Shikonin Inhibited Growth and N-acetylation of 2-aminofluorene in Bacteria Strains *Staphylococcus aureus* and *Klebsiella pneumoniae*

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Objectives. N-acetyltransferase (NAT) is the first step in the metabolism (N-acetylation) of arylamine carcinogens (2-aminofluorene) and drugs (sulfamethazine). Angiogenesis is critical for tumor growth and inflammation, and shikonin has been reported to inhibit angiogenesis and induce apoptosis *in vivo* and *in vitro*. Therefore, our objective was to investigate the effect shikonin has on growth and N-acetylation of 2-aminofluorene in bacteria.
Methods. In this study, growth inhibition of *S. aureus* and *K. pneumoniae* was determined by measuring absorbance by an optical density method (OD at 650 nm) using a Beckman Spectrophotometer (DU 6401). We examined arylamine N-acetyltransferase (NAT) activity in the bacteria *S. aureus* and *K. pneumoniae* collected from patients and examined the levels of N-acetylation of 2-aminofluorene by high performance liquid chromatography.
Results. Shikonin elicited dose-dependent bacteriostatic activity in both examined bacteria cultures. Cytosols and suspensions of *S. aureus* and *K. pneumoniae* with and without specific concentrations of shikonin co-treatment showed different percentages of 2-aminofluorene acetylation. The data indicated that the decrease in N-acetylation of 2-aminofluorene was associated with increased levels of shikonin in both examined bacteria cytosols and intact cells. The apparent values of Km and Vmax decreased after co-treatment with 4 μM shikonin.
Conclusions. Shikonin induces inhibition of growth and inhibition of arylamine NAT activity (N-acetylation of 2-aminofluorene) in *S. aureus* and *K. pneumoniae*. **(Mid Taiwan J Med 2003;8:119-26)**

Key words

2-aminofluorene, *K. pneumoniae*, N-acetyl-2-aminofluorene, N-acetyltransferase, *S. aureus,* shikonin

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INTRODUCTION

Exposure to environmental and occupational chemical carcinogens is an important cause of human cancer. Arylamines are one of the most potent carcinogens which induce

tumors in humans. They need to be activated by NAT to form metabolites which are capable of combining covalently with cellular DNA in order to form tumors. N-acetylation is the first step in the metabolic pathway for arylamine drugs and carcinogens and is catalyzed by cytosolic arylamine N-acetyltransferase (NAT) using acetyl coenzyme A as a cofactor [1]. When 2-aminofluorene (AF), a type of arylamine carcinogen, is N-acetylated, it forms 2-acetylaminofluorene (AAF). Its further metabolic activation by other enzymes induces carcinogenesis in target tissues and organs [2]. Humans exhibit a genetic polymorphism which effects NAT activity and results in rapid and slow acetylator phenotypes [3,4]. Statistical and epidemiological studies have indicated a higher risk for the rapid acetylator phenotype in colorectal cancer [5,6], as well as the slow acetylator phenotype in bladder cancer [7]. Thus, the genetic variations in N-acetyltransferase activity for arylamine-induced neoplasms within target organs may indicate different risks among the human population.

Shikonin, an ingredient of Zicao plants such as *Lithospermum Erythrohizon*, has been reported to be able to inhibit angiogenesis *in vivo* and *in vitro* [8]. It is well known that angiogenesis is critical for tumor growth and inflammation reactions. Shikonin has been shown to induce apoptosis in the HL-60 human premyelocytic leukemia cell line [9], topoisomerase II-mediated DNA cleavage *in vitro* [10], and to stimulate glucose uptake in 3T3-L1 adipocytes via an insulin-independent tyrosine kinase pathway [11]. Recently, it was reported that shikonin could induce the inhibition of human telomerase [12]. N-acetyltransferase activitiy has been demonstrated to exist in some human gastrointestinal flora [13]. Until now, there has been no available information which addresses the effects of shikonin on growth and NAT activity (N-acetylation of 2-aminofluorene) in *S. aureus* and *K. pneumoniae*. Thus, the present study was performed to determine whether or not shikonin effects growth and NAT activity in *S. aureus* and *K. pneumoniae*

MATERIALS AND METHODS

Chemicals and Reagents

Shikonin was obtained from Ichimaru Pharcos Co. Ltd (Gifu-Pref, Japan). Ethylenediaminetetraacetic acid (EDTA), acetyl carnitine, 2-aminofluorene (AF), 2-acetyl-aminofluorene (AAF), Tris, leupeptin, bovine serum albumin (BSA), phenylmethyl-sulfonylfluoride (PMSF), dithiothreitol (DTT), carnitine acetyltransferase, and acetyl-Coenzyme A (Ac-CoA) were obtained from Sigma Chemical Co. (St. Louis. MO). Acetic acid, acetonitrile, dimethyl sulfoxide (DMSO), and potassium phosphates were obtained from Merck Co (Darmasstadt, Germany). The API NH kit for identification of bacteria strains was obtained from BioMerieux Vitek, Inc. (Hazelwood, MO). The Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). All chemicals used were reagent grade.

Preparation of Bacteria Cytosol and Intact Suspensions

S. aureus and *K. pneumoniae* were isolated from patients at the China Medical University Hospital and identified by the API NH kit. *S. aureus* and *K. pneumoniae* were incubated seperately in 100 mL trypticase soy broth at 35°C for 2 days, then centrifuged at 3500 g at 4°C for 20 min. The pellet was washed twice in cold phosphate buffered saline and used for the following two procedures: 1) For the cytosol NAT assay, the bacterial pellet (about 1 mL) was placed immediately in 2 mL of lysis buffer [20 mM tris-HCl, pH 7.5 (at 4°C), 1 mM DTT, 1 mM EDTA, 50 mM PMSF, and 10 mM leupeptin], and disrupted by a sonicator and centrifuged for 30 min at 10,000 rpm. The supernatant was kept on ice until assayed for NAT activity. 2) The pellet was resuspended in 2 mL trypticase soy broth for all intact bacteria assays. The optical density (OD) of the intact bacterial cell suspension was determined in triplicate by a Beckman Spectrophotometer DU 6401. The Colony Forming Unit (CFU) was derived from a standard

curve correlating OD with plate counts.

Bacteriological Part

Effects of Various Concentrations of Shikonin on Growth of S. aureus and K. pneumoniae. About 1×10^8 bacteria were placed in individual tubes containing 1 mL trypticase soy broth with different concentrations of shikonin (0.04, 0.4, 4 and 40 μM) (study group) and without shikonin (control group). The culture tubes were incubated at 37°C with 5% CO_2 and checked for growth after 24 h. The determination of the effects of shikonin on *S. aureus* and *K. pneumoniae* strains was based on the measurement of the absorbance by an optical density method (OD at 650 nm) using a Beckman Spectrophotometer (DU 6401). The control groups were prepared under the same conditions as the shikonin treated groups except for the addition of shikonin. All experiments and controls were run in triplicate. Growth inhibition (%) was calculated by the following equation [13].

$$\text{Growth inhibition (\%)} = 1 - \left(\frac{\text{Original OD-final OD (including shikonin)}}{\text{Original OD}} \times 100\right)$$

Biochemical Part

Preparation of S. aureus and K. pneumoniae Cytosols. A group of 10×10^{10} colony forming units (CFU) of *S. aureus* and an equal group of *K. pneumoniae* were washed twice in cold phosphate buffered saline (PBS) and then immediately placed in 1 mL of lysis buffer [20 mM tris-HCl, pH 7.5 (at 4°C), 1 mM DTT, 1 mM EDTA, 50 mM PMSF, and 10 mM leupeptin] respectively. The cell suspensions were disrupted in a sonicator (Hert Systems. Inc. Farmingdale, NY; USA) and centrifuged for 30 min at 10,000 rpm. The supernatant was kept on ice until assayed for NAT activity (N-acetylation of AF) and for protein determination [13,14].

NAT Activity Determination. The determination of total amounts of acetylated and nonacetylated substrate (AF) was carried out by high performance liquid chromatography as described by Chung et al [13].

Protein Determination. Protein concentrations of *S. aureus* and *K. pneumoniae* cytosols were determined with bovine serum albumin as the standard according to the method by Bradford at al [15]. All of the samples were assayed in triplicate.

Effects of Various Concentrations of Shikonin on NAT Activity (N-acetylation of AF) in S. aureus and K. pneumoniae Cytosols. Shikonin was dissolved in DMSO in serial concentrations (0.04, 0.4, 4 and 40 μM). The reaction mixtures consisted of 50 μL cytosols, 20 μL of recycled mixture containing 22.5 μM AF, and 10 μL of shikonin. The reactions were started by the addition of Ac-CoA. The control reactions contained 20 μL distilled water in place of Ac-CoA. Following these steps, NAT activity was determined as described above [13,14].

Effects of Various Concentrations of Shikonin on NAT Activity (N-acetylation of AF) in Intact Bacteial Cells. For the intact cell study, 3×10^9 CFU of bacteria in 1 mL trypticase soy broth were incubated with 22.5 μM AF for 24 h with selected concentrations of shikonin for the study group and without shikonin for the control group. Following incubation, the cells and media suspensions were removed and centrifuged. The supernatant was immediately extracted with ethylacetate/methanol (95:5), the solvent evaporated under speed vacuum, and the residue redissolved in methanol and assayed by HPLC. All samples were run in triplicate.

Effects of Shikonin on Kinetic Constants of NAT from S. aureus or K. pneumoniae Cytosols. Cytosols of *S. aureus* and *K. pneumoniae* cotreated with and without 4 μM shikonin and selected concentrations (5.625, 11.25, 22.5, 45, and 90 μM) of AF and untreated cytosols were analyzed for NAT activity as described above.

Statistical Analysis

Statistical analysis of the data was performed by the unpaired Students *t* test. The kinetic constants were calculated with the Cleland HYPER Program [16] which performs linear regression using the least-squares method. The

Table 1. Effect of shikonin on the growth of *S. aureus* (N = 3)

Strains	Concentrations of shikonin (μM)				
	0	0.04	0.4	4	40
1	0*	6	22	56	89
2	0	2	18	49	84
3	0	4	20	54	92
4	0	4	18	48	86
5	0	6	24	58	94
6	0	6	20	58	92
7	0	7	26	60	91
8	0	8	28	59	92

*Percentage of inhibition.

Table 2. Effect of shikonin on the growth of *K. pneumoniae* (N = 3)

Strains	Concentrations of shikonin (μM)				
	0	0.04	0.4	4	40
1	0*	4	16	46	78
2	0	0	14	42	74
3	0	2	16	42	82
4	0	2	15	41	76
5	0	4	16	43	78
6	0	4	18	44	76

*Percentage of inhibition.

velocity (1/V) versus substrate (1/S) data were linearized by plotting 1/S *vs* 1/V.

RESULTS

Bacteriological Part

Effect of Various Concentrations of Shikonin on the Growth of S. aureus and K. pneumoniae. The growth data of intact bacteria cells (*S. aureus* and *K. pneumoniae*) cotreated with various concentrations of shikonin (% inhibition) and growth data of untreated cells are presented in Tables 1 and 2. The growth of *S. aureus* and *K. pneumoniae* was inhibited by shikonin in a dose-dependent manner. When the concentration of shikonin reached 40 μM the percent inhibition reached about 86% for *S. aureus* and 77% for *K. pneumoniae*. Therefore, the concentrations of shikonin which produced IC_{50} values were 4 μM for *S. aureus* and 12.3 μM for *K. pneumoniae*. The most significant effect in the shikonin treated group was a decrease in the percentage of viable bacterial cells when compared with the control group.

Biochemical Part

Effect of Various Concentrations of Shikonin on the NAT Activity (N-acetylation of AF) of S. aureus and K. pneumoniae. The possible effects of shikonin on NAT activity (N-acetylation of AF) in cytosols of *S. aureus* and *K. pneumoniae* and intact bacteria were examined by high performance liquid chromatography which assessed the percentage of N-acetylation of AF. The percentage of AF acetylation (Tables 3, 4; Figure) differed based on the concentration of shikonin co-treatment (0 to 40 μM) which indicates that shikonin induced a dose-dependent effect in both cytosols and suspensions examined; i.e. the higher the concentration of shikonin, the higher the inhibition of AF acetytlation.

Effect of Various Concentrations of Shikonin on the Kinetic Constants of NAT of S. aureus and K. pneumoniae. The kinetic data for acetylation of 2-amino in *S. aureus* and *K. pneumoniae* are shown in Tables 5 and 6. For the cytosol studies, the values of Km and Vmax were 2.94 ± 0.84 mM and 16.72 ± 3.69

Table 3. Effects of shikonin on N-acetyltransferase activity in *S. aureus*

Concentrations of shikonin (μM)	AAF (nmol/min/mg protein)
Control	1.06 ± 0.22
0.04	0.94 ± 0.18
0.4	0.82 ± 0.16
4	0.46 ± 0.09*
40	0.24 ± 0.04**

*difference between 4 μM shikonin and control ($p < 0.05$); **difference between 40 μM shikonin and control ($p < 0.01$). All experiments and control were run in triplicate. Values are mean ± SD (n = 3).

Table 4. Effects of shikonin on N-acetyltransferase activity in *K. pneumoniae*

Concentrations of shikonin (μM)	AAF (nmol/min/mg protein)
Control	0.94 ± 0.24
0.04	0.88 ± 0.20
0.4	0.74 ± 0.16
4	0.56 ± 0.12*
40	0.32 ± 0.06**

*difference between 4 μM shikonin and control ($p < 0.05$); **difference between 40 μM shikonin and control ($p < 0.01$). All experiments and control were run in triplicate. Values are mean ± SD (n = 3).

Table 5. Kinetic data for acetylation of 2-aminofluorene in *S. aureus*

	In cytosol	
	Km (mM)	Vmax (nmol/min/mg)
Control	2.94 ± 0.84	16.72 ± 3.69
Shikonin	1.16 ± 0.46*	6.83 ± 2.28**

*difference between 4 μM shikonin and control ($p < 0.05$); **difference between 4 μM shikonin and control ($p < 0.05$). Values are mean ± SD (n = 3). All experiments and controls were run in triplicate.

Table 6. Kinetic data for acetylation of 2-aminofluorene in *K. pneumoniae*

	In cytosol	
	Km (mM)	Vmax (nmol/min/mg)
Control	2.86 ± 0.68	15.28 ± 2.54
Shikonin	1.04 ± 0.34*	6.09 ± 1.95**

*difference between 4 μM shikonin and control ($p < 0.05$); **difference between 4 μM shikonin and control ($p < 0.05$). Values are mean ± SD (n = 3). All experiments and controls were run in triplicate.

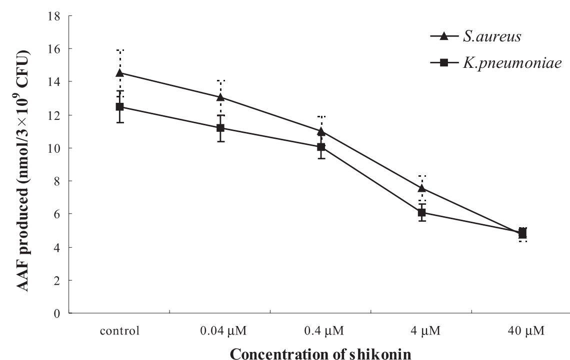

Figure. Percentage of N-acetylation of 2-aminofluorene by *S. aureus* and *K. pneumoniae* N-acetyltransferase with or without specific concentrations of shikonin. Values are mean ± SD (n = 3). All experiments and controls were run in triplicate. *difference between shikonin and control ($p < 0.05$).

nmol/min/protein for *S. aureus* and 2.86 ± 0.68 mM and 15.28 ± 2.54 nmol/ min/protein for *K. pneumoniae*. When 4 μM shikonin was added to the reaction mixtures, the values of Km and Vmax decreased by 60% and 59% for *S. aureus* and 63% and 60% for *K. pneumoniae*.

DISCUSSION

Many reports have mentioned the role of NAT in N-acetylation of AF to explain the observed effects of shikonin on growth and NAT activities (N-acetylation of AF) of *S. aureus* and *K. pneumoniae*. NAT enzyme activity has been shown in laboratory animals and humans [1,2] to be involved in some forms of carcinogenesis [17,18]. The genetically mediated variations in NAT activities within target organs for arylamine-induced neoplasms may indicate differential risks among the human population [19]. It has been reported that some enzymes of enteric bacteria are known to contribute to the metabolic activation of chemical carcinogens in animal studies [20,21]. One animal study demonstrated that NAT in mice can acetylate several arylamine carcinogens and form N-acetyl derivatives [22]. NAT activity has also been demonstrated to exist in some human gastrointestinal flora [23]. Our previous studies showed that many kinds of enteric bacteria such as *K. pneumoniae*, *Salmonella* group B, *S. aureus*, *E. coli, N. gonorrhoreae*, and *H. pylori* exhibit

NAT activity. Shikonin has the ability to inhibit cancer formation [8-11]. *S. aureus* exists naturally in humans and is known to cause disease in many tissues and *K. pneumoniae* attachment in the respiratory tract causes pneumonia. Therefore, the present study focused on the effect shikonin has on growth and NAT activity of *S. aureus* and *K. pneumoniae*.

The data presented in our study clearly demonstrate that shikonin inhibites NAT activity (N-acetylation of AF) and growth (decrease viable bacteria cells) of *S. aureus* and *K. pneumoniae*. The results also show that shikonin decreases NAT activity in *S. aureus* and *K. pneumoniae* in a dose-dependent manner. Therefore, shikonin can be used as an *S. aureus* and *K. pneumoniae* bactericide. Because shikonin elevates NAT activity of *S. aureus* and *K. pneumoniae*, the kinetic constants (Km and Vmax of NAT) were also examined by selecting AF as a substrate. The decrease in kinetic constants suggests that shikonin may act like an uncompetitive inhibitor. Enzyme inhibitors can be divided into 3 types: type 1 is a competitive inhibitor which competes with the substrate for binding at the same site on the enzyme, which leads to a change in the value of Km but not the value of Vmax; type 2 is a noncompetitive inhibitor which binds to a different site but blocks the conversion of the substrate to products which leads to a change in Vmax but not Km; and type 3 is an uncompetitive inhibitor that binds only to the enzyme-substrate complex which leads to a change in the values of Km and Vmax [24]. The important finding is that shikonin induced inhibition of *S. aureus* and *K. pneumoniae* growth. Further investigations are needed to explain the mechanism of the shikonin affect on the growth of *S. aureus* and *K. pneumoniae.*

The reason for selecting AF for N-acetylation of *S. aureus* and *K. pneumoniae* was based on several factors: 1) our previous studies demonstrated that most human intestinal bacterial species show higher N-acetylation activity of AF than PABA; 2) AF is the common substrate for NAT1 and NAT2. Therefore, we selected AF as the substrate for kinetic constant studies; 3) AF is a more convenient substrate for determining the quantity of AF and AAF by HPLC. Other investigators have already reported that the intestinal microflora in mice plays an important role in absorption and metabolic activation of 1-nitropyrene [21]. But in humans, 1-nitropyene can be metabolized to 1-aminopyrene and N-formyl-1-aminopyrene by human microflora, and the acetyltransferase enzyme of human enteric bacteria may contribute to the metabolic activation of 2-amino-3-methylimidazo [4,5] and quinoline [20,21]. Based on our findings, the bacteria examined in our studies could acetylate AF after the individual was exposed to the arylamine carcinogens (AF). It is after that process that acetylated AF may be absorbed by the human gut. *S. aureus* and *K. pneumoniae* are involved in either the metabolic activation or the detoxification of arylamine carcinogens in humans. Further work is required to explain the mechanism by which shikonin reduces N-acetylation of AF. Other reports have demonstrated that increased levels of NAT activity may be associated with increased sensitivity to the mutagenic affects of many arylamines [25]. Furthermore, the attenuation of NAT activity in the liver is associated with several disease processes such as breast and bladder cancer [1,2].

Therefore, this finding shows that shikonin decreases the risk of carcinogenesis due to arylamine amines.

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紫草醌抑制金黃色葡萄球菌和肺炎克雷白氏菌的生長及二胺螢素的N-乙醯化作用

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目的 N-乙醯轉移酵素(NAT，N-acetyltransferase)的作用相當明確，它是芳香胺致癌物和藥物代謝的第一步驟。紫草醌(shikonin)在體內及體外實驗都發現會造成癌細胞計畫性死亡(apoptosis)，以及抑制血管新生(angiogenesis)作用，而血管新生是癌細胞生長及發炎作用的重要步驟。因此，本研究目的是檢測紫草醌(shikonin)引響細菌的生長和N-乙醯化2-aminofluorene的量。

方法 利用Beckman的分光光度計來測量650 nm光譜吸光值來決定紫草對金黃色葡萄球菌及肺炎克雷白氏菌生長抑制的影響。芳香胺的N-乙醯轉移酵素活化物和二胺螢素是利用高效率液相層析法(HPLC，high pressure liquid chromatography)測量，金黃色葡萄球菌及肺炎克雷白氏菌是由病人身上收集培養。

結果 用不同濃度的紫草醌作用在金黃色葡萄球菌及肺炎克雷白氏菌的胞質液和懸浮液，造成二胺螢素的N-乙醯化程度不同。研究結果顯示，紫草醌的濃度愈高，對細菌本身及其胞質液中二胺螢素的N-乙醯化作用的抑制愈顯著。紫草醌並能降低金黃色葡萄球菌及肺炎克雷白氏菌的NAT酵素的動能常數(apparent values of Km and Vmax)。

結論 紫草醌能降低Km和Vmax的值，紫草醌有抑制細菌生長及芳香胺的N-乙醯轉移酵素活化物的作用。(**中台灣醫誌 2003;8:119-26**)

關鍵詞

二胺螢素，肺炎克雷白氏菌，N-乙醯二胺螢素，乙醯轉移酵素，金黃色葡萄球菌，紫草醌

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