As shown in Figure 1C, the number of small, medium, and large polyps on small intestines were significantly prevented by 31.0% ($p < 0.05$ *vs*. the vehicle control), 45.8% ($p < 0.01$ *vs*. the vehicle control), and 52.5% ($p < 0.01$ *vs*. the vehicle control), respectively, in the AKBA-treated mice; by 15.2% ($p > 0.05$ *vs*. the vehicle control), 35.6% ($p < 0.01$ *vs*. the vehicle control), and 18.7% ($p < 0.05$ *vs*. the vehicle control), respectively, in the aspirin-treated mice. In colons, AKBA strongly reduced the number of small, medium, large polyps and super large polyps by 78.7% ($p < 0.01$ *vs*. the vehicle control), 65.7% ($p < 0.01$ *vs*. the vehicle control), 76.8% ($p < 0.01$ *vs*. the vehicle control), and 84.5% ($p < 0.01$ *vs*. the vehicle control), respectively; by 38.5% ($p > 0.05$ *vs*. the vehicle control), 28.6% ($p > 0.05$ *vs*. the vehicle control), 22.2% ($p > 0.05$ *vs*. the vehicle control), 45.3% ($p < 0.05$ *vs*. the vehicle control), respectively, by aspirin (Figure 1 D). A significant difference existed between these two groups of data from AKBA- and aspirin-treated mice ($p < 0.05$).

3.3. *AKBA exhibited more potential than aspirin in prevention of tumorigenesis and malignant progression of intestinal polyps*

We examined the intestinal polyps by histopathologic analysis. Both AKBA and aspirin possessed the activity against tumorigenesis and malignant progression of intestinal polyps. In $APC^{Min/+}$ mice, small intestinal polyps were determined to be adenomas, showing the crowded pencil-shaped hyperchromatic nuclei with preserved polarity and diminished mucin (Figure 2A). In colonic polyps, larger polyps were determined to be advanced adenomas, showing focal high grade dysplasia and intramucosal carcinomas demonstrated marked nuclear pleopmorphism with lack of nuclear polarity (Figure 2B). The number of dysplastic cells and degree of dysplasia in each polyp was obviously reduced in the AKBA-treated mice (Figure 2C). High grade dysplasia or intramucosal carcinoma was not found in the AKBA-treated polyps. The interdigitated normal appearing intestinal villi were observed in these intestinal polyps (Figure 2C). AKBA' effect, *i.e*. the decrease of polyp size and degree of dysplasia, was more prominent in the colonic polyps (Figure 2D). Aspirin also showed the effect of prevention tumorigenesis in small intestinal polyps and colonic polyps, whereas, its effect was not as well as AKBA in the prevention of intestinal tumorigenesis and malignant progression (Figures 2E and 2F).

3.4. *AKBA had greater effect than aspirin in induction of apoptosis in intestinal adenocarcinomas*

Examination of intestinal polyps by TUNEL staining assay indicated the apoptotic effect of AKBA and aspirin

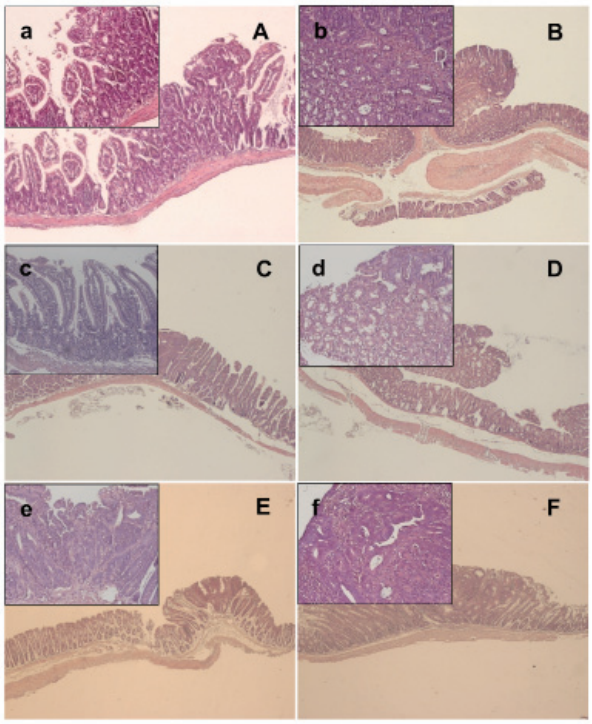

Figure 2. The representative figures of the H&E-stained intestinal sections from control mice, AKBA- or aspirin-treated mice. (A): The crater-shaped adenomatous polyps in small intestine of control $APC^{Min/+}$ mice (×100). a' (inset): adenomatous epithelium shows the enlarged, hyperchromatic, elongated and crowded dysplastic nuclei (×200). **(B)**: The adenomatous polyps with focal high grade dysplasia in colon of control $APC^{Min/+}$ mice (×100). **b'** (inset): the crypt architecture shows complex and the nuclei are pleomorphic with frequent mitoses. A lack of nuclear polarization was observed in the glands (×200). **(C)**: The small intestine in the AKBA-treated mice (×100). **c'** (inset): Crypt architecture shows mostly normal with unremarkable epithelial nuclei (×200). **(D)**: The colon in the AKBA-treated mice (×100). **d'** (inset): The structure and nuclei of epithelium shows mostly normal (×200). **(E)**: The small intestine in the aspirin-treated mice (×100). **e'** (inset): part of crypt architectures were in their normal shape with unremarkable nuclei (×200). **(F)**: Colon in the aspirin-treated mice (×100). **f'** (inset): some of the structure and nuclei of epithelium were in normal shape, whereas not as well as in the AKBA-treated colons (×200).

on adenocarcinomas. As shown in Figure 3A, AKBA strongly increased the number of TUNEL-positive cells by 269.7% ($p < 0.01$ *vs*. untreated control) in small intestinal polyps and 252.5% ($p < 0.01$ *vs*. the vehicle control) in colonic polyps. Aspirin increased TUNEL staining cells by 56.6% ($p < 0.05$ *vs*. the vehicle control), in small intestinal polyps, and by 54.2% ($p < 0.05$ *vs*. the vehicle control), in colonic polyps. A significant difference existed between AKBA and aspirin ($p < 0.05$).

Further analysis by western blotting assay suggested that these effects by AKBA might arise from its role of modulating the apoptotic-related proteins. As shown in Figure 3B, the levels of Topo IIIβ and PCNA in the AKBA-treated adenomatous polyps were significantly reduced by 54.7% ($p < 0.01$ *vs*. the vehicle control), 68.7% ($p < 0.01$ *vs*. vehicle control), respectively, in small intestinal polyps; by 72.7% ($p < 0.01$ *vs*. the vehicle control), 55.3% ($p < 0.01$ *vs*. the vehicle control),

respectively, in colonic polyps. Aspirin reduced the level of Topo IIβ and PCNA by 41.6% ($p < 0.05$ *vs*. the vehicle control), 40.8% ($p < 0.01$ *vs*. the vehicle control), respectively, in small intestinal polyps and by 55.0% ($p < 0.01$ *vs*. the vehicle control), 30.1% ($p < 0.05$ *vs*. the vehicle control), respectively, in colonic polyps. Analysis of caspase-9, caspase-3, and cleaved PARP in the adenomatous polyps confirmed the AKBA's activity. As shown in Figure 3C, the levels of caspase-9, caspase-3, and survivin in the AKBA-treated polyps were significantly reduced by 65.4% ($p < 0.01$ *vs*. the vehicle control), 41.2% ($p < 0.01$ *vs*. the vehicle control) and 40.4% ($p < 0.01$ *vs*. the vehicle control), respectively, in small intestinal polyps; and by 67.4% ($p < 0.01$ *vs*. the vehicle control), 53.3% ($p < 0.01$ *vs*. the vehicle control) and 28.3% ($p < 0.05$ *vs*. the vehicle control), respectively, in colonic polyps. The level of cleaved PARP were strongly increased by 63.8% ($p < 0.01$ *vs*. the vehicle control), 103.1% ($p < 0.01$ *vs*. the vehicle control), respectively, in small intestinal polyps and colonic polyps. In the aspirin-treated mice, the levels of caspase-9, caspase-3 and survivin were reduced by 20.1% ($p > 0.05$ *vs*. the vehicle control), 29.5% ($p < 0.05$ *vs*. the vehicle control) and 36.0% ($p < 0.01$ *vs*. the vehicle control), respectively, in small intestinal polyps; and by 32.7% ($p < 0.05$ *vs*. the vehicle control), 19.7% ($p > 0.05$ *vs*. the vehicle control), and 15.4 % ($p > 0.05$ *vs*. the vehicle control), respectively, in colonic polyps. The level of cleaved PARP were increased by 25.4% ($p < 0.05$ *vs*. the vehicle control), 56.9% ($p < 0.01$ *vs*. vehicle control), respectively, in small intestinal polyps and colonic polyps.

3.5. *Modulation of the Wnt/β-catenin signaling pathway by AKBA*

The expression levels of proteins in the Wnt/β-catenin signaling pathway were determined by the assays of immunochemical staining and western blotting. AKBA possessed the activity of modulating the Wnt/β-catenin pathway, whereas, aspirin did not significantly affect these proteins in this signaling pathway. In the AKBA-treated polyps, the level of β-catenin was strongly reduced by 49.0% ($p < 0.01$ *vs*. the vehicle control) in small intestinal polyps, by 46.8% ($p < 0.01$ *vs*. the vehicle control) in colonic polyps (Figure 4A). The

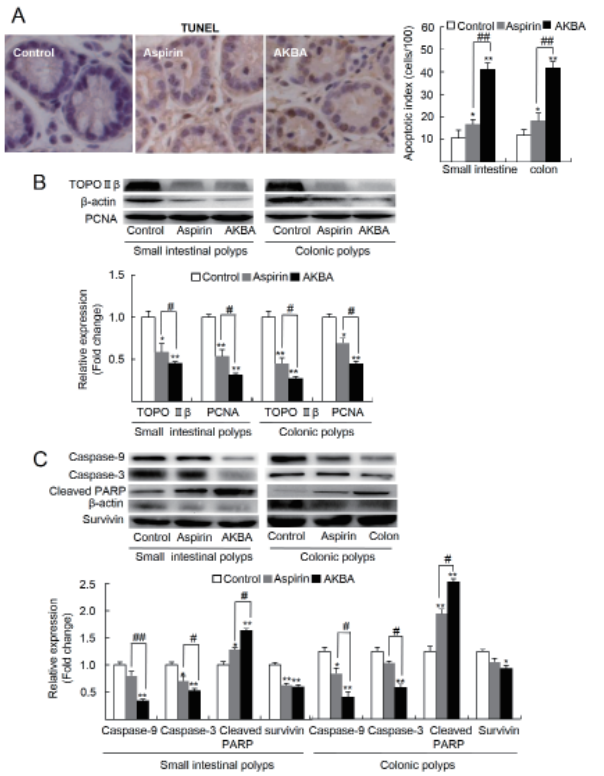

Figure 3. AKBA and aspirin induced apoptosis of intestinal polyps. (A): AKBA and aspirin increased TUNEL-positive cells in intestinal polyps as estimated by immunohistochemical staining assay (×400). The bars represent mean ± S.D. of six mice. **(B)** and **(C)**: AKBA and aspirin modulated the expression of apoptotic related proteins in intestinal polyps as estimated by western blotting assay. Experiments were performed in triplicate separately. *, $p < 0.05$; **, $p < 0.01$ versus the vehicle control; #, $p < 0.05$; ##, $p < 0.01$ versus aspirin.

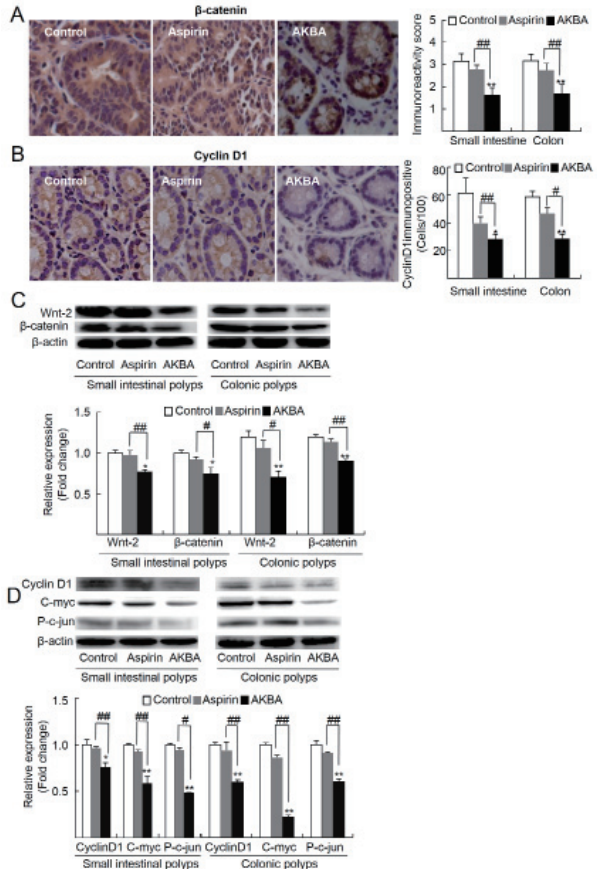

Figure 4. AKBA modulated the Wnt/β-catenin pathway and its targets. (A) and **(B)**: The expressions of β-catenin and cyclin D1 in epithelia of intestinal polyps were examined by immunohistochemical assay (×400). The bars represent mean ± S.D. of six mice. **(C)** and **(D)**: The expressions of Wnt-2, β-catenin and targets cyclin D1, c-Myc and p-c-jun in intestinal polyps were determined by western blotting assay. Experiments were performed in triplicate separately. *, $p < 0.05$; **, $p < 0.01$ versus the vehicle control; #, $p < 0.05$; ##, $p < 0.01$ versus aspirin.

level of cyclin D1 was significantly reduced by 54.9% ($p < 0.05$ *vs*. the vehicle control) in small intestinal polyps, and by 50.6% ($p < 0.01$ *vs*. the vehicle control) in colonic polyps, respectively (Figure 4B).

Western blotting assay further determined the levels of proteins in the Wnt/β-catenin signaling pathway and its downstream targets. In the AKBA-treated adenomatous polyps, the levels of Wnt-2 and β-catenin were significantly reduced by 33.1% ($p < 0.05$ *vs*. the vehicle control) and 35.7% ($p < 0.05$ *vs*. the vehicle control), respectively, in small intestinal polyps, and 41.2% ($p < 0.01$ *vs*. the vehicle control) and 34.4% ($p < 0.01$ *vs*. the vehicle control), respectively, in colonic polyps (Figure 4C). Figure 4D showed the levels of the downstream targets of the Wnt/β-catenin pathway. In the AKBA-treated adenomatous polyps, the levels of cyclin D1, c-Myc and p-c-jun were significantly decreased by 34.4% ($p < 0.05$ *vs*. the vehicle control), 41.8% ($p < 0.01$ *vs*. the vehicle control), 53.3% ($p < 0.01$ *vs*. the vehicle control), respectively, in small intestine polyps; by 40.5 % ($p < 0.01$ *vs*. the vehicle control), 78.0% ($p < 0.01$ *vs*. the vehicle control), and 49.8% ($p < 0.01$ *vs*. the vehicle control), respectively, in colonic polyps. The levels of these proteins did not show significant changes in the aspirin-treated adenomatous polyps both in small intestines and colons ($p > 0.05$ *vs*. the vehicle control).

3.6. *AKBA had a great effect on suppression of inflammatory factors*

We evaluated the inhibitory effect of AKBA in the suppression of inflammatory cytokines in adenomatous polyps. Immunochemical staining showed that both AKBA and aspirin possessed the activity of inhibition on COX-2, and 5-LOX expression (Figures 5A and 5B). We compared the density of immunochemical staining of COX-2 and 5-LOX in these adenomatous polyps. A greater inhibitory effect of AKBA on these inflammatory cytokines was observed as compared to that of aspirin ($p < 0.05$). Similar profiles of the inhibitory effect on these cytokines were also determined in the AKBA- and aspirin-treated adenomatous polyps as evaluated by western blotting assay (Figure 5C). As shown in Figure 5D, the levels of p-NF-κBSer^{536} and NF-κB in the adenomatous polyps by AKBA were significantly reduced by 51.6% ($p < 0.01$ *vs*. the vehicle control), 42.5% ($p < 0.01$ *vs*. the vehicle control), respectively, in small intestines; by 74.4% ($p < 0.01$ *vs*. the vehicle control), 52.3% ($p < 0.01$ *vs*. the vehicle control), in colons. The levels of p-NF-κBSer^{536} and NF-κB in the aspirin-treated adenomatous polyps were reduced by 31.4% ($p < 0.05$ *vs*. the vehicle control), 22.2% ($p < 0.05$ *vs*. the vehicle control), respectively, in small intestines and 57.2% ($p < 0.01$ *vs*. the vehicle control), 42.6% ($p < 0.01$ *vs*. the vehicle control), respectively, in colons. These results indicated that AKBA possessed

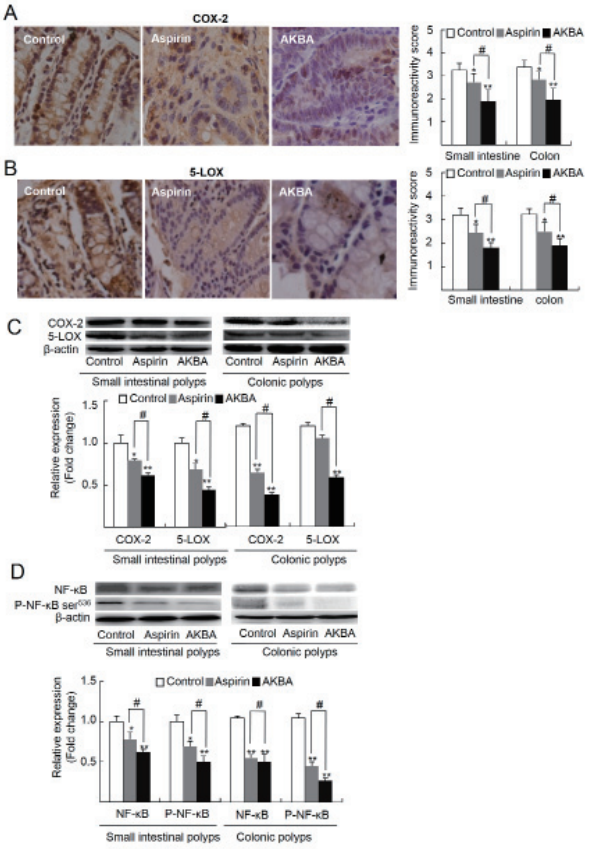

Figure 5. AKBA and aspirin suppressed inflammatory mediators in intestinal polyps. (A) and **(B)**: Immunohistochemical staining indicated the decrease of COX-2 **(A)** and 5-LOX **(B)** expressions in intestinal polyps (×400). The bars represent mean ± S.D. of six mice. **(C)** and **(D)**: Western blotting showed the decrease of COX-2, 5-LOX, p-NF-κB ser^{536} and NF-κB in the intestine polyps of AKBA- or aspirin-treated mice. Experiments were performed in triplicate separately.*, $p < 0.05$; **, $p < 0.01$ versus the vehicle control; #, $p < 0.05$; ##, $p < 0.01$ versus aspirin.

greater activity than aspirin in the suppression these inflammatory stimuli in intestinal adenomatous polyps.

4. Discussion

AKBA, a naturally occurring, orally active pentacyclic triterpenoid extracted from *boswellia serrata*, was initially determined for its anti-inflammatory properties and used for the treatment of many chronic inflammation diseases (*18*). Recent studies have highlighted its role in anti-cancer properties. AKBA by oral administration in mice could change intestinal microenvironment, prevent stroma and epithelial cell growth (*12*). In this study, we determined the inhibitory effect of AKBA in the adenomatous polyposis coli multiple intestinal neoplasia ($APC^{Min/+}$) mouse model and then compared these results with those of aspirin. AKBA was found to have higher efficacy than aspirin in the prevention of intestinal adenomatous polyposis development. Our statistical analysis suggested that the inhibitory effect of AKBA might arise from its multiple actions including anti-proliferation, induction

of apoptosis, modulation of the Wnt/β-catenin signaling pathway, and anti-inflammation. These results supported our previous results that AKBA could develop as a promising agent for the prevention of intestinal tumorigenesis (*12*).

The development of intestinal tumorigenesis is a gradual series of histological changes, called the 'adenomatous polyps-carcinoma' sequence. This sequence has been mostly considered to arise from the mutation of adenomatous polyposis coli (*APC*) gene (*19*). In the $APC^{Min/+}$ mouse model, the mutation of APC gene leads to the dysregulation of the Wnt/β-catenin signaling pathway in which accumulated β-catenin in nucleus leads to transcription of many its target genes. These target genes are amplified following the activation of the aberrant Wnt/β-catenin signaling pathway (*20,21*). Among the targets of β-catenin, PCNA is a marker of cell proliferation and is highly expressed during the 'adenomatous polyps-carcinoma' sequence. Cyclin D1 is a cell cycle protein frequently overexpressed in adenomatous polyps. A high level of c-Myc stimulates cell proliferation and prevents apoptosis in adenomatous polyps. c-Myc and cyclin D1 have also been identified as the transactivation targets of the β-catenin–TCF complex through binding site in their promoter region. Survivin is a prominent anti-apoptotic molecule widely expressed in adenomatous cells. Overexpression of survivin could lead to adenomatous proliferation (*22,23*). The $APC^{Min/+}$ mouse model is thus widely used for evaluation of chemopreventive agents. AKBA possessed strong activity of modulating the Wnt/β-catenin pathway and many of its target proteins, including cyclin D, PCNA, survivin, c-Myc, and p-c-jun. In this study, we did not find significant changes of proteins in Wnt/β-catenin pathway in the aspirin-treated adenomatous polyps. We thus suggested that AKBA might be an inhibitor of the Wnt/β-catenin signaling pathway.

In colonic adenocarcinomas, the inflammatory microenvironment is characterized by the presence of inflammatory stimuli including cytokines and network of growth factors both supporting stroma and adenomatous areas (*24,25*). These stimuli contribute to adenocarcinoma expansion, and even facilitate the metastatic process. Important inflammatory stimuli include COX-2, NF-κB, 5-LOX, PGEs, LTB4, IL-6, TNF-α, FGF-2, and iNOS *etc*. (*12,26*). NF-κB is considered as a 'first responder' to various types of cellular stress. NF-κB activation could induce IκB phosphorylation, targeting it for degradation through an ubiquitin-proteasome pathway, releasing NF-κB to enter the nucleus, where it regulates gene expression. COX-2 is an immediate-early growth response gene product. In response to stimulation of these cytokines, the intercellular communications of the NF-κB-COX-2 pathway take place and lead to adenocarcinoma growth. The accumulation of these molecules further stimulates the Wnt/β-catenin signaling pathway, leading to expansion of adenomatous polyps (*27*). 5-LOX is a key enzyme in metabolizing arachidonic acid to leukotrienes which act quickly to promote the progression of intestinal polyps through activation of NF-κB. High levels of 5-LOX directly stimulate angiogenesis, proliferation and anti-apoptosis in adenomatous polyps (*26*). In addition, COX-2 and 5-LOX are usually co-expressed and up-regulated in the intestinal adenomatous polyps (*12*). Aspirin has long been considered to have the activity of anti-inflammation through the mechanisms of suppressing COX-2 and prostaglandin E2 (PGE2) (*5,6*). Recent studies suggested that aspirin might possess the activity against intestinal adenocarcinomas. The mechanism of aspirin action has been considered to be associated with its anti-inflammation activity. In this study, we compared the efficacy and mechanisms of AKBA with those of aspirin. AKBA possessed a greater activity than aspirin in the suppression of these inflammatory cytokines. These findings suggest that administration of AKBA might have a more beneficial effect than aspirin in prevention of colon adenocarcinoma growth.

In conclusion, AKBA significantly prevented the formation of intestinal adenomatous polyposis without toxicity to mice. Our comparative study suggested that AKBA possess more potential than aspirin in anti-proliferation, induction of apoptosis, modulation of the Wnt/β-catenin pathway, and anti-inflammation. These results support our previous results that AKBA could develop as a promising agent in prevention of colorectal adenocarcinomas.

Acknowledgements

This project was supported by the Natural Science Foundation of China (Grant nos. 91229113, 81173090, and 81072665).

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(Received January 21, 2014; Accepted February 1, 2014)

Original Article

DOI: 10.5582/ddt.8.33

Combination treatment of ligustrazine piperazine derivate DLJ14 and adriamycin inhibits progression of resistant breast cancer through inhibition of the EGFR/PI3K/Akt survival pathway and induction of apoptosis

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Summary **A ligustrazine (TMP) derivative, (E)-2-(2, 4-dimethoxystyryl)-3,5,6-trimethylpyrazine (DLJ14) was synthesized for the improvement of low bioavailability and short half-life of ligustrazine. We have observed potential reversal effects of DLJ14 on adriamycin (Adr)-resistant human myelogenous leukemia cells (K562/A02) and Adr-resistant human breast cancer cells (MCF-7/A) *in vitro* or *in vivo* in previous studies. The aim of the present study was to investigate the underlying molecular mechanism of DLJ14 and Adr combination treatment on Adr-resistant human breast cancer. Inhibition of cancer cell growth was estimated by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell cycle distribution was analyzed by flow cytometry and apoptosis determined using Annexin V-FITC/propidium iodide (PI) double staining and Hoechst 33258 nuclear staining. The expression of proteins in the epidermal growth factor receptor (EGFR)/phosphatidylinositol-3 kinase (PI3K)/Akt survival pathway and mitochondrial-mediated apoptosis pathway were measured by Western blotting analysis. Results showed that DLJ14 and Adr combination treatment exhibited stronger inhibition of the survival of MCF-7/A cells than Adr treatment alone. This effect might be associated with its role in cell cycle arrest and apoptosis induction. DLJ14 combined with Adr induced cell cycle arrest in the G2/M-phase by activating $p21^{wafl/cip1}$ and p53 in mitochondria and increased cleavage of caspase-9 and caspase-3, and Bax/Bcl-2 ratio. Mitochondrial membrane potential (MMP) disruption and cytochrome c (Cytc) release from mitochondria to cytosol suggested that apoptosis induction might be mediated by the mitochondrial pathway. Moreover, the combination of DLJ14 and Adr could down-regulate the expression of EGFR, p-EGFR, PI3K, and p-Akt in MCF-7/A cells. Overall, DLJ14 and Adr combination treatment may inhibit proliferation of Adr-resistant human breast cancer cells through inhibition of the EGFR/PI3K/Akt survival pathway and induction of apoptosis *via* the mitochondrial-mediated apoptosis pathway.**

Keywords: Ligustrazine piperazine derivative DLJ14, resistant human breast cancer, EGFR/PI3K/Akt, mitochondrial-mediated apoptosis pathway, cell cycle

1. Introduction

The development of multidrug resistance (MDR) represents a major obstacle for successful cancer chemotherapy (*1*). For example, the severe MDR of adriamycin (Adr), which is one of the most effective anticancer drugs for various types of cancers, has limited its therapeutic effectiveness (*2*). Great efforts have been made in development of agents which could improve the efficacy of Adr against Adr-resistant cancer cells.

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(E)-2-(2,4-dimethoxystyryl)-3,5,6-trimethylpyrazine (DLJ14), a novel ligustrazine (TMP) piperazine derivate, was synthesized for the improvement of low bioavailability and short half-life of ligustrazine. Our preliminary results showed that DLJ14 efficiently enhanced anti-proliferative properties of Adr against Adr-resistant human myelogenous leukemia cells (K562/A02) and Adr-resistant human breast cancer cells (MCF-7/A) *in vitro* and *in vivo* through modulating the glutathione S-transferase (GSTπ)-mediated pathway (*3,4*). However, the molecular mechanisms underlying the enhancement effects of DLJ14 and Adr combination treatment on cancer cells have not been thoroughly investigated.

In previous studies, we observed that DLJ14 significantly increased the expression of cellular c-Jun NH_2-terminal kinase (JNK) and p-JNK in both K562/A02 cells and MCF-7/A cells, especially in the presence of Adr. The JNK signaling pathway is mainly activated by epidermal growth factor receptor (EGFR) (*5*). EGFR is over-expressed in all subtypes of breast cancer and could activate the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway. In cancer cells, dysregulation of PI3K/Akt signaling pathways is associated with deficiency of apoptosis and the phenotype of multidrug resistance (*6*). PI3K/Akt might exert many anti-apoptotic functions, such as Bax, Bad, and caspase-9 as well as inactivation of pro-apoptotic transcription factors like p53 (*7*). Apoptosis has been accepted as a fundamental component in the pathogenesis of cancer. It is mediated by caspases which can be activated through two pathways, the extrinsic pathway characterized by activation of cell-surface death receptors (tumor necrosis factor receptor, Fas) and the intrinsic pathway depending on release of mitochondrial factors (*8*).

In this study, we investigated the effects of DLJ14 on the survival pathway of EGFR/PI3K/Akt and the Adr-induced mitochondrial-mediated apoptosis pathway in MCF-7/A cells. Our results showed that DLJ14 and Adr combination treatment may inhibit proliferation of Adr-resistant human breast cancer cells through inhibition of the EGFR/PI3K/Akt survival pathway and induction of apoptosis *via* the mitochondrial-mediated apoptosis pathway.

2. Materials and Methods

2.1. *Chemical*

(E)-2-(2,4-dimethoxystyryl)-3,5,6-trimethylpyrazine (DLJ14, CAS1000672-75-2) was synthesized at the Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, China. It was dissolved in dimethylsulfoxide (DMSO, St. Louis, MO, USA) for *in vitro* assays.

2.2. *Drugs and reagents*

Adriamycin (Adr) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. (Zhejiang, China). RPMI-1640 medium was purchased from GIBCO BRL (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), propidium iodide (PI), RNase, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Hoechst 33258 was purchased from Sigma (St. Louis, MO, USA). Annexin V-FITC and PI apoptosis detection kit was purchased from Life Technologies Corporation (Grand Island, NY, USA). Mitochondria/cytosol kit and 5,5',6,6'-tetrachlo-1,1',3,3'-tetraethyl benzimidazolcarbocyanine (JC-1) kit were purchased from Beyotime Biotech Inc. (Shanghai, China). Monoclonal antibody of phosphor-JNK was purchased from Santa Cruz Biotechnology (Santa Cruz, Califonia, USA). Monoclonal antibodies against Bax, Bcl-2, caspase-9, cytochrome c, p53, $p21^{wafl/cip1}$, EGFR, phosphor-EGFR, Akt, phosphor-Akt, PI3K, and cyclin A were purchased from Cell Signaling Technology (CST, Boston, MA, USA). Monoclonal antibody against β-actin was purchased from ZS Bio (Beijing, China). Caspase-3 polyclonal antibody was purchased from Boster Biotech Inc. (Wuhan, Hubei, China).

2.3. *Cell lines and cell culture*

The Adr-resistant human breast cancer cells (MCF-7/A) were obtained from the Institute of Hematology of Chinese Academy of Medical Sciences (Tianjin, China). MCF-7/A cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humid atmosphere (5% CO_2 - 95% air) The cells were maintained in medium containing 1 μmol/L adriamycin and cultured in drug-free medium for 2 weeks prior to experiments.

2.4. *Cell proliferation assay*

Cell viability was measured by MTT assay as described elsewhere (*9*). Briefly, MCF-7/A cells were seeded into 96-well plates (3.0×10^3 /well) and cultured in the presence or absence of DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h. MTT solution (5 mg/mL) was added to each well for 4 h. Then 150 μL DMSO was added to dissolve the formazan precipitate before absorbance was measured at 570 nm using a THERMOmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. *Analysis of cell apoptosis by change of nuclear morphology*

Hoechst 33258 staining was used to visualize change of nuclear morphology and apoptotic body formation. MCF-7/A cells were seeded into 96-well plates (3.0×10^3 /well) and cultured in the presence or absence of DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h.

Then cells were washed twice with PBS and fixed with methanol-glacial acetic acid (3:1) for 10 min, followed by staining with Hoechst 33258 in the dark at room temperature for 10 min. After three washes with PBS, cells were visualized with the fluorescence microscope (excitation, 340 nm; emission, 460 nm) (NIKON, Ti-U, Tokyo, Japan). Viable cells were identified by intact nuclei with blue fluorescence, apoptotic cells by chromosome condensation and fragmentation of nuclei, exhibiting highlight staining (*10*).

2.6. *Analysis of cell apoptosis by annexin V cell surface binding*

MCF-7/A cells seeded in 6-well plates (2.0×10^5/well) were incubated with DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h in the presence or absence of 40 μM SP600125 (a JNK inhibitor). Both floating and attached cells were harvested and washed with cold PBS. Cell surface phosphatidylserine in apoptotic cells was quantitatively estimated using an Annexin V/FITC and PI apoptosis detection kit according to the manufacturer's instructions with a FACSVantage flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA) using emission filters of 525 and 575 nm, respectively (*11*).

2.7. *Cell cycle analysis*

MCF-7/A cells were synchronized by 24 h of growth in 0.5% serum medium, then exposed to 10% serum medium containing DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h. Cells were harvested, washed twice with cold PBS and fixed with precooled 70% ethanol at 20°C overnight. Fifty μg/mL DNase-free RNase A was then added for 30 min at 37°C before addition of PI, at a final concentration of 50 μg/mL for DNA staining in the dark at 4°C for 45 min. For each sample, more than 1×10^4 cells were analyzed using a FACScan Flow cytometer. The distribution of cell cycle stages was determined using ModFit LT software 3.0 (Varity Software House, Topsham, USA).

2.8. *Measurement of mitochondrial membrane potential (MMP)*

Mitochondrial membrane potential ($\Delta\Psi m$) was measured using the fluorescent, lipophilic and cationic probe JC-1 according to manufacture's directions. Cells seeded in 6-well plates (2.0×10^5/well) were treated with DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h. Cells were harvested and stained with JC-1 for 20 min at 37°C in the dark. Then stained cells were centrifuged and the pellet was washed with serum free medium and resuspended in JC-1 staining buffer. Intracellular fluorescence intensity of JC-1 was quantified by FACScan Flow cytometer.

2.9. *Preparation of mitochondria and cytosol*

A mitochondria/cytosol isolation kit was used to separate mitochondria and cytosol of MCF-7/A cells according to the manufacture's protocol. After treatment with DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h, cells (1.0×10^7) were collected and suspended in 400 μL of isolation buffer containing protease inhibitors and lysed on ice for 10 min. After mechanical homogenization with a Dunce grinder, a mixture containing unbroken cells, debris and nuclei was separated by centrifugation at 800× g for 10 min at 4°C. The supernatants were centrifuged at 12,000 g for 15 min at 4°C to obtain pellets of mitochondria which were dissolved in 30 μL of lysis buffer and cytosol supernatant. The mitochondria and cytosol were used for analysis of cytochrome c by Western blotting assay.

2.10. *Western blotting assay*

MCF-7/A cells seeded in 6-well plates (3.0×10^5/well) were treated with DLJ14 (10 μM, 20 μM) and/or Adr (5 μM) for 48 h. Cells were harvested and cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) followed by protein transfer to a PVDF membrane and probed with monoclonal antibodies against phosphor-JNK, Bax, Bcl-2, caspase-3, caspase-9, cytochrome c, p53, $p21^{wafl/cip1}$, EGFR, phosphor-EGFR, Akt, phosphor-Akt, PI3K, cyclin A, and β-actin. Immunoblots were developed with horseradish peroxidase-conjugated secondary antibodies, and visualized using an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) and quantified by densitometry using a ChemiDoc XRS (Bio-Rad, Berkeley, California, USA). The band density was normalized to β-actin. The percentage increase or decrease of the proteins was estimated by comparison to vehicle control (100%).

2.11. *Statistical analysis*

Data are expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests using the SPSS/Win 13.0 software. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. *Anti-proliferation effect of DLJ14 and Adr combination treatment in vitro*

The inhibitory effect of DLJ14 and Adr combination treatment on MCF-7/A cells was evaluated using MTT assay. As shown in Figure 1A, combination treatment of DLJ14 (20 μM) and Adr (5 μM) on MCF-7/A cells resulted in significant inhibition of cell proliferation ($p <$

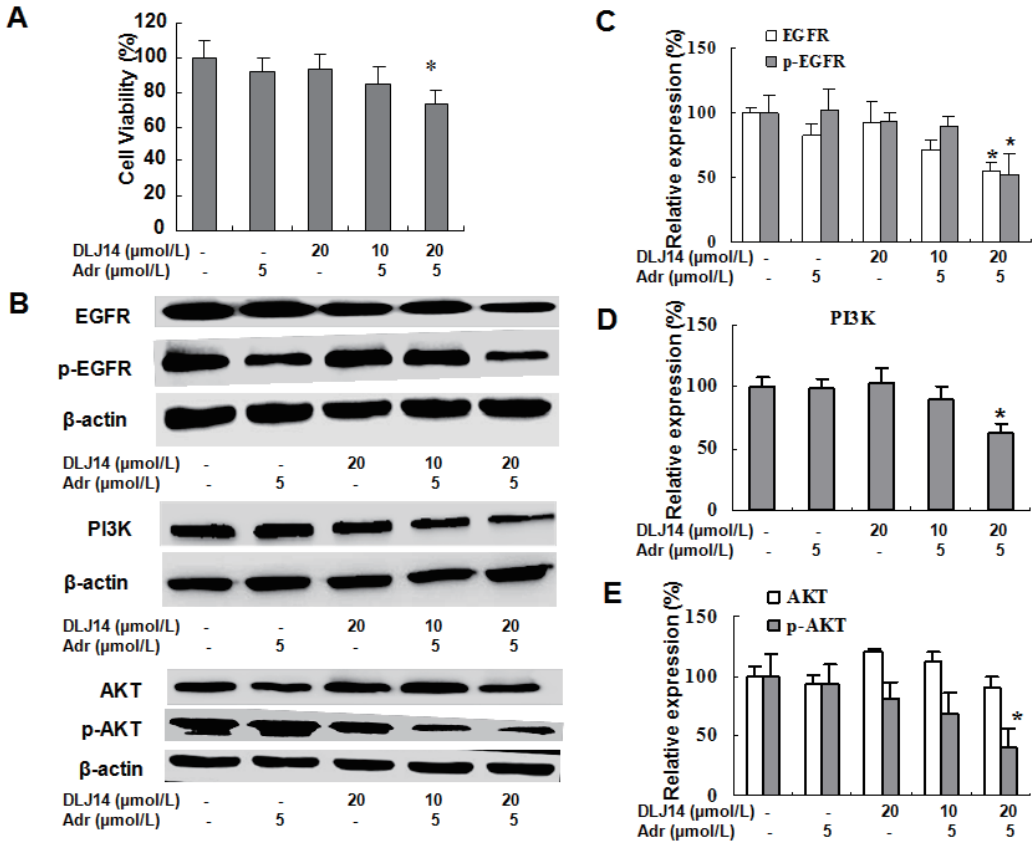

Figure 1. DLJ14 enhanced Adr-mediated anti-proliferation on MCF-7/A cells and regulated the expressions of EGFR, PI3K, and AKT. MCF-7/A cells were treated with DLJ14, Adr, or the combination treatment of DLJ14 and Adr for 48 h and cell viability was determined by MTT assay **(A)**. The expression of EGFR, p-EGFR, PI3K, total Akt, and p-Akt in MCF-7/A cells were detected by Western blotting method. Representative blots of three independent experiments are shown in **(B)**. Quantification of the protein levels of EGFR and p-EGFR, PI3K, and total Akt and p-Akt were normalized to the expression of β-actin and are shown respectively in **(C)**, **(D)**, and **(E)**. Data are expressed as means ± SEM ($n = 3$). * $p < 0.05$ *vs.* Adr treatment alone.

0.05 *vs*. the Adr treatment alone group).

3.2. *DLJ14 and Adr combination treatment inhibits the EGFR/PI3K/Akt survival pathway in MCF-7/A cells*

In order to investigate the effect of DLJ14 and Adr combination treatment on the EGFR/PI3K/Akt survival pathway in MCF-7/A cells, expression of related proteins was determined by Western blotting analysis. As shown in Figures 1B-1E, either DLJ14 or Adr has no significant effect on the expression of EGFR, p-EGFR, PI3K, Akt, and p-Akt. However, DLJ14 and Adr combined treatment could significantly decrease the expression of EGFR, p-EGFR, PI3K, and p-Akt when compared with the Adr treatment alone group except for the expression of total Akt.

3.3. *Arrest of MCF-7/A cells in the G2/M Phase*

The anti-proliferative effect of DLJ14 and Adr combination treatment on MCF-7/A cells was also determined by cell cycle analysis. Flow cytometry analysis showed that DLJ14 treatment alone at 20 μmol/L had no obvious effect on cell cycle progression. Adr treatment alone at 5 μmol/L slightly arrested MCF-7/A cells in the G2/M phase. However, combination treatment of DLJ14 (10 μmol/L, 20 μmol/L) and Adr (5 μmol/L) significantly arrested MCF-7/A cells in the G2/M phase compared to Adr treatment alone (Figures 2A and 2C).

The arrest of the MCF-7/A cell cycle by combination treatment of DLJ14 and Adr was associated with upregulation of $p21^{Waf1/Cip1}$ (a cell cycle regulator gene) expression, and downregulation of cyclin A expression. Moreover, we also observed that the expression of p53 was increased significantly by this combination treatment (Figure 2B and 2D). Activation of p53 could up-regulate transcription of $p21^{Waf1/Cip1}$.

3.4. *Induction of apoptosis by DLJ14 and Adr combination treatment*

The effect of DLJ14 and Adr combination treatment on the apoptosis of MCF-7/A cells was first determined by detecting cell morphology. Treatment of MCF-7/A cells with DLJ14 and Adr combination treatment for 48 h followed by nucleus staining with DNA-binding fluorochrome Hoechst 33258 showed an increased number of cells with reduced nuclear size, chromosome

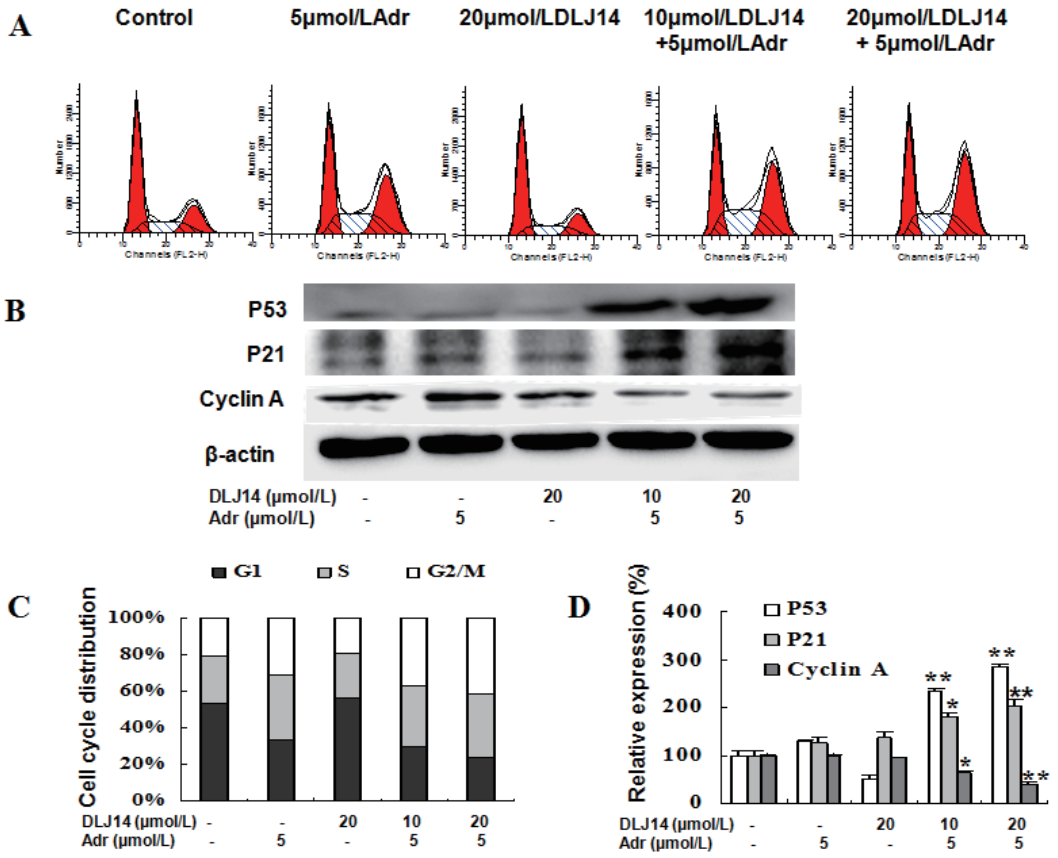

Figure 2. DLJ14 potentiates G2/M phase arrest of Adr in MCF-7/A cells by regulating the expression of p53, p21, and Cyclin A. MCF-7/A cells were treated with DLJ14, Adr, or the combination treatment of DLJ14 and Adr for 48 h before the cells were harvested, fixed, and stained with PI and analysed by flow cytometry **(A)**. Percentage of cells in different stages is shown in **(C)**. The expression of p53, p21, and Cyclin A in MCF-7/A cells were detected by Western blotting method. Representative blots of three independent experiments are shown in **(B)**. Quantification of the protein levels of p53, p21, and Cyclin A were normalized to the expression of β-actin and are shown in **(D)**. Data are expressed as mean ± SEM (n = 3). * $p < 0.05$, ** $p < 0.01$ *vs*. Adr treatment alone.

condensation, and nuclear fragmentation, which are characteristics of apoptosis in comparison to Adr-treated cells (Figure 3A).

Annexin V cell surface staining followed by flow cytometry analysis also showed a significant increase of annexin V positive cells following treatment of the cells with 20 μmol/L DLJ14 and 5 μmol/L Adr, while 5 μmol/L Adr alone had no obvious effect. Interestingly, 20 μmol/L DLJ14 treatment alone induced an obvious increase of early apoptotic cells (Annexin V^+/PI^- cells) in comparison to the control cells, while the combination treatment induced a dramatic increase of necrosis/late apoptotic cells (Annexin V^+/PI^+) (Figures 3B and 3C).

Further analysis suggested that the apoptotic effect of DLJ14 and Adr combination treatment was associated with alteration of apoptotic proteins in MCF-7/A cells. As shown in Figures 3D and 3F, this combination treatment caused a marked increase of the expression of caspase-3 and caspase-9 and also a marked increase of cleavage of caspase-3 and caspase-9 compared to Adr-treatment alone. Figures 3D and 3E show the regulation of the expression of Bax and Bcl-2 in MCF-7/A cells by the combinationtreatment, showing up-regulation of Bax and down-regulation of Bcl-2. Statistical analysis indicated that the ratio of Bax/Bcl-2 was obviously increased.

3.5. *Induction of mitochondrial membrane potential collapse and release of cytochrome c*

To investigate the mechanism of apoptosis, mitochondrial functions were examined by determining mitochondrial activity such as mitochondrial membrane potential and cytochrome c in the mitochondrion and cytosol of MCF-7/A cells. The change of mitochondrial membrane potential was analyzed using JC-1 which could selectively enter into the mitochondrion, and its color could change reversibly from red to green as membrane potential decreased (*12*). Cell apoptosis usually accompanies mitochondrial depolarization. JC-1 release in depolarized mitochondria causes the reduction of red fluorescence intensity. As shown in Figure 4A, the percentage of cells with depolarized mitochondria was significantly increased to 16.2% by DLJ14 and Adr combination treatment, which was 4.48% in the Adr alone treated group. Since mitochondrial membrane disruption is often associated with the release of mitochondrial proteins into cytosol,

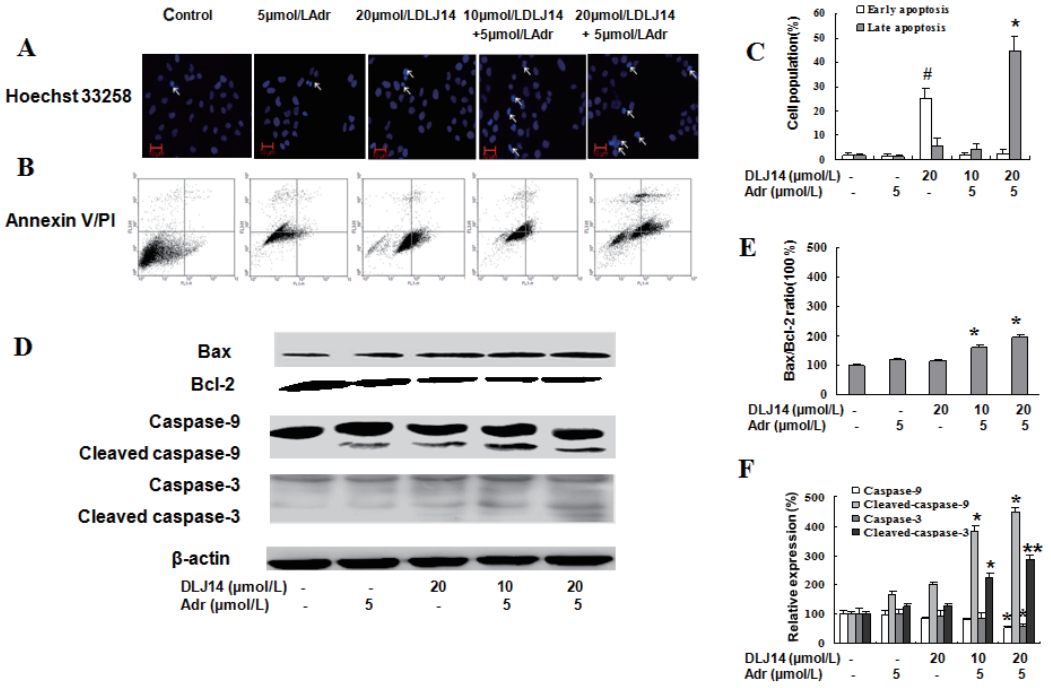

Figure 3. DLJ14 induces the apoptosis of MCF-7/A cells treated with Adr. MCF-7/A cells were treated with DLJ14, Adr, or the combination treatment of DLJ14 and Adr for 48 h, and then stained with DNA-binding fluorochrome Hoechst 33258 staining. Representative images of MCF-7/A cells are shown in **(A)**. Arrows indicate characteristic apoptotic features. Scale bar = 10 μm. Cell apoptosis was analyzed by flow cytometry following Annexin V/FITC and PI double-staining. The FL1 axis shows the Annexin V intensity, and the FL2 axis shows PI staining **(B)**. The percentages of apoptosis and necrosis cells were scored in three separate experiments and the results are shown in **(C)**. The expression of Bax, Bcl-2, caspase-9, cleaved caspase-9, caspase-3, and cleaved caspase-3 in MCF-7/A cells were detected by Western blotting method. Representative blots of three independent experiments are shown in **(D)**. Quantification of the protein levels were normalized to the expression of β-actin and are shown in (**E** and **F**). Data are expressed as mean ± SEM ($n = 3$). $^{\#}$ $p < 0.05$ *vs.* untreated cells, * $p < 0.05$, ** $p < 0.01$ *vs*. Adr treatment alone.

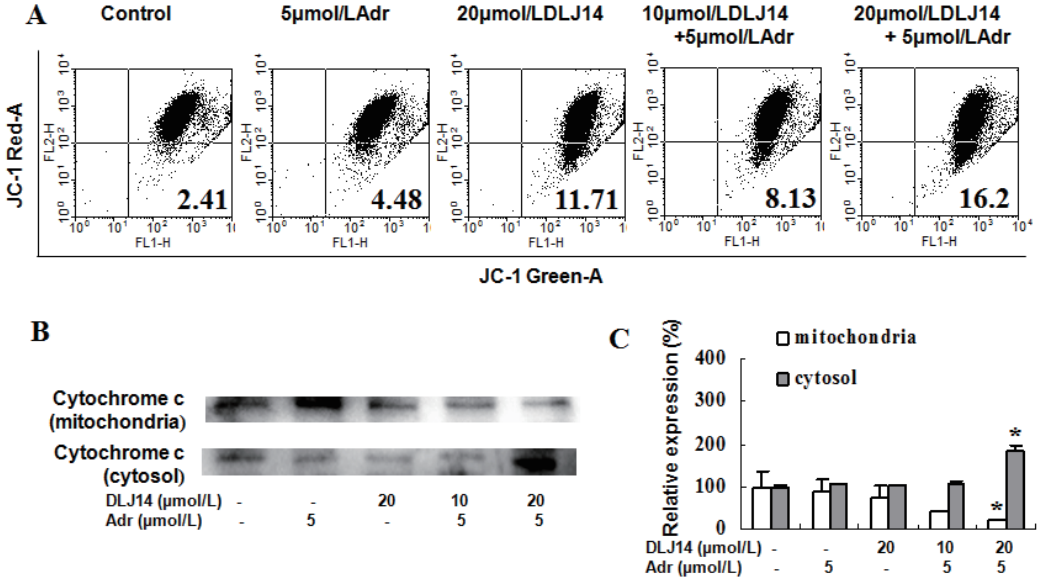

Figure 4. DLJ14 increases mitochondrial membrane potential collapse and the release of cytochrome c in MCF-7/A cells induced by Adr. MCF-7/A cells were treated with DLJ14, Adr, or the combination treatment of DLJ14 and Adr for 48 h, and then stained with JC-1 (the red passage: excitation 325 nm, emission 590 nm and the green passage: excitation 488 nm, emission 530 nm) to determine the depolarized cells by flow cytometry **(A)**. The expression of mitochondrial cytochrome c and cytosol cytochrome c in MCF-7/A cells were detected by Western blotting method. Representative blots of three independent experiments are shown in **(B)**. Quantification of the protein levels were normalized to the expression of β-actin and are shown in **(C)**. Data are expressed as mean ± SEM ($n = 3$). * $p < 0.05$, ** $p < 0.01$ *vs.* Adr treatment alone.

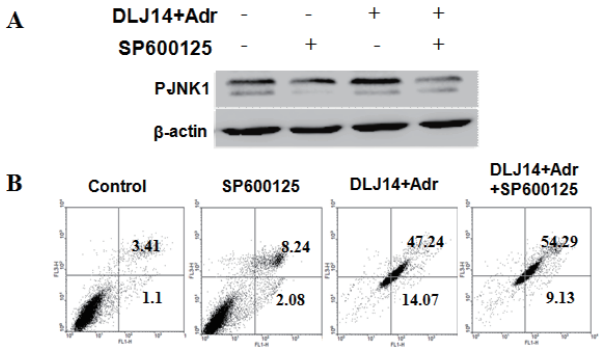

Figure 5. Effect of DLJ14 and Adr combination treatment on apoptosis induction in MCF-7/A cells. MCF-7/A cells were pretreated with or without specific JNK inhibitor (SP600125, 40μmol/l) for 30 min, and then exposed to the combination treatment of DLJ14 and Adr for 48 h. The expression of P-JNK1 was determined by Western blotting method. Representative blots of three independent experiments are shown in **(A)**. Cell apoptosis was analysed by flow cytometry following Annexin V/FITC and PI double-staining **(B)**.

the subcellular distribution of cytochrome c was then examined. Figures 4B and 4C showed that the level of cytochrome c in mitochondria was significantly decreased and the level of cytochrome c in cytosol was obviously increased in MCF-7/A cells by DLJ14 and Adr combination treatment compared to Adr treatment alone.

3.6. *Induction of apoptosis by DLJ14 and Adr combination treatment is not mainly due to the activation of the JNK1 signal pathway*

Our previous results showed that JNK1 phosphorylation was significantly elevated in MCF-7/A cells after DLJ14 treatment. In order to further explore the contribution of JNK signaling pathway to the enhanced therapeutic effect of DLJ14 and Adr combination treatment, SP600125, a small molecule JNK inhibitor, was used to block the JNK signaling pathway (*13*). We examined the regulation of JNK1 phosphorylation and the apoptosis induction of DLJ14 and Adr combinationtreatment. Results showed that the increase of phospho-JNK1 (p-JNK1) expression induced by the combination treatment was significantly blocked by SP600125 (Figure 5A). However, the apoptosis induction of MCF-7/A cells by the combination treatment was obviously not inhibited by SP600125, and the percentage of apoptotic cells was 61.31% or 63.42% in the DLJ14 and Adr combination treatment group without or with SP600125 (40 μM) (Figure 5B).

4. Discussion

TMP, one of active ingredients of the Chinese herb *Ligusticum chuanxiong* Hort, has been widely used for cerebrovascular and cardiovascular diseases in China (*14*). Moreover, TMP has been suggested to be useful as an adjuvant agent to reverse multidrug resistance of tumor cells (*15*). DLJ14, a novel TMP piperazine derivate, was synthesized for the improvement of low bioavailability and short half-life of TMP. In preliminary studies, DLJ14 enhanced therapeutic effects of Adr on resistant cancer cells K562/A02 or MCF-7/A *in vitro* or *in vivo*. In this study, we investigated the underlying molecular mechanism of DLJ14 and Adr combination treatment on Adr-resistant human breast cancer. In cancer cells, dysregulation of PI3K/Akt signaling pathways is associated with deficiency of apoptosis and the phenotype of multidrug resistance. PI3K/Akt is a major downstream signaling cascade of EGFR which is over-expressed in all subtypes of breast cancer. Thus, the effect of DLJ14 and Adr combination treatment on the EGFR/PI3K/Akt survival pathway in MCF-7/A cells was determined. Results showed that DLJ14 and Adr combination treatment effectively decreased the protein expression of EGFR, p-EGFR, PI3K, and p-Akt, but not total Akt, as compared to only Adr treatment. This indicated that DLJ14 and Adr combination treatment inhibited the survival pathway of EGFR/PI3K/Akt to enhance anti-tumor effects.

PI3K/Akt signaling pathway plays an important role in cancer cell growth. Activation of PI3K/Akt pathway not only promotes cell survival, but also exerts anti-apoptotic function *via* the mitochondrial pathway. It phosphorylates Bad for degradation and thus increases Bcl-2 activity (*16*). Moreover, PI3K/Akt pathway also blocks p53 *via* activation of Mdm2 (*17*). Therefore, inhibition of PI3K/Akt pathway could promote tumor cell apoptosis. Apoptosis has been accepted as a fundamental component in the pathogenesis of cancer. Apoptosis mediated by mitochondria can be triggered by MMP collapse, which is a decisive event in the process of cytochrome c release. The collapse of MMP can initiate the release of molecules from space between the outer and inner mitochondrial membranes into cytosol, and triggercaspase cascade and other apoptotic processes (*18*). Release of cytochrome c is also associated with decrease of Bcl-2 and increase of Bax followed by activating caspase-9 and caspase-3 (*19*). In this study, we observed that DLJ14 and Adr combination treatment increased Bax/Bcl-2 ratio and collapse of MMP, subsequently induced cytochrome c release from mitochondria to cytosol and caspase-9 and caspase-3 activation. These results indicated that DLJ14 and Adr combination treatment might induce apoptosis in MCF-7/A cells *via* activating the mitochondria-mediated intrinsic pathway.

Cell cycle is also a main regulatory mechanism of cell growth and many chemical compounds could trigger apoptosis in tumor cells accompanied by cell cycle arrest (*20,21*). Flow cytometry analysis showed that DLJ14 and Adr combinationtreatment induced G2/M phase arrest of MCF-7/A cells compared with Adr treatment alone. $p21^{Waf1/Cip1}$ is known as a cell cycle inhibitor involved in G2/M phase progression and its

up-regulation has been linked to cell cycle arrest at G1 or G2/M phase. Activated of p53 is able to regulate transcription of the cell cycle regulator gene $p21^{Waf1/Cip1}$ (*22*). Cyclin A is also related to cell cycle arrest at G2 phase through forming Cyclin A/Cdk2 complexes (*23*). This study, in parallel with flow cytometery analysis, showed that DLJ14 and Adr combination treatment could activate p53, upregulate the expression of $p21^{Waf1/Cip1}$ and downregulate the expression of Cyclin A.

JNK primarily contributes to pro-apoptotic cell death or tumor suppression in response to a variety of stress, inflammatory or oncogenic signals and a is major downstream signal pathway of EGFR (*24*). We used SP600125, a specific JNK inhibitor, to explore the role of JNK1 pathway in the enhanced therapeutic effects of the combination of DLJ14 and Adr treatment. Results showed that the apoptosis induction of MCF-7/A cells by the combination treatment was not inhibited significantly by SP600125. This result indicated that the JNK1 pathway might not play a crucial role in the apoptosis induction from DLJ14 and Adr combination treatment.

In summary, the combination treatment of DLJ14 and Adr enhanced anti-proliferation effects on MCF-7/A cells through inhibiting EGFR/PI3K/Akt survival pathway and induced cell apoptosis *via* the mitochondrial-mediated intrinsic pathway accompanied with G2/M cell cycle arrest. Moreover, JNK1 pathway activation induced by DLJ14 and Adr combination treatment might not be involved in apoptosis induction. DLJ14 therefore has the potential to be developed as a promising agent for treatment of cancers with Adr resistant cells.

Acknowledgements

This work was funded by the National Science Foundation of China Grants (81373450) and was supported by Program for Changjiang Scholars and Innovative Research Team in University "PCSIRT" (IRT13028).

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(Received December 21, 2013; Revised February 15, 2014; Accepted February 21, 2014)

Original Article

DOI: 10.5582/ddt.8.42

Fibroblast growth factor-2 inhibits mineralization of osteoblast-like Saos-2 cells by inhibiting the functioning of matrix vesicles

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Summary **Fibroblast growth factor-2 (FGF2) inhibits osteoblast mineralization, but the mechanism by which it does so is not fully understood. Matrix vesicles (MVs) play an essential role in the initiation of mineralization, so the current study examined the effect of FGF2 on the functioning of MVs to investigate this mechanism. This study found that FGF2 significantly inhibited differentiation and mineralization of osteoblast-like Saos-2 cells, as indicated by down-regulation of mRNA expression of the osteogenic master regulator runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), and collagen 1 alpha 1 (Colla1), and by decreasing the formation of bone nodules. MVs were isolated from Saos-2 cells cultured in osteogenic medium supplemented with and without FGF2 and their presence was verified using electron microscopy and Western blotting. FGF2 markedly reduced the ALP activity of and in vitro mineralization by MVs. These findings suggest that FGF2 inhibits osteoblast mineralization by limiting the capacity of MVs.**

Keywords: Fibroblast growth factor-2, mineralization, matrix vesicles, Saos-2 cells

1. Introduction

Osteoblasts produce and secrete a variety of growth factors such as fibroblast growth factor-2 (FGF2), transforming growth factor β (TGFβ), insulin-like growth factor-1 (IGF1), platelet-derived growth factor (PDGF), and prostaglandin E2 (PGE2) that regulate osteoblast proliferation and differentiation in an autocrine and paracrine manner (*1*). FGF2 has been reported to play an important role in bone and callus formation (*2,3*). Disruption of the *FGF2* gene in mice leads to decreased bone mass and decreased bone formation (*4*). However, transgenic mice overexpressing FGF2 were found to have decreased osteoblast differentiation and impaired bone formation (*5*). *In vitro*, continuous treatment with a high concentration of exogenous FGF2 inhibits expression of osteogenic marker gene collagen type 1 (Col1), it reduces alkaline phosphatase (ALP) activity, and it decreases matrix mineralization in osteoblasts (*6-8*). Although FGF2 is reported to act by regulating expression of various genes involved in osteoblast proliferation and differentiation and activating signal transduction pathways including extracellular signal-regulated kinase (ERK) (*9,10*), the mechanism by which FGF2 inhibits mineralization is still understood poorly. Its effect on extracellular components of osteoblasts has not been explored.

Matrix vesicles (MVs) are secreted by mature osteoblasts into the extracellular region. These small vesicles have a diameter ranging from 30 to 400 nm and they contain abundant phospholipid and proteins. MVs play an important role in the initial stage of bone mineralization by promoting the deposition of hydroxyapatite (HA) crystals (*11,12*). MVs manage mineral nucleation, they regulate the inorganic phosphate (Pi)/inorganic pyrophosphate (PPi) ratio in the intra- and extra-cellular fluid, and they control calcium ion and Pi homeostasis (*13*).

Therefore, MVs are presumably involved in FGF2 inhibiting osteoblast mineralization. The current study used osteoblast-like Saos-2 cells to observe the effect

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of FGF2 on expression of osteogenic factors and mineralization. MVs were isolated and their presence was then verified using electron microscopy and biomarker detection. Assays of the ALP activity of and mineralization by MVs were also performed in order to determine the effect of FGF2 on the capacity of MVs and to provide a better understanding of the mechanism by which FGF2 inhibits osteoblast mineralization.

2. Materials and Methods

2.1. *Cell culture and treatment*

Human osteoblast-like Saos-2 cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in McCoy's 5A (Gibco, Carlsbad, CA, USA) supplemented with 15% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA) with 1% penicillin-streptomycin (Beyotime, Haimen, China) at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. To induce mineralization, subconfluent Saos-2 cells were cultured in osteogenic medium supplemented with 7.5 mM β-glycerophosphate (β-GP) (Sigma, MO, USA) and 50 μg/mL ascorbic acid (AA) (Sigma, St. Louis, MO, USA). To study the effect of FGF2 treatment, human recombinant FGF2 (Peprotech, Rocky Hill, NJ, USA) was added to a final concentration of 50 ng/mL in osteogenic medium (containing β-GP and AA) (*7*). Media were replaced every three days with the same type of fresh media.

2.2. *Analysis of mineralization*

The mineralization of Saos-2 cells was determined in 12-well plates using Alizarin red S staining during osteogenic induction (3, 6, and 9 days). The cells were fixed with 4% paraformaldehyde for 10 min after they were washed with PBS, and then they were stained with 0.5% (w/v) alizarin red S solution for 1 h. Dye was thoroughly washed from wells using PBS. For quantitative analysis, the cells were incubated in 10% (w/v) cetylpyridium chloride at 37°C for 1 h, and then the optical density of the supernatant was measured at 562 nm.

2.3. *RNA isolation and quantitative real-time polymerase chain reaction (RT-qPCR)*

RNA was isolated from 12-well plates using Trizol reagent (Gibco, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. RNA was then used for cDNA synthesis with a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). After the RT reaction, cDNA was used as the template for RT-qPCR of ALP, runt-related transcription factor 2 (Runx2), and collagen 1 a1 (Colla1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control. RT-qPCR was performed using a SYBR Green qPCR Kit (Toyobo, Osaka, Japan) in a real-time PCR detection system with a LightCycler 480 thermocycler (Roche Applied Science, Mannheim, Germany) with gene-specific primers: 5'-CCG TGG CAA CTC TAT CTT GG-3' and 5'-GCC ATA CAG GAT GGC AGT GA-3' for ALP, 5'-AGC AAG GTT CAA CGA TCT GAG AT-3' and 5'-TTT GTG AAG ACG GTT ATG GTC AA-3' for Runx2, 5'-CCC TGG AAA GAA TGG AGA TGA T-3' and 5'-ACTGAA ACC TCT GTG TCC CTT CA-3' for Col1a1, and 5'-CAC CAT CTT CCA GGA GC-3' and 5'-AGT GGA CTC CAC GAC GTA-3' for GAPDH.

2.4. *Isolation of MVs*

After induction for 9 days, Saos-2 cells were washed twice with Hank's balanced salt mixture (Solabio, Shanghai, China) and then digested with 1 mg/mL collagenase Type IA (Sigma, St. Louis, MO, USA) at 37°C for 3 h. The supernatant was collected by centrifugation at 3,000 g for 30 min and concentrated through a 100K Amicon Ultra filter (Millipore Corporation, Billerica, MA, USA) to about 1 mL. The concentrated supernatant was mixed with Exoquick™ (System Biosciences Inc, Carlsbad, CA, USA). The mixture was refrigerated at 4°C overnight and centrifuged at 3,000 g for 30 min to collect MVs that were located at the bottom of the tubes (*13*).

2.5. *Transmission electron microscopy of MVs*

Freshly collected MVs were centrifuged at 8,000 g for 30 min to yield pellets that were then fixed with 2.5% glutaraldehyde at 4°C for 2 h and incubated in 1% osmium tetroxide phosphate buffer solution for 1 h in turn. The samples were dehydrated in a graded ethanol series with acetone before they were embedded in epoxy resin. Seventy-five-nm-thick semithin sections were prepared and mounted on copper grids and stained with a uranyl acetate solution and lead citrate solution to enhance the contrast. An H800 transmission electron microscope (TEM) (Hitachi Electronic Instruments, Japan) was used to obtain electron micrographs.

2.6. *Western blotting*

MV pellets were lysed with radio-immunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) on ice for 60 min and then centrifuged at 14,000 g for 15 min at 4°C. The amount of MV lysate protein was determined using a Bradford assay (BioRad Laboratories, Carlsbad, CA, USA). Equal amounts (25 μg) of MV protein from each sample were fractioned on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to

PVDF membranes (Millipore Corporation, Billerica, MA, USA). After blocking, the membrane was incubated with anti-CD63 and CD9 antibodies (1:1,000) (Carlsbad, CA, USA) at 4°C overnight. Primary antibodies were immunostained with goat anti-rabbit IgG peroxidase-conjugated secondary antibodies. Blots were developed with enhanced chemiluminescence (ECL) (Millipore Corporation, Billerica, MA, USA) and exposed to X-ray film.

2.7. *Analysis of the ALP activity of MVs*

P-nitrophenyl phosphate (p-NPP) (Sigma, St. Louis, MO, USA), as substrate, was used to determine the ALP activity of MVs. MV pellets were lysed with an appropriate volume of lysis buffer containing 25 mM Tris-HCl (pH 7.4) and 0.5% Triton X-100. One microliter of MV lysate was incubated with 100 µL p-nitrophenyl phosphate. The reaction was stopped by addition of 50 µL NaOH (3 M) and absorbance was measured at 405 nm. ALP activity was normalized to the protein content of MVs.

2.8. *In vitro biomineralization by MVs*

Calcium precipitation by MVs was assayed in synthetic cartilage lymph (SCL) (100 mM NaCl, 12.7 mM KCl, 0.57 mM $MgCl_2$, 1.83 mM $NaHCO_3$, 0.57 mM Na_2SO_4, 3.42 mM NaH_2PO_4, 2 mM $CaCl_2$, 5.55 mM D-glucose, 63.5 mM sucrose, and 16.5 mM TES, pH = 7.5). An appropriate volume of fresh MVs was added to the aforementioned SCL buffer and the mixture was incubated for 12 h at 37°C. After centrifugation at 8,800 g for 15 min, the calcium phosphate mineral complex was washed twice with water. The precipitant was dissolved in 0.1 M HCl for 3 h. The calcium content of the supernatant was determined using a Calcium Assay Kit (Bioassay Systems, Carlsbad, CA, USA) and was normalized to total protein content.

2.9. *Statistical analysis*

For quantitative data, results are expressed as the mean ± S.D. To determine the differences between groups, an unpaired Student's *t*-test was performed, with $p < 0.05$ being considered statistically significant.

3. Results and Discussion

3.1. *The effect of FGF2 on mineralization of Saos-2 cells*

To evaluate calcium deposition in the Saos-2 cell matrix, alizarin red staining was performed and staining was quantified using a colorimetric analysis based on solubilization of the red matrix precipitate with cetylpyridinium chloride after 3, 6, and 9 days of osteogenic induction. Obvious mineralized nodules were noted after 3 days of induction and increased in a time-dependent manner, becoming extensive after 6 and 9 days (Figure 1A). Cetylpyridinium chloride analysis revealed significantly decreased mineralization in Saos-2 cells treated with FGF2 after 3, 6, and 9 days of induction compared to that in cells cultured in osteogenic medium (Figure 1B). This finding is consistent with those of previous studies (*14,15*).

3.2. *The effect of FGF2 on the expression of osteogenic marker genes in Saos-2 cells*

Osteoblast differentiation and mineralization requires the expression of Runx2, ALP, and Colla1. Runx2, which is a crucial transcriptional factor and expressed in the earliest stage of osteogenic differentiation, regulates the expression of major bone matrix protein genes and osteoblast differentiation and function (*16*). ALP is an early marker of osteogenic differentiation, it hydrolyzes pyrophosphate, and it provides inorganic phosphate to promote mineralization (*17*). Colla1 also plays an important role in osteogenesis by laying the foundation for bone matrix mineralization (*18*).

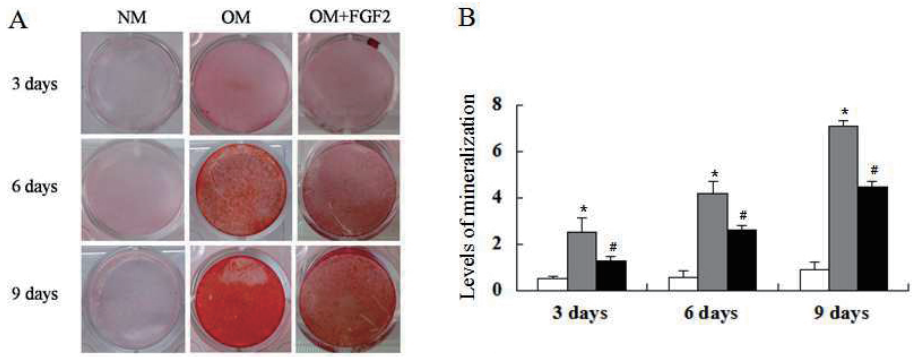

Figure 1. Effect of FGF2 on matrix mineralization of Saos-2 cells. (A) Saos-2 cells were incubated in normal medium (NM), osteogenic medium (OM) and OM supplemented with 50 ng/mL FGF2 for 3, 6, and 9 days. The mineralization of cells was detected using Alizarin Red S staining. **(B)** Cultured cells stained with Alizarin Red S were incubated in cetylpyridinium chloride and staining was quantified at 562 nm. Results are shown as the mean ± S.D., $n = 3$. * $p < 0.05$, *vs.* cells incubated in NM; # $p < 0.05$ *vs.* cells incubated in OM.

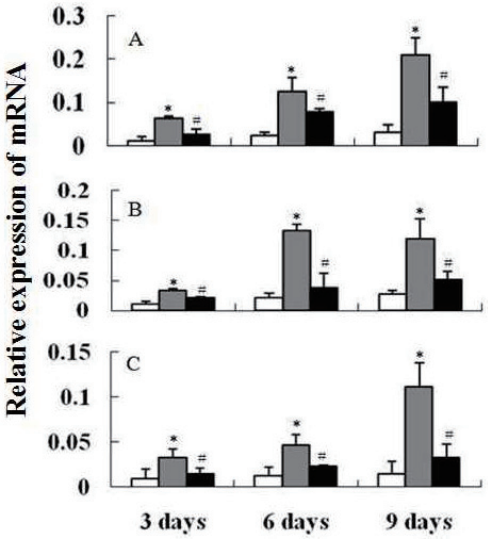

Figure 2. Effect of FGF2 on expression of osteoblast differentiation marker genes in Saos-2 cells. RT-qPCR was used to measure mRNA levels of ALP **(A)**, Runx2 **(B)**, and Collal **(C)** in Saos-2 cells incubated in NM (blank columns), OM (gray columns) and OM with 50 ng/mL FGF2 (black columns) for 3, 6, and 9 days. The expression was normalized to the expression of GAPDH. The experiments were repeated three times and obtained similar results. Results are the mean ± S.D., $n = 3$. * $p < 0.05$ *vs.* cells incubated in NM; # $p < 0.05$ vs. cells incubated in OM.

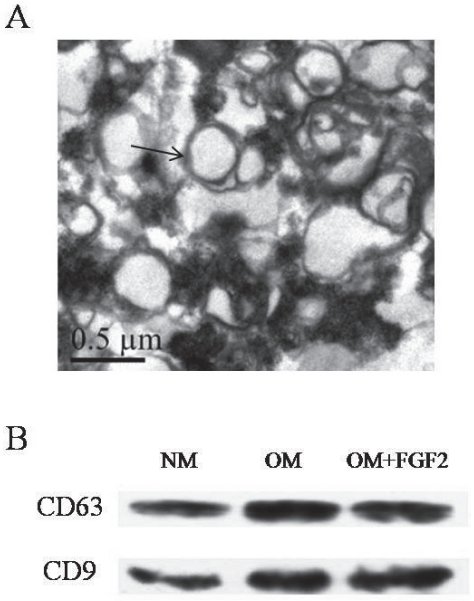

Figure 3. Verification of the presence of MVs. (A) In this TEM image of MVs isolated from Saos-2 cells, the black arrow shows MVs that were membrane-bounded bodies. **(B)** Western blots confirmed the presence of MVs and exosomal protein markers CD63 and CD9 in all of the MVs.

In the current study, levels of mRNA of 3 mineralization-related genes (*ALP*, *Runx2*, and *Col1a1*) were measured using RT-qPCR. Treatment with FGF2 markedly suppressed the expression of all 3 genes at different time points (Figure 2) in comparison to cells incubated in osteogenic medium, suggesting that FGF2 inhibits the osteogenic differentiation of Saos-2 cells.

3.3. *Isolation and characterization of MVs*

Exoquick™ reagents were used to successfully isolate MVs from Saos-2 cells after 9 days of induction, and the presence of MVs was verified using transmission electron microscopy and biomarker detection. As seen in Figure 3A, MVs were apparent as spherical membrane-bounded vesicle structures with a diameter ranging from 30 to 400 nm. Moreover, CD63 and CD9, which are biomarkers of MVs, were present in isolated MVs (Figure 3B).

3.4. *Effect of FGF2 on the ALP activity of and mineralization by MVs*

MVs are released by osteoblasts into the extracellular environment and serve as nucleation sites to accumulate calcium and Pi, thus stimulating the formation of HA crystals and initiating mineralization (*19*). One important role of MVs is to regulate the Pi/PPi ratio in extra-cellular fluid *via* their rich content of pyrophosphate/phosphate-regulating proteins, tissue-nonspecific ALP, ecto-nucleotide pyrophosphatase phosphodiesterase 1 (NPP1), phosphatase orphan 1

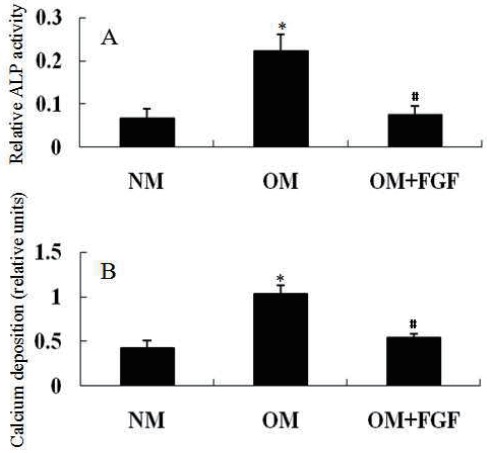

Figure 4. The effect of FGF2 on the capacity of MVs. (A) The effect of FGF2 on the ALP activity of MVs. Saos-2 cells were treated with FGF2 at 3-day intervals for a total of 9 days, resulting in MVs with decreased ALP activity compared to MVs isolated from OM. **(B)** The effect of FGF2 on MV mineralization. MVs from Saos-2 cells cultured in OM supplemented with FGF2 are capable of less calcium precipitation than MVs isolated from OM. Results are the mean ± S.D., $n = 3$. * $p < 0.05$ *vs.* cells incubated in NM; # $p < 0.05$ *vs.* cells incubated in OM.

(PHOSPHO1), and sodium-dependent Pi symporters (Pit1/2) (*20*). The current study found that Saos-2 cells treated with FGF2 yielded MVs with decreased ALP activity compared to untreated cells (Figure 4A). ALP associated with MVs may generate Pi by hydrolyzing pyrophosphate in the extracellular matrix (ECM). A decrease in the ALP activity of MVs may reduce hydrolysis of PPi and consequently fail to yield sufficient Pi to promote the growth of apatite crystals in the ECM. FGF2 reduced the ALP activity

of MVs, indicating this fibroblast growth factor's potential to prevent apatite growth at sites distant from osteoblasts. Furthermore, a biomineralization assay revealed a dramatic decrease in calcium precipitation by MVs derived from Saos-2 cells treated with FGF2 (Figure 4B). FGF2 may act on both the ALP activity of and calcium precipitation by MVs to decrease mineralization in the ECM of osteoblasts.

The regulatory effect of FGF2 on osteoblasts is mediated through the activation of the 4 FGF receptors (FGFRs) FGFR-1, FGFR-2, FGFR-3, and FGFR-4 (*21*). Enhanced FGF signaling caused by mutations in FGFRs is responsible for the aberrant mineralization phenotype of craniosynostosis syndromes (*22*). However, the pathogenesis of the craniosynostosis syndromes is still poorly understood. MVs are reportedly involved in several mineralization-related diseases such as atherosclerosis and osteoarthritis (*23*). In the current study, altered FGF signaling changed the functioning of MVs. This finding may help to elucidate the pathogenesis of FGF/FGFR-associated craniosynostosis syndromes.

In conclusion, this study has described a mechanism by which FGF2 regulates osteoblast mineralization. This study found that FGF2 significantly inhibited the differentiation and mineralization of Saos-2 cells. MVs yielded by Saos-2 cells treated with FGF2 had decreased ALP activity and limited capacity to precipitate calcium. These findings help to further understand the mechanism by which FGF2 inhibits osteoblast mineralization and they suggest that MVs may be involved in the pathogenesis of FGF/FGFR-related craniosynostosis syndromes.

Acknowledgment

This work was supported by the Key Project for Drug Research and Development of the Ministry of Science and Technology of China (Grant No. 2010ZX09401-302-5-07).

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(Received January 11, 2014; Revised January 28, 2014; Accepted February 1, 2014)

Original Article

DOI: 10.5582/ddt.8.48

A combination of oral uracil-tegafur plus leucovorin (UFT + LV) is a safe regimen for adjuvant chemotherapy after hepatectomy in patients with colorectal cancer: Safety report of the UFT/LV study

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Summary **The use of adjuvant systemic chemotherapy for resectable liver metastases from colorectal cancer (CRC) is controversial because no trial demonstrated its benefit. We conducted the phase III trial to evaluate UFT/leucovorin (LV) for colorectal liver metastases (CRLM). The primary endpoint has not been available until 2014, we first report the feasibility and safety data of UFT/LV arm. In this multicenter trial, patients who underwent curative resection of liver metastases from colorectal cancer were randomly assigned to receive surgery alone or surgery followed by adjuvant chemotherapy with UFT/LV. The primary endpoint was relapse-free survival. Secondary endpoints included overall survival and safety. A total of 180 patients were enrolled, 90 were randomly assigned to receive UFT/LV therapy. Eighty two of whom were included in safety analyses. In the UFT/LV group, the completion rate of UFT/LV was 54.9%, the relative dose intensity was 70.8% and grade 3 or higher adverse events occurred in 12.2% of the patients. Elevated bilirubin levels, decreased hemoglobin levels, elevated alanine aminotransferase levels, diarrhea, anorexia were common. Most other adverse events were grade 2 or lower and tolerable. In conclusions, UFT/LV is a safe regimen for postoperative adjuvant chemotherapy in patients who have undergone resection of liver metastases from colorectal cancer. Further studies are warranted to improve completion rate, but UFT/LV is found to be a promising treatment in this setting.**

Keywords: Adjuvant treatment, colorectal cancer, randomized controlled trial, resection of liver metastases, UFT/LV

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1. Introduction

Hepatectomy is widely acknowledged to be therapeutically useful in patients with liver metastases from colorectal cancer (CRC), with a resection rate of 10% to 40% and a 5-year survival rate of 30% to 45%

(*1-5*). The aggressive extension of surgical indications has led to long-term survival even in patients with unfavorable prognostic factors (*6,7*). However, relapse is common and occurs in approximately 75% of the patients (*8*).

Kokudo and his colleagues retrospectively analyzed 132 patients who had liver resection for colorectal metastasis at their hospital, they showed that adjuvant chemotherapy significantly improved surgical and disease-free survival after hepatic resection for colorectal metastases (*9*). Postoperative adjuvant chemotherapy is considered useful for inhibiting recurrence in the residual liver and the development of micrometastasis in patients who undergo resection of liver metastases. Several phase III clinical trials have previously compared surgery alone with surgery plus postoperative adjuvant chemotherapy, but clear-cut evidence demonstrating the effectiveness of postoperative adjuvant chemotherapy has yet to be obtained. This is reflected in the 2010 guidelines for the management of colorectal cancer issued by the Japanese Society for Cancer of Colon and Rectum. Despite this situation, FOLFOX (oxaliplatin, 5-fluorouracil, and leucovorin (LV)) therapy, which was shown to be effective for stage III and unresectable stage IV CRC (*10-14*), has been widely used in routine medical practice. However, the feasibility and safety of postoperative FOLFOX therapy in patients undergoing hepatectomy has yet to be firmly established. Because the usefulness, safety, and feasibility of FOLFOX therapy has not been adequately demonstrated after resection of liver metastases in patients with CRC, investigators in Japan and other countries have criticized its indiscriminant use in patients after hepatectomy.

UFT (Taiho Pharmaceutical Company, Tokyo, Japan) is an oral 5-fluorouracil preparation combining tegafur and uracil in a molar ratio of 1:4. Tegafur is metabolized to 5-fluorouracil in the liver, and uracil competitively inhibits dihydropyrimidine dehydrogenase (DPD), the main metabolizing enzyme of 5-fluorouracil, thereby increasing serum concentrations of 5-fluorouracil and enhancing antitumor activity. The National Surgical Adjuvant Breast and Bowel Project (NSABP) C-06 trial demonstrated that UFT/LV therapy is noninferior to 5-fluorouracil/LV therapy as postoperative adjuvant chemotherapy for stage II or III colon cancer, establishing UFT/LV as a standard therapy of stage III CRC in Japan (*15*). It was speculated that UFT/LV would be a candidate as a novel treatment strategy for CRLM.

For this reason, we focused on UFT/LV adjuvant therapy which was approved in 2003 and initiated a phase III clinical trial to compare the effectiveness and safety of postoperative adjuvant chemotherapy with UFT/LV with those of surgery alone in Japanese patients who underwent resection of liver metastases from colorectal cancer from 2004. This study is registered in the UMIN Clinical Trials Registry (registration ID number: UMIN: C000000013, *http://www.umin.ac.jp/ctr/index-j.htm*). Although the primary endpoint (3-year relapse free survival (RFS)) is not found until 2014, we report the results of an interim analysis of the treatment completion rate, relative dose intensity, and safety of UFT/LV therapy. Because the safety and feasibility of adjuvant chemotherapy after hepatectomy remain unclear, reporting on safety in this study is expected to contribute to the optimal use of adjuvant chemotherapy after resection of liver metastases.

2. Materials and Methods

2.1. *Patients*

The trial was approved by the medical ethics committees of all participating centres and was conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all patients before enrollment.

Eligible patients had to satisfy the following criteria: an age of 20 to younger than 80 years; a histopathologically confirmed diagnosis of liver metastasis from CRC; surgical resection of liver metastasis; macroscopically curative hepatectomy; initial treatment for liver metastasis or one previous resection of liver metastasis (either synchronous or metachronous); no extrahepatic lesions; no previous local or systemic chemotherapy or radiotherapy for liver metastasis; adequate organ functions at the start of treatment after surgery (white-cell count 4,000-12,000/μL, platelet count $\geq 100 \times 10^3$/μL, hemoglobin level $\geq$ 9.0 g/dL, total bilirubin level $\leq$ 1.5 mg/dL, aspartate aminotransferase and alanine aminotransferase levels $\leq$ 100 IU/L, prothrombin activity $\geq$ 50%, serum creatinine level $\leq$ 1.5 mg/dL, blood urea nitrogen level $\leq$ 25 mg/dL, total protein level $\geq$ 5.9 g/dL, albumin level $\geq$ 3.0 g/dL, C-reactive protein level $\leq$ 2.1 ng/mL); and a performance status of 0 to 2.

Patients were excluded if they had another active cancer, a clearly positive surgical margin at the time of hepatectomy, or serious postoperative complications. Pregnant or breast-feeding women were excluded. Patients with any of the following concurrent conditions were also excluded: receiving insulin treatment; poorly controlled diabetes mellitus or hypertension; a history of myocardial infarction within the past 6 months or unstable angina; liver cirrhosis; or interstitial pneumonia, pulmonary fibrosis, or pulmonary emphysema).

2.2. *Procedures*

Protocol treatment was started within 8 weeks after surgery. In the surgery alone group, patients were postoperatively followed up with further no treatment until metastasis or recurrence was confirmed. In the UFT/LV group, UFT (300 mg/m^2/day as tegafur) and LV (75 mg/day) were simultaneously given after meals 3 times per day for 28 days, followed by a 7-day rest.

This was regarded as 1 course of treatment. This cycle was repeated until patients had received 5 courses (25 weeks) of UFT/LV therapy. The treatment criteria for UFT/LV therapy were as follows: white-cell count ≥ 4,000/μL, platelet count ≥ 100 × 10^3/μL, aspartate aminotransferase and alanine aminotransferase levels < 100 IU/L, total bilirubin level ≤ 1.5 mg/dL, and no grade 1 or higher nonhematologic toxicity, with the exception of constipation and hair loss. If the treatment criteria were not met because of adverse events at the scheduled time of starting a course of therapy, treatment was postponed until the criteria were satisfied. If the treatment criteria were not met during a course of therapy, the study treatment was discontinued and resumed when the criteria were met again. If the following criteria were met during a course of therapy, treatment with UFT was discontinued at the scheduled time of treatment resumption according to predesignated criteria: white-cell count ≤ 1,000/μL or platelet count < 25 × 10^3/μL, grade 3 or higher nonhematologic toxicity, or the criteria for the resumption of treatment were met from after 9 days to 15 days after discontinuing therapy. Once the dose of UFT was reduced, it was not increased again, even if toxicity resolved. The dose of LV was not changed. Protocol treatment with UFT/LV therapy was discontinued in the event of any of the following conditions: recurrence occurred; treatment could not be resumed for more than 15 days because of toxicity; the dose had to be reduced by more than one level because of toxicity; the patient requested withdrawal of the protocol treatment; death occurred during the protocol treatment; the protocol treatment was violated; the patient was found to be ineligible; or the physician in charge considered it difficult to continue the protocol treatment.

2.3. *Evaluation of safety*

Adverse events were monitored until 30 days after the final treatment and were evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0. The worst grade of each adverse event was recorded.

2.4. *Statistical analysis*

The primary endpoint of the study was 3-year relapse-free survival. The sample size was planned approximately 20% for the surgery arm and 35% for the UFT/LV arm with power 75% at the 2-sided 5% significance level, requiring 180 patients. Rates of relapse-free survival were estimated by the Kaplan-Meier method and compared by the logrank test. Secondary endpoints were overall survival, relapse-free period in the residual liver, and relapse-free period in other organs. The relative dose intensity (RDI) of UFT/LV therapy was calculated as follows: RDI = total administered dose divided by the total planned dose according to the study protocol × 100 (%).

3. Results

3.1. *Patients characteristics*

From January 2004 through December 2010 a total of 180 patients were enrolled at 11 hospitals. Ten patients were excluded, and the other 170 were included in safety analysis (88 in the surgery alone group and 82 in the UFT/LV group) (Figure 1). The reasons for exclusion were as follows: 2 patients assigned to the surgery alone group mistakenly received UFT/LV

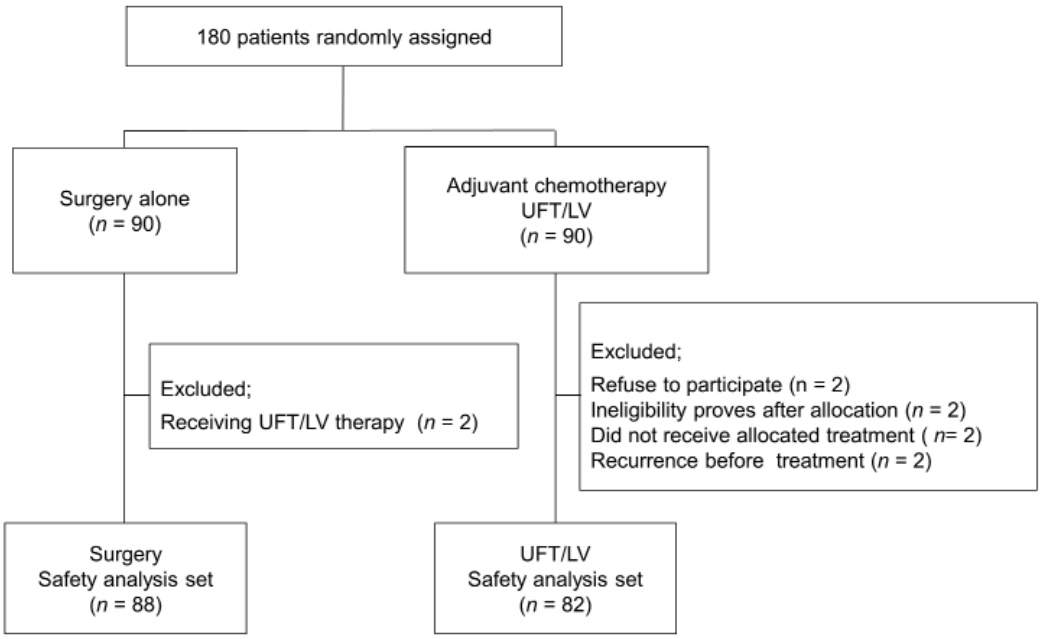

Figure 1. CONSORT diagram

Table 1. Baseline characteristics

Characteristics	UFT/LV (n = 90) n (%)	Surgery alone (n = 90) n (%)
Sex		
Male	59 (65.6)	63 (70.0)
Female	31 (34.4)	27 (30.0)
Age (years), mean (S.D.)	62.2 (8.5)	64.5 (9.2)
Location of primary tumor		
Colon	54 (60.0)	59 (65.6)
Rectum	36 (40.0)	31 (34.4)
Tumor number		
Single	38 (42.2)	44 (48.9)
Multiple	52 (57.8)	46 (51.1)
Size of largest tumor (mm)		
≤ 30	46 (51.1)	49 (54.4)
30 < ≤ 50	23 (25.6)	23 (25.6)
50 <	21 (23.3)	18 (20.0)
Timing of liver metastasis		
Synchronous	39 (43.3)	40 (44.4)
Metachronous	51 (56.7)	50 (55.6)
Type of hepatectomy		
Partial resection	61 (67.8)	61 (67.8)
Subsegmentectomy	2 (2.2)	6 (6.7)
Segmentectomy	13 (14.4)	7 (7.8)
Lobectomy	14 (15.6)	16 (17.7)

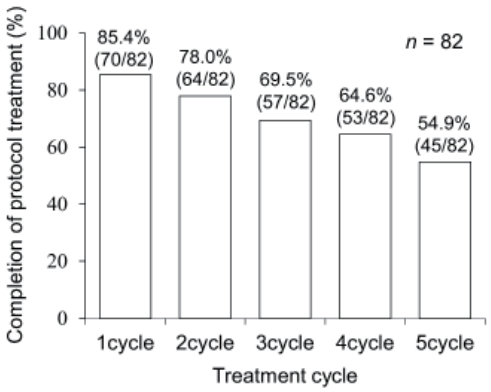

Figure 2. Completion of protocol treatment including those who had recurrence or discontinued treatment.

therapy, and 8 patients assigned to the UFT/LV group did not receive the study drugs. Table 1 shows the baseline characteristics of all enrolled patients (Table 1).

3.2. *Treatment status*

Among the 82 patients who received UFT/LV therapy, 45 (54.9%) completed the protocol treatment (5 courses). The proportion of patients according to the number of completed courses of protocol treatment was 85.4% (70 patients) for 1 course, 78.0% (64 patients) for 2 courses, 69.5% (57 patients) for 3 courses, and 64.6% (53 patients) for 4 courses (Figure 2). The protocol treatment was discontinued in 37 patients. The main reasons for treatment withdrawal were adverse events in 26 patients (70.3%), 19 of which discontinued because of the patient's or physician's discretion, recurrence in 8 (21.6%) and unknown reasons in 3 (8.1%). The most common cause of treatment withdrawal due to adverse events was grade 3 or 4 diarrhea, and treatment withdrawal at the patient's or physician's discretion were grade 1 or 2 mild adverse events (grade 2: diarrhea was common; grade 1: anorexia, stomatitis, diarrhea were common). The RDI of UFT/LV therapy was 70.8%, with a median value of 90.0% (Figure 3).

3.3. *Safety*

Among the 82 patients in the UFT/LV group who were included in the safety analysis, 67 (81.7%) had adverse events (all grades), and 10 (12.2%) had grade 3 or 4 adverse events. Table 2 shows the adverse event profiles of the patients who were included in safety analysis. Grade 3 or 4 hematologic toxicity developing after UFT/LV therapy comprised decreased hemoglobin levels in 3 patients (3.7%) and febrile neutropenia in 1 (1.2%). Grade 3 or 4 nonhematologic toxicity

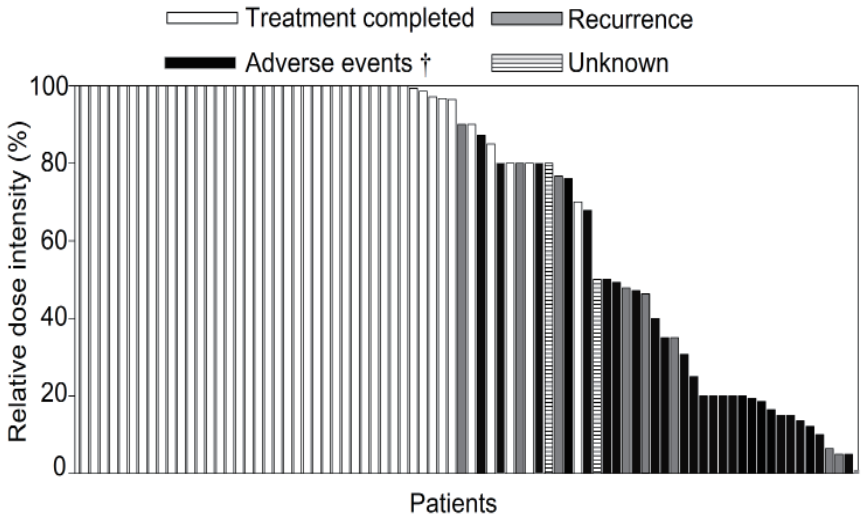

Figure 3. Relative dose intensity in the UFT/LV group including patients with recurrence. Each bar represents the percentage of relative dose intensity in each patient. The main reasons for treatment withdrawal were adverse events in 26 (70.3%, black bar), recurrence in 8 (21.6%, gray bar) and unknown in 3 (8.1%, gray bar with horizontal line). † 19 patients discontinued treatment because of the patient's or physician's judgment.

Table 2. Frequency of common toxic effects (worst grade)

Adverse events	UFT/LV (*n* = 82)				Surgery (*n* = 88)			
	Grade1, 2		Grade3, 4		Grade1, 2		Grade3, 4	
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)
Leukocytes	12	14.6	0	0	3	3.4	0	0
Platelets	10	12.2	0	0	7	8.0	0	0
Haemoglobin	13	15.9	3	3.7	7	8.0	1	1.1
Fibrile neutropenia	0	0	1	1.2	0	0	0	0
AST	13	15.9	2	2.4	4	4.5	0	0
ALT	18	22.0	1	1.2	9	10.2	0	0
Total bilirubin	22	26.8	1	1.2	5	5.7	0	0
ALP	3	3.7	0	0	1	1.1	0	0
Diarrhea	18	22.0	4	4.9	1	1.1	0	0
Anorexia	21	25.6	2	2.4	0	0	0	0
Nausea	9	11.0	2	2.4	1	1.1	0	0
Vomiting	0	0	0	0	1	1.1	0	0
Stomatitis	9	11.0	0	0	0	0	0	0
Fever	4	4.9	0	0	2	2.3	0	0
Hand-foot skin reaction	3	3.7	0	0	0	0	0	0
Hyperpigmentation	1	1.2	0	0	0	0	0	0
Dysgeusia	4	4.9	0	0	0	0	0	0
Neuropathy	1	1.2	0	0	0	0	0	0
Fatigue	5	6.1	0	0	0	0	0	0

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

comprised elevated aspartate aminotransferase levels in 2 patients (2.4%), elevated alanine aminotransferase levels in 1 (1.2%), elevated bilirubin levels in 1 (1.2%), diarrhea in 4 (4.9%), anorexia in 2 (2.4%), and nausea in 2 (2.4%). There was no treatment-related death in the UFT/LV group.

4. Discussion

Patients undergoing curative resection of primary and metastatic liver tumors have been reported to achieve approximately 35% (*17*). But relapse is common after resection with two thirds of patients (*18,19*). Adjuvant chemotherapy improves survival in patients with stage III CRC, but the role of adjuvant chemotherapy after resection of CRLM is still unknown. In this paper, we reported the safety and feasibility data from the multi-center phase III study of 180 patients with CRLM, who underwent UFT/LV or surgery alone. UFT/LV is one of the most widely used regimens and is recommended as a standard care for postoperative adjuvant chemotherapy for CRC in Japan.

In the NSABP C-06 trial, conducted in the United, 95.3% of the 774 patients who received UFT/LV therapy had adverse events (grade 3 or higher adverse events, 38.2%) (*15*). In the ACTS-CC trial (Clinical Trials.gov: No. NCT00660894), a phase III controlled study designed to verify the noninferiority of S-1 to UFT/LV, a total of 1,535 patients have been enrolled, among whom 748 received UFT/LV therapy. Mochizuki *et al*. have reported on safety in the ACTS-CC trial (*20*). In the UFT/LV group, the incidence of adverse events was 73.7% for all grades and 14.4% for grades 3 or higher. The completion rate of UFT/LV therapy was 73.4%, and the RDI was 76.0%. Recently presented data suggest outcome (*21*).

In the JCOG0205 trial (*22*), the 3-year disease-free survival, the primary endpoint of the study, was 79.3% in the UFT/LV group and 77.8% in the 5-fluorouracil/LV group (hazard ratio = 1.016, 91.3% confidence interval, 0.838 to 1.232, one-sided p = 0.0236), demonstrating the non-inferiority of UFT/LV therapy to 5-fluorouracil/LV therapy. The completion rate of protocol treatment was 78% in both groups combined, indicating good treatment continuity. As for safety, the incidence of grade 3 or 4 increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels was higher in UFT/LV, whereas the incidences of diarrhea and anorexia were similar in the groups. The results of this study showed that the safety of UFT/LV for CRLM is similar when used as postoperative adjuvant chemotherapy in patients with stage III CRC.

The question of whether postoperative chemotherapy should be useful may be considered marginal. Portier *et al*. performed a controlled study (FFCD9002 trial) to compare surgery alone with 6 months of treatment with 5-fluorouracil/LV in patients who underwent curative resection of liver metastases. The 5-year disease free survival (DFS) was significantly better in the 5-fluorouracil/LV group, but there was no significant difference between the groups in overall survival. Although protocol treatment was completed in 54 (66.7%) of 81 patients, 20 patients (24.7%) in the 5-fluorouracil/LV group had grade 3 or higher adverse events such as hematologic toxicity, stomatitis, nausea, and diarrhea. Twelve patients (14.8%) experienced more than grade 3 to 4 toxicity (*23*). In the CPT-

Table 3. Feasibility of different chemotherapeutic regimens in previous studies in patients with initially reserved liver metastases from colorectal cancer

References	Number of patients	Randomised postoperative treatments	Complete treatment rate
Portier G *et al*., 2006 (*23*)	86 *vs*. 87	Systemic FU/FA *vs*. surgery alone	66.7% —
Ychou M *et al*., 2009 (*24*)	153 *vs*. 153	Systemic FU/FA *vs*. FOLFIRI	82% 75%
	Number of patients	Randomised perioperative treatments	Complete treatment rate
Nordlinger B *et al*., 2008 (*17*)	182 *vs*. 182	PeriOpCT *vs*. surgery alone	84% (preOp) 70% (postOp)*

Abbreviations: FU, fluorouracil; FA, folinic acid; PeriOpCT, perioperative chemotherapy with FOLFOX4; preOp, preoperative chemotherapy; postop, postoperative chemotherapy. * 115 patients started postoperative chemotherapy, of whom 80 (70%) received six cycles.

GMA-301 trial (*24*), which compared the usefulness of FOLFIRI (folinic acid, 5-fluorouracil, and irinotecan) therapy with that of 5-fluorouracil/LV therapy, there was no statistically significant difference in the primary endpoint of disease-free survival between the groups. The incidence of grade 3 or 4 toxicity was 30% in the 5-fluorouracil/LV group and 47% in the FOLFIRI group. In the FOLFIRI group, 22 patients (14%) had grade 3 or 4 diarrhea, and 36 (23%) had grade 3 or 4 neutropenia. Even in the 5-fluorouracil/LV control group, 11 patients (7%) had grade 3 or higher diarrhea, and 10 (7%) had neutropenia (Table 3). For these reasons, our results indicate that treatment UFT/LV after curative resection of liver metastases is associated with a lower incidence of grade 3 or 4 adverse events than conventional 5-fluorouracil/LV therapy and is well tolerated.

Further improvements in treatment completion and adherence are required for postoperative adjuvant chemotherapy with UFT/LV to contribute to patient outcomes in clinical practice. In the FFCD trial and CPT-GMA-301 trial, the protocol treatment completion rate was 65% to 80%. These results suggested that there is room for further improvement in the treatment completion rate of this regimen. In our study, the protocol treatment completion rate was 54.9% (45 of 82 patients, including those who discontinued treatment because of recurrence and 60.8% (45/74) when patients who discontinued treatment because of recurrence were excluded. Only 7 patients discontinued treatment because of grade 3 or higher adverse events that met the criteria for the withdrawal of protocol treatment. In about half of the patients who discontinued protocol treatment, therapy was withdrawn at the patient's request or physician's discretion because of grade 1 or 2 adverse events. Patient enrollment in our study was started in 2004. When the study began, UFT/LV therapy was not recognized to be a standard regimen for postoperative adjuvant chemotherapy in patients with colorectal cancer. Consequently, treatment was withdrawn in some patients because of relatively mild grade 2 or lower adverse events, leading to a treatment completion rate of only 55%. Despite the debatable results, the RDI including patients who had recurrence or discontinued treatment was 70.8%, a median value of 90.0%, and without recurrence during chemotherapy was 73.2% and 97.9%, respectively. As for mild adverse events, however, the compliance of individual patients can most likely be improved by obtaining fully informed consent before treatment and appropriate dose modification of drugs. UFT/LV therapy is thus considered a promising regimen for postoperative adjuvant chemotherapy in patients who undergo resection for liver metastases from colorectal cancer.

Recently, Nordlinger *et al*. reported the EORTC trial (40983) (*17*). In that study, secondary evaluations of eligible patients and those who underwent hepatectomy showed that the 3-year progression-free survival (PFS) significantly differed between the perioperative chemotherapy group and the surgery alone group. However, an intention-to-treat analysis revealed that the 3-year PFS did not differ significantly between the groups (28.1% *vs*. 35.4%, $p = 0.058$). The final results for the secondary endpoint of overall survival were presented at the 2012 annual meeting of American Society of Clinical Oncology (ASCO). The addition of perioperative chemotherapy to resection led to no significant improvement in long term survival (HR 0.87, 0.66-1.14, $p = 0.303$), but there was a mere 4% improvement in the FOLFOX4 arm after 5-years (*25*). Perioperative FOLFOX therapy is considered a high-risk chemotherapeutic regimen in terms of safety. In a previous study, the completion rate of preoperative FOLFOX therapy according to protocol was 84% (143/171). A total of 115 patients could receive postoperative adjuvant chemotherapy, but only 80 (44%) were able to complete postoperative FOLFOX therapy (Table 3) (*17*). Grade 3 or 4 adverse events occurring during preoperative and postoperative therapy were leukopenia (preoperative chemotherapy 6% *vs*. postoperative chemotherapy 12%), neutropenia (18% *vs*. 35%), diarrhea (8% *vs*. 5%), nausea (4% *vs*. 4%) and peripheral neuropathy (2% *vs*. 10%). These results indicate that perioperative chemotherapeutic regimens are far from being safe (Table 4). Moreover, the incidence of postoperative complications was

Table 4. Reported incidences of adverse events with other regimens

Items	Portier *et al*., 2006 FFCD trial (*23*)		Ychou *et al*., 2009 CPT-GMA-301 trial (*24*)				Nordlinger *et al*., 2008 EORTC40983 trial (*17*)			
	FU/LA (*n* = 81)	Surgery alone (*n* = 85)	FU/LA (*n* = 152)		FOLFIRI (*n* = 154)		Preoperative chemotherapy (*n* = 171)		Postoperative chemotherapy (*n* = 115)	
	Grade 3/4 (%)	Grade 3/4 (%)	All grades (%)	Grade 3/4 (%)	All grades (%)	Grade 3/4 (%)	All grades (%)	Grade 3/4 (%)	All grades (%)	Grade 3/4 (%)
Leukocytes	} 7.4%*	—	7	1	18	3	—	6	—	12†
Neutropenia		—	16	7	41	23	—	18	—	35
Haemoglobin		—	—	—	—	—	—	1†	—	1†
Diarrhea	8.6	—	51	7	60	14	—	8†	—	5†
Constipation	—	—	15	0	24	1	—	—	—	—
Stomatitis	7.4	—	26	0	21	1	—	7†	—	0
Nausea	7.4	—	59	3	75	3	—	4†	—	4†
Vomiting	—	—	24	3	42	5	—	4	—	3†
Anorexia	—	—	12	0	20	1	—	—	—	—
Anemia	—	—	12	0	12	0	—	—	—	—
Fatigue	—	—	16	1	21	1	—	—	—	—
Neuropathy	2.5	—	—	—	—	—	—	2†	—	10†
Dysgeusia	—	—	—	—	—	—	—	2†	—	4†
Hand-foot skin syndrome	—	—	—	—	—	—	—	0	—	1

† No grade 4 reported. * Hematologic event was 7.4%.

significantly higher in the chemotherapy group (25% *vs*. 16%, $p = 0.04$).

In particular, liver disorders caused by irinotecan-based regimen (FOLFIRI) and oxaliplation-based regimen (FOLFOX) include fatty liver, steatohepatitis (yellow liver) (*26-28*), and sinusoidal dilation (blue liver) (*29,30*). Despite these findings, the results of the EORTC 40983 trial led to the recognition of "preoperative/ postoperative chemotherapy plus surgery" as a standard therapy for resectable liver metastases in Europe (*31*). The National Comprehensive Cancer Network guidelines recommend multidisciplinary treatment combining hepatectomy and chemotherapy such as FOLFOX for the management of liver metastases (*32*). At present, the Japan Clinical Oncology Group (JCOG) is currently conducting a randomized phase II/III study (JCOG0603) comparing surgery alone with surgery plus mFOLFOX6 therapy after curative resection of liver metastasis from colorectal cancer (*33*).

UFT/LV do not require the placement of a central venous port or continuous intravenous infusion, thereby reducing system patients' stress associated with port placement, decreasing complications, and prolonging the interval between hospital visits. From the viewpoint of medical professionals, the use of oral anticancer agents reduces the time and effort required to set up infusion systems and is thus more convenient and economical.

Because patients have to receive oral medication on their own initiative, they should be instructed that it is essential to take medication as directed, and efforts should be made to show that oral anticancer agents have different adverse event profiles from those of injectable preparations. Even after the starting treatment, efforts to improve patient care by providing supportive therapy and instruction on drug administration management are required to improve adherence to treatment regimens and thereby promote the continuation of treatment while maintaining patients' quality of life. The development of adjuvant chemotherapy that prevents postoperative recurrence and substantially improves outcomes after resection of liver metastases in patients with colorectal cancer is an urgent task.

In conclusion, our results suggest that oral UFT/LV therapy is a therapeutically useful regimen. The final analysis of the data from our study is scheduled to be performed in 2014, and further detailed results are awaited.

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(Received January 28, 2014; Revised February 24, 2014; Accepted February 26, 2014)

Original Article

DOI: 10.5582/ddt.8.57

A lesson from Japan: Research and development efficiency is a key element of pharmaceutical industry consolidation process

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Summary **Scholarly attention to pharmaceutical companies' ability to sustain research and development (R&D) productivity has increased as they increasingly handle business challenges. Furthermore, the deterioration of R&D productivity has long been considered a major cause of mergers and acquisitions (M&As). This study attempts to investigate quantitatively the possible causes of the deterioration and the relationship between the deterioration and M&As by examining the Japanese pharmaceutical industry. Japan from 1980 to 1997 is an ideal case because of the availability of official data, but more importantly the significant changes in its business environment at the time. Using the Malmquist Index and data envelopment analysis, we measured the deterioration of R&D productivity from 1980 to 1997 based on a sample of 15 Japanese companies. Two lessons can be learned from Japan's case. First, to sustain R&D productivity over the long term, companies should use licensing activities and focus on the dominant therapeutic franchises. Second, if a company fails significantly to catch up with the benchmark, it is likely to pursue an M&A or seek an alternative way to improve R&D productivity. These findings appear similar to the current situation of the global pharmaceutical industry, although Japan pursued more licensing activities than M&A to improve R&D productivity.**

Keywords: R&D productivity, industry consolidation, Japanese pharmaceutical industry, data envelopment analysis

1. Introduction

Scholarly attention to pharmaceutical companies' ability to sustain research and development (R&D) productivity has increased as they increasingly handle challenges such as escalating R&D expenditure, a lack of new molecule entities (NMEs), and cost containment schemes by payors (*1,2*). Indeed, R&D expenditure in the pharmaceutical industry has increased rapidly (*3,4*), but the number of NMEs entering the market has declined (*5-11*). Some argue that the rising cost was due to the complex nature of clinical trials while development risk remained fairly stable from the 1970s to 1990s (*12-14*).

Among Japan, Europe, and the United States, R&D spending declined most in Japan. Japanese companies spent 5,161 million yen in 1990 and 12,760 million yen in 2010. European companies spent 7,766 million euros in 1990 and 27,796 million euros in 2010. U.S. companies spent 6,803 million dollars in 1990 and 40,688 million dollars in 2010. Meanwhile, R&D productivity in terms of NME development declined most in Japan as well. Japanese, European, and U.S. pharmaceutical companies developed 74, 88, and 49 NMEs, respectively between 1990 and 1994, and 36, 89, and 77 NMEs between 1995 and 2000 (*15*). Consequently, in an attempt to address the deterioration of R&D productivity, Japanese pharmaceutical companies started pursuing mergers and acquisitions (M&As) since 1995 (Table 1).

However, although the deterioration of R&D productivity has long been considered a major cause of M&As (*16-18*), few studies have investigated quantitatively the possible causes of the deterioration and the relationship between the deterioration and M&As. This study attempts to address this gap in

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Table 1. List of antibiotics approved in Japan from 1980 to 1999

	Subclass code					Total
	611	612	613	614	624	
1980-1989						
Japan Origin	2	3	17	2	3	24
Import	0	1	10	0	1	11
Licensed-in	4	1	10	0	2	15
Total	6	5	37	2	6	50
1990-1999						
Japan Origin	1	1	7	1	3	10
Import	1	0	2	2	0	5
Licensed-in	0	0	2	0	0	2
Total	2	1	11	3	3	17
Total						
Japan Origin	3	4	24	3	6	34
Import	1	1	12	2	1	16
Licensed-in	4	1	12	0	2	17
Total	8	6	48	5	9	67

the literature by examining the case of the Japanese pharmaceutical industry.

In analyzing the relationship between the deterioration of R&D productivity and industry consolidation, the Japanese pharmaceutical industry is an ideal example for at least four reasons. First, the deterioration of R&D productivity in this industry accelerated after the 1990s. Second, except when Merck obtained a minority share in Banyu in 1982, the industry did not have M&As until 1997. Third, Japanese companies developed 30 globally available NMEs in the 1980s and 1990s. The main interest of their R&D programs shifted from antibiotics in the 1980s to drugs for lifestyle diseases such as high cholesterol, hypertension, and diabetes in the 1990s (Table 2). Finally, Japan provides many official data sources. It has a universal health care coverage system, and the Ministry of Health and Welfare (MHLW) approves and sets the price for each drug to be reimbursed by patients. The MHLW also provides ethical drug production statistics for 34 efficacy classes and 177 subclasses (Table 3). Finally, pharmaceutical companies must complete and submit an interview form to the MHLW, disclosing detailed information on their approved drugs such as the origin of NMEs, in order for those drugs to be listed under the MHLW's reimbursement list.

Table 4 is a list of the number of NMEs approved by the MHLW. Antibiotics represented more than 10% of the total NMEs approved in the 1980s. However, this share dropped sharply in the 1990s because of the pharmaceutical companies' focal shift to lifestyle diseases. In this study, we verify the relationship between lifestyle drug franchises and the deterioration of R&D productivity. We consider antibiotics, digestive system, and various cardiovascular and metabolism franchises as lifestyle disease drugs.

Several studies have discussed the changes in R&D efficiency of Japanese pharmaceutical companies. One study showed that the Japanese domestic environment for pharmaceuticals changed radically from 1975 to 1995, which degraded the innovative capability of the companies (*19*). Another study emphasized the importance of understanding the dynamics of R&D investment strategies between 1975 and 1990 (*20*). Finally, one study measured and observed the deterioration of R&D productivity of Japanese pharmaceutical companies from 1983 to 1992 using a quantitative method (*21*). This study aims to investigate the possible causes of the deterioration of R&D productivity in the Japanese pharmaceutical industry in the 1990s and its consequences, using data envelopment analysis (DEA) and the Malmquist Index (*22,23*). Based on the scores from the Malmquist Index calculation, one-way ANOVA and Tukey-Kramer testing were conducted to identify the possible causes of R&D productivity deterioration from 1980 to 1997. The relationship between the deterioration of R&D productivity and M&As was also discussed.

2. Materials and Methods

2.1. *Three approaches to measure R&D productivity*

There are at least three approaches to measure R&D productivity: ratio analysis, least squares regression, and DEA. DEA is a mathematical programming approach for measuring relative efficiency, utilizing multiple inputs and outputs, while ratio analysis handles single inputs and outputs. The fundamental difference between the statistical and DEA approaches is that the former reflects the average or central tendency behavior of the observations, while the latter deals with the best performance and evaluates all performances by deviations from the efficient frontier. DEA offers at least two advantages as an empirical tool in measuring R&D efficiency. First, it does not require a data normalization process, unlike in an econometric approach. Second, it is a non-parametric approach and does not require an explicit specification of inputs and outputs.

2.2. *Variables used in this paper*

In our DEA, we select one input and three output variables to measure R&D productivity: the actual R&D expenditure as the sole input, and the accumulated number of weighted NMEs approved by the MHLW, sales, and operating profit as the three output variables. Some studies employed a multiple-variable model with the number of patent and publication submissions as input (*21*). However, the publication strategy may vary among companies, and there is little relationship between these variables and actual sales. Thus, these variables are not satisfactory indicators of input. We,

Table 2. Breakdown of antibiotics production by code number

Code number	Description	% In antibiotics production amount in each year			
		1976	1981	1991	1997
611	Antibiotic preparations acting mainly on gram-positive bacteria	2.2%	0.5%	3.0%	4.5%
612	Antibiotic preparations acting mainly on gram-positive bacteria	0.3%	3.0%	4.8%	2.8%
613	Antibiotic preparations acting mainly on gram-positive, gram-negative bacteria	68.4%	79.2%	70.3%	63.9%
614	Antibiotic preparations acting mainly on gram-positive bacteria and mycoplasma	10.5%	6.3%	3.6%	9.8%
615	Antibiotic preparations acting mainly on gram-positive, gram-negative bacteria, rickettsia and chlamydia	10.1%	3.5%	1.6%	1.7%
616	Antibiotic preparations acting mainly on acid-fast bacteria	4.5%	3.2%	0.6%	0.4%
617	Antibiotic preparations acting mainly on acid-fast bacteria	0.3%	0.3%	0.8%	0.7%
619	Other antibiotic preparations (including mixed antibiotic preparations)	1.8%	2.2%	0.1%	0.1%
624	Synthetic antibacterials (after 1991)	0.0%	0.0%	15.3%	16.2%
618	Antibiotic preparation acting mainly on a malignant tumor (before 1990)	2.0%	2.0%	0.0%	0.0%

Table 3. List of Japanese originated drugs sold over 20 countries

Period	Company	Generic name	Category
1980s	Toyama Chemical	Piperacillin	Antibiotics
	Toyama Chemical	Cefoperazone	Antibiotics
	Yamanouchi	Nicardipine	Hypertension drug
	Shionogi	Oxacephalosporin	Antibiotics
	Chugai	Nicorandil	Angina drug
	Yamanouchi	Ceftizoxime	Antibiotics
	Yamanouchi	Cefotetan	Antibiotics
	Danippon	Enoxacin	Antibiotics
	Fujisawa	Cefixime	Antibiotics
	Daiichi	Ofloxacin	Antibiotics
	Kyorin	Norfloxacin	Antibiotics
	Yamanouchi	Famotidine	Digestive drug
	Sankyo	Cefpodoxime	Antibiotics
	Sankyo	Pravastatin sodium	Cholesterol lowering drug
1990s	Taisho	Clarithromycin	Antibiotics
	Chugai	Lenograstim	Immunostimulator
	Shionogi	Ceftibuten	Antibiotics
	Takeda	Lansoprazole	Digestive drug
	Takeda	Leuprorelin acetate	Cancer drug
	Fujisawa	Tacrolimus	immunosuppressive drug
	Yamanouchi	Tamusulosin	Urinary drug
	Dainippon	Sparfloxacim	Antibiotics
	Tanabe	Imidapril Hydrochloride	Hypertension drug
	Daiichi	Levofloxacin	Antibiotics
	Yakult	Irinotecan	Cancer drug
	Sumitomo	Meropenem	Antibiotics
	Eisai	Rabeprazole	Digestive drug
	Takeda	Candesartan cilexetil	Hypertension drug
	Eisai	Donepezil	Alzheimer drug
	Takeda	Pioglitazone	Diabetics drug

Table 4. List of industry consolidation events in Japan

Year	Events	Companies
1982	Minority share acquisition	MSD; Banyu
1998	Meger (Domestic)	Yoshitomi; Green Cross
1998	Majority share acquisition	Japan Tobacco; Torii Pharmaceutical
1999	Meger (Domestic)	Mitsubishi Chemical; Tokyo Tanabe
2000	Merger (Cross border)	Schering; Mitsui Pharmaceutical
2000	Majority share acquisition	Boehringer Ingelheim; SS Pharmaceutical
2001	Merger (Domestic)	Mitsubishi Chemical; Yoshitomi
2001	Majority share acquisition	Roche; Chugai
2002	Majority share acquisition	Taisho Pharmaceutical; Toyama Chemical
2003	Merger (Cross border)	MSD; Banyu
2003	Merger (Cross border)	Abbott; Hokuriku
2005	Merger (Domestic)	Yamanouchi; Fujisawa
2005	Merger (Domestic)	Sumitomo Chemical; Dainippon
2007	Merger (Domestic)	Daiichi; Sankyo
2007	Merger (Domestic)	Mitsubishi Chemical; Tanabe
2007	Merger (Cross border)	Eisai; Morphotek
2007	Merger (Cross border)	Astellas; Agensys
2008	Merger (Cross border)	Eisai; MGI Pharma
2008	Merger (Cross border)	Takeda; Amgen Japan
2008	Merger (Cross border)	Takeda; Millennium Pharmaceuticals
2008	Majority share acquisition	Daiichi Sankyo; Ranbaxy
2008	Merger (Cross border)	Shionogi; Sciele Pharma
2008	Merger (Cross border)	Fuji Film Holdings/ Taisho; Toyama Chemical
2009	Merger (Cross border)	Dainippon Sumitomo; Sepracor
2009	Merger (Cross border)	Hisamitsu; Noven Pharmaceuticals
2009	Merger (Cross border)	Eisai; AkaRx
2010	Merger (Cross border)	Astellas; OSI Pharmaceuticals
2011	Merger (Cross border)	Shionogi; C&O Pharmaceutical Technology
2011	Merger (Cross border)	Kyowa Hakko Kirin; ProStrakan
2011	Merger (Cross border)	Daiichi Sankyo; Plexxikon
2011	Merger (Cross border)	Takeda; Nycomed
2011	Merger (Cross border)	Taisho Pharmaceutical; Hoepharma

instead, use the actual number of NMEs approved by the MHLW. The R&D expenditure of a particular year was averaged over three years to consider accounting time delay of R&D expenditure. The time lag between the R&D expenditure and its outcome was assumed to be eight years (*24,25*). The "Annual Statistical Survey on Trends in Pharmaceutical Production" published by the MHLW was employed to determine the number of drugs. An interview form provided by the company that seeks approval from the MHLW was employed to identify the originator of the drugs for each NME under consideration. To distinguish between internal and licensed NMEs, cost allocation among the clinical phases was considered. The average expected cost of the clinical period was 60.6 million dollars in 2000, and the expected cost in Phase III was 27.1 million dollars or 44.7% of the total clinical cost (*10*). There are two basic methods for a company to receive approval from the MHLW: *i*) registering as an original drug developer

and *ii*) registering as a co-development partner. Because there was little information on the clinical stage of the licensed NMEs, we set the weight for a licensed-in NME as 50% and a co-development NME as 20% of the R&D expenditure prior to the NME's approval.

2.3. *Definition of the Malmquist Index and its components*

The Malmquist Index was employed to identify the historical change in R&D productivity since a historical trend of DEA scores of R&D productivity does not reveal the causes of changes (*23*). The Malmquist Index score (MI score) was 1.00 if there was no change in R&D productivity, less than 1.00 if there was any improvement in R&D productivity, and greater than 1.00 if there was any deterioration in R&D productivity. The MI score can be decomposed into two mutually exclusive scores: the efficiency change (EC) and frontier shift (FS) scores. The EC score measures changes in how companies catch up to the industry benchmark from one period to another. The FS score measures changes in the efficient frontier, which is an industry-based R&D productivity benchmark in a given year. If R&D productivity deteriorates, both scores are greater than 1.00. The Bartlett test of homogeneity of variances, ANOVA, and Tukey-Kramer test were conducted to identify causes of the deterioration of R&D productivity.

2.4. *Data exclusion criteria*

We selected 24 companies originally but obtained a final sample of 15 companies after applying the following exclusion criteria: *i*) availability of financial data and *ii*) significant change in management control. We selected 1980 as the start of the study period because this was when the MHLW started the current approval system and 1997 as the end of the period because this marked the end of the M&A period in Japan; data on R&D expenditure, sales, and operating profit after 1997 may be distorted due to post-M&A processes such as restructuring and R&D reviews.

3. Results and Discussion

3.1. *Deterioration of the R&D productivity of the Japanese companies from 1980 to 1997*

Table 5 shows that the R&D productivity of the 15 Japanese companies declined from 1980 to 1997 and that R&D expenditures that were 2.10 times greater were required in 1997 to generate the same level of output in 1980 (MI score = 2.10). This finding is similar to those of Hashimoto and Haneda (*3*). This deterioration was mainly due to the decline of the industry benchmark (FS score = 2.08) and the efforts of companies to catch up (EC score = 1.01).

3.2. *A relationship between the R&D productivity and antibiotics R&D strategy in 1980s*

The results of the ANOVA tests show that the changes in R&D productivity differed among companies that developed antibiotics in the 1980s ($p < 0.05$) and among companies that developed different antibiotics subclasses, that is, '613 and '624' ($p < 0.05$). However, continuing antibiotics research did not explain the dispersion of R&D productivity among the 15 Japanese companies (Table 6). Table 7 shows that antibiotics approvals in the 1980s explained the dispersion of R&D productivity deterioration, but companies' approaches toward antibiotics (*i.e.*, internally or using licensing activities) did not explain the dispersion. Table 8 shows similar results but does not show that a shift from one

Table 5. List of financial data for 15 companies in 1980 and 1997

Name	1980			1997		
	Sales	R&D Expense	Operating Profit	Sales	R&D Expense	Operating Profit
Chugai	71,353	2,531	11,293	164,102	21,986	17,098
Daiichi	73,596	2,880	10,201	232,565	22,951	42,125
Dainippon	53,195	2,204	3,265	137,595	10,511	6,508
Eisai	103,365	4,012	18,575	258,655	30,473	45,711
Fujisawa	155,906	4,841	27,230	215,162	28,262	19,772
Kaken	19,394	990	1,667	63,519	5,676	2,846
Nippon Shinyaku	34,636	1,194	4,238	48,201	6,513	3,299
Sankyo	187,196	4,135	21,422	462,551	33,583	126,002
Shionogi	142,304	5,837	17,345	211,679	25,518	15,363
Takeda	430,883	11,858	37,199	640,094	54,770	104,250
Tanabe	114,544	4,217	16,116	181,976	19,777	16,156
Tokyo Tanabe	22,936	326	2,921	43,414	3,475	3,485
Toyama Chemical	31,865	775	5,299	42,776	5,581	3,912
Yamanouchi	76,601	3,169	12,090	317,780	28,607	67,175
Yoshitomi	44,106	1,998	5,617	109,170	10,099	12,001
Average	104,125	3,398	12,965	208,616	20,519	32,380
St. Dev.	103,751	2,836	10,162	165,811	13,992	38,551

Table 6. List of drug developers and names of major approved products

Name	Sales in JPY million	Antibiotics			Lifestyle disease drug	Digestive drug	Major drug approved between 1980 and 1997
		Internally developed (I), Licensed (L), or None (N)	Internally developed between 1980 and 1997	Focus on subclass	Internally developed (I), Licensed (L), or None (N)	Internally developed (I), Licensed (L), or None (N)	
Chugai	> 50	N	N	No development	I	L	Epoetin β
Daiichi	> 50	I	Y	New quinolone	I	L	Levofloxacin
Dainippon	> 50	I	Y	New quinolone	I	L	Flomoxef sodium
Eisai	> 100	N	N	No development	I	I	Teprenone
Fujisawa	> 100	I	Y	Cepham	I	L	Tacrolimus hydarate
Kaken	< 50	L	Y	Cepham	N	N	Beraprost sodium
Nippon Shinyaku	< 50	N	N	No development	N	I	Irsogladine maleate
Sankyo	> 100	I	Y	Both cepham and new quinolone	I	I	Pravastatin sodium
Shionogi	> 100	I	Y	Both cepham and new quinolone	L	L	Latamoxef sodium
Takeda	> 100	I	Y	Cepham	I	I	Lansoprazole
Tanabe	> 100	I	Y	Cepham	N	L	Imidapril hydorchoride
Tokyo Tanabe	< 50	N	N	No development	N	N	Ranimustine
Toyama Chemical	< 50	I	Y	Both cepham and new quinolone	L	I	Cefetram pivoxil
Yamanouchi	> 50	I	Y	Cepham	I	I	Famotidine
Yoshitomi	< 50	L	N	Cepham	I	L	Etizolam

Table 7. MI score of the R&D productivity for 15 Japanese companies in 1997 and its components of MI score

Company	Malmquist Index	Efficiency Change	Frontier Shift
Chugai	3.19	1.24	2.56
Nippon Shinyaku	2.69	1.06	2.55
Tokyo Tanabe	2.57	1.09	2.36
Fujisawa	2.50	1.32	1.90
Eisai	2.42	1.17	2.07
Toyama Chemical	2.29	1.12	2.04
Takeda	2.15	1.04	2.06
Sankyo	2.05	1.00	2.05
Tanabe	2.04	0.98	2.08
Kaken	1.86	0.84	2.21
Dainippon	1.68	0.81	2.06
Daiichi	1.61	1.00	1.61
Yoshitomi	1.57	0.79	1.99
Yamanouchi	1.51	0.82	1.84
Shionogi	1.40	0.75	1.86
Average	2.10	1.00	2.08

Table 8. Summary of statistical results on R&D productivity

	Barlett Testing	ANOVA
Size Effect	0.376	0.768
Antibiotics Approval in 1980s	0.811	0.010***
Lifestyle diseases drug approval in 1980s	0.818	0.579
Digestive drug approval in 1980s	0.407	0.823
Antibiotics approval in 1980s and 1990s	0.696	0.914
Antibiotics Subclasses	0.347	0.011**

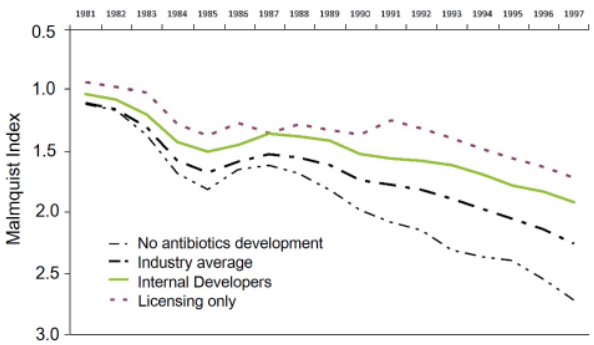

Figure 1. Trends of MI indices of R&D productivity grouped by antibiotics development strategies

subclass to another was a factor.

The results of our analysis suggest that the deterioration of R&D productivity was a major issue in the Japanese pharmaceutical industry and that involvement in antibiotics R&D helped sustain the R&D productivity of Japanese pharmaceutical companies in the 1980s. Figure 1 shows that the R&D productivity of companies utilizing licensing activities deteriorated, although the deterioration from 1980 to 1997 was not statistically significant.

3.3. *Interpretations of the R&D deterioration among Japanese companies using the Malmquist Index*

Table 9 shows the decomposition of the Malmquist Index into two components. It illustrates that while the R&D productivity of companies with no approved antibiotics deteriorated significantly, through licensing activities, they were able to catch up with the industry benchmark with an 18% improvement (EC score = 0.82), and internal efforts to develop antibiotics were slightly helped (EC score = 0.98). These results suggest that licensing activities were more useful than internal development for Japanese companies in sustaining R&D productivity in the 1980s.

Furthermore, Table 10 shows that the development of a new subclass of antibiotics also helped sustain R&D productivity (EC score = 0.91) even though the Tukey-Kramer test did not show this factor was statistically significant. The development of subclass '613', the dominant subclass in the 1980s, had a marginal impact on the ability to sustain R&D productivity (EC score = 0.97).

Pharmaceutical company Chugai, which had the worst MI score, merged with Roche in 2000. Similarly, Tokyo Tanabe, which had the third-worst MI score, merged with Mitsubishi Chemical in 1999 (Table 5). This finding is consistent with those of LaMattina (*17*), which suggest that without an appropriate R&D strategy or improvement of R&D productivity, the industry will continue to pursue M&As in the near future. However, an M&A is not always an appropriate solution since the best fit may not be available at the time of decision making. Fujisawa, which had the fourth-worst MI score, withdrew its generic drug business from the United States in 1998. This study showed that the R&D productivity deterioration in the industry may explain why companies with the worst productivity scores entered into M&As within a few years of the deterioration. We conclude that the deterioration of R&D productivity was a possible cause of industry consolidation in the 1990s in Japan, albeit further study may be required to verify the causal relationship between these two phenomena.

3.4. *Implications for the current pharmaceutical industry*

Two lessons can be learned from Japan's case. First, to sustain R&D productivity over the long term, companies should use licensing activities and focus on the dominant therapeutic franchises, even on only the most advanced subclass. Second, if a company fails significantly to catch up with the benchmark, it is likely to pursue an M&A or seek an alternative way to improve R&D productivity.

Though the study focused on the Japanese pharmaceutical industry from 1980 to 1997, it made a few interesting observations that can be applied to today's global pharmaceutical industry. The global industry seems to have entered a similar situation, but this assumption needs to be verified quantitatively. In the 1990s, research focus shifted from antibiotics to lifestyle disease drugs. Recently, this focus shifted to cancer and vaccine franchises. The number of NMEs approved by the U.S. Food and Drug Administration declined from 1996 to 2010. Thus, just as Japanese companies pursued licensing in the late 1980s to improve their R&D productivity, global companies sought M&As in the 2000s to sustain their R&D productivity. For example, Roche acquired leading cancer drug developer Genentech in 2008. To improve its R&D capability in anti-cancer drugs, Takeda acquired U.S. bioventure Millennium Pharmaceuticals in 2008 for 1 billion dollars. Likewise, to accelerate its vaccine research, Pfizer acquired Wyeth in 2009.

Due to the issue of data availability, we excluded NMEs undergoing clinical trials, even though such NMEs are an important component of R&D productivity. Thus, this study shows only the R&D productivity of companies positioning themselves within the industry.

Table 9. Statistical results of antibiotics development involvement in 1980s

		Tukey-Kramer		
		Subgroup 1	Subgroup 2	Subgroup 3
Subgroup 1	No antibiotics approval in 1980s	--	--	--
Subgroup 2	Approved licensed-in antibiotics in 1980s	0.017**	--	--
Subgroup 3	Approved internally developed antibiotics in 1980s	0.007***	0.758	--

Table 10. Average score of MI Index and its components, with subgroups defined by the company's antibiotics development strategy

	Malmquist Index	Efficiency Index	Frontier Shift Index
Average of companies with internally developed antibiotics	1.91	0.98	1.95
Average of companies licensed in antibiotics	1.71	0.82	2.10
Average of companies with only subclass 624 development	1.65	0.91	1.84
Average of companies with only subclass 613 development	1.95	0.97	2.02
Average of companies with both subclass 613 and 624 development	1.84	0.94	1.95
Average of companies with no antibiotics approval product	2.72	1.14	2.38

However, if internal data for ongoing R&D programs for each therapeutic franchise can be obtained, it is possible to monitor changes in R&D productivity within a company such as by using the net present value of each NME in the R&D expenditure by therapeutic class. In this way, management can not only monitor changes in R&D productivity relative to the industry benchmark but also analyze how each R&D program affects the company's overall R&D productivity regularly. This study also helps health care professionals and scientists monitor the progress of each R&D program using the same parameters and understand the reasons for any dispersion from the benchmark. The outcomes may help management allocate resources efficiently.

Sustaining R&D productivity has become a top priority of pharmaceutical companies. The methodology developed in this paper would enable management to monitor changes in R&D productivity relative to the benchmark, understand causes of any dispersion, and consider appropriate measures to resolve issues.

This study illustrated the importance of focusing on dominant therapeutics and the usefulness of licensing activities, and identified a possible cause of deterioration of R&D productivity in the Japanese pharmaceutical industry. The study also found that the deterioration of R&D productivity is a possible cause of M&As, albeit there may be other causes. Tools for monitoring R&D productivity within a company and the industry have become more important as the R&D productivity of global pharmaceuticals continues to decline. Our methodology will enable management to monitor changes in R&D productivity quantitatively and identify an appropriate R&D strategy.

3.5. *Limitations*

Despite using the DEA and Malmquist Index approaches, this study has at least two limitations. First, DEA does not measure absolute efficiency and is sensitive to data selection. Second, we selected the Japanese industry due to data availability. To obtain generalizable results on the relationship between the deterioration of R&D productivity and M&As, future studies should use a more recent global industry data set.

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(Received January 8, 2014; Revised January 20, 2014; Re-revised January 21, 2014; Accepted January 24, 2014)

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