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Changes in and shortcomings of drug stockpiling, vaccine development and related policies during outbreaks of avian influenza A H5N1, H1N1, and H7N9 among humans

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ABSTRACT: The purpose of this paper is to provide a reference for the future stockpiling of drugs and developing vaccines for treatment of emerging infectious diseases by summarizing the status of drug stockpiling, vaccine development, and related policies during three major outbreaks of avian influenza among humans (H5N1 in 2003, H1N1 in 2009, and H7N9 in 2013). Documents regarding drug stockpiling and vaccine development during three influenza outbreaks have been reviewed. Results indicated that the response to pandemic influenza outbreaks has improved markedly in terms of stockpiles of antivirals and vaccine development. These improvements also suggest advances in related policy planning. These trends also foreshadow better prospects for prevention and control of emerging infectious diseases. However, the rationality of drug stockpiling and international cooperation still needs to be enhanced.

Keywords: Rapid-response stockpile, guidelines, timetable

1. Introduction

The experience of the 2003 SARS outbreak in Asia emphasized the need to enhance the capacity of stockpiling drugs and developing vaccines. This paper seeks to provide a reference for the process of stockpiling drugs, developing vaccines, and the related policies of emerging infectious diseases by summarizing the status and shortcomings of drug stockpiling, vaccine development, and related policies during three major outbreaks of avian influenza among humans (H5N1 in 2003, H1N1 in 2009, and H7N9 in 2013).

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2. Drug stockpiling, vaccine development and related policies during outbreaks of influenza A (H5N1)

Outbreaks of the highly pathogenic H5N1 avian influenza in humans are now known to have begun in parts of South-east Asia in 2003. So far, 15 countries reported a total of 622 laboratory-confirmed human cases and 371 deaths due to H5N1 avian influenza *(1)*.

2.1. *Drug stockpiling during outbreaks of influenza A (H5N1)*

Many researchers believed that oseltamivir, an antiviral drug, combined with draconian measures can stop the virus from further developing *(2)*. Soon after the outbreak, antiviral drugs had been stocked worldwide. Until August 2009, United states had stocked 50 million courses, United Kingdom had stocked 30 million courses, South Korea had stocked more than 21 million courses, and Canada had stocked 1.4 million courses of antiviral medications *(3)*.World Health Organization (WHO) had stocked 2 million treatment courses of oseltamivir in January 2006 *(4)*. In addition, Roche announced that another 3 million treatment courses were ready to be shipped to sites of pandemic influenza outbreaks in April 2006. Table 1 shows the timetable of the antiviral stockpile against H5N1 of the World Health Organization (WHO). As this information shows, amassing effective drug stockpiles took three years.

2.2. *Vaccine development during outbreaks of influenza A (H5N1)*

Table 1 also presents the timetable of development of vaccines against H5N1. WHO obtained the wild-type

Table 1. Timetable of drug stockpiling and vaccine development of human infection with H5N1, H1N1 and H7N9

Time	Event
H5N1	
February 2003	First outbreak of H5N1 in Hong Kong, China.
April 2004 (14 months after first outbreak)	Isolation of wild type viruses of H5N1.
August 2005 (30 months after first outbreak)	Valid result during the first stage of vaccine's adults experiment.
April 2006 (38 months after first outbreak)	Rapid response stockpile of oseltamivir gets ready.
H1N1	
April 2009	First outbreak of H1N1 in Mexico.
April 2009 (same month of first outbreak)	Deploying rapid-response stockpile of drug.
May 2009 (1 month after first outbreak)	Isolation of wild type viruses of H1N1.
July 2009 (3 months after first outbreak)	Vaccination against pandemic H1N1 influenza first implemented in China.
H7N9	
March 2013	First outbreak of H7N9 in China.
April 2013 (1 month after first outbreak)	Isolation of wild type viruses of H7N9.
April 2013 (1 month after first outbreak)	CFDA approved the production of a new anti-influenza drug which is effective in fighting influenza H7N9

H5N1 virus and provided the virus to the National Institute of Allergy and Infectious Diseases (NIAID) for developing a vaccine against H5N1 infection among humans in April 2004 *(5)*. In August 2005, NIAID declared that the vaccine had proven effective during the first phase of adult experiments. The US Department of Health and Human Services (HHS) announced that the U.S. government had stockpiled enough vaccine (against former avian Influenza virus) to treat more than 3 million patients in 2006. U.S. Food and Drug Administration (FDA) approved the first U.S. vaccine for humans against the avian Influenza virus H5N1 in April 2007 *(6)*. China food and drug administration (CFDA) also approved China's first vaccine for humans against H5N1 in April 2008 *(7)*. However, the continual mutation of H5N1 limited the practicability of existing vaccines. Therefore, candidate vaccines to prevent H5N1 infection had been developed, but they were not ready for widespread use.

2.3. *Related policies of drug stockpiling and vaccine development during outbreaks of influenza A (H5N1)*

Researchers raised the idea of stockpiling oseltamivir for better controlling H5N1 in 2005 for the first time. WHO also proposed the idea of an oseltamivir stockpile and added oseltamivir as an essential medicine to treat H5N1 *(8)*. This idea had supportfrom many countries.

However, vaccine, as a more effective prevention and control measure, gained more attention. WHO came up with the idea to develop a global plan to increase the supply of pandemic influenza vaccine in 2006 *(9)*. This global plan focused on the poor resource countries which considered the H5N1 virus to be a national security and public health threat. As Table 2 shows, WHO also issued a series of guidelines, containing information about production, control and use of vaccines, related to the development of human H5N1 vaccines.

2.4. *Shortcomings of drug stockpiling, vaccine development, and related policies during outbreaks of influenza A (H5N1)*

Drug stockpiling and vaccine development had played a positive role during the process of controlling the outbreak of H5N1 among humans as the number of human infections was reduced and the influenza controlled. However, there were still some shortcomings that should be noted.

First, the policy's focus on drug stockpiling and vaccine development had not been issued on time. As the information mentioned above, the majority of related policies had been published in 2005, which indicated a two year delay. Second, the stockpiling of effective drugs was still relatively slow. As mentioned above, amassing an effective drug stockpile by WHO took more than three years. Finally, the process of vaccine development was unfavorable. According to the recent research mentioned above, no vaccine was available to civilian populations, nor produced in quantities sufficient to protect more than a tiny fraction of the Earth's population in the event of an H5N1 pandemic.

3. Drugstockpiling, vaccinedevelopment and related policies during outbreaks of influenza A (H1N1) 2009

The emergence of H1N1 virus in 2009 caused the first influenza pandemic of the 21st century *(10)*. By

Table 2. Guidelines related to antiviral stockpiling and vaccinedevelopment against pandemic influenza A (H5N1) published by WHO

Publication date	NO.	Guidelines
2004	1	Guidelines for the use of seasonal influenza vaccine in humans at risk of H5N1 infection.
	2	WHO Guidelines on the use of vaccines and antivirals during Influenza Pandemics.
2005	1	Strengthening pandemic influenza preparedness and response.
	2	WHO guidance on development of influenza vaccine reference viruses by reverse genetics.
	3	Recommendations for the production and control of influenza vaccine (inactivated).
	4	WHO biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines.
2006	1	WHO rapid advice guidelines on pharmacological management of humans infected with avian influenza A (H5N1) virus.

Table 3. Guidelines related to antiviral stockpiling and vaccinedevelopment against pandemic influenza A (H1N1) 2009 published by WHO

Publication date	NO.	Guidelines
2009	1	Recommendations of the Strategic Advisory Group of Experts (SAGE) on Influenza A (H1N1) vaccines.
	2	Characteristics of the emergent influenza A (H1N1) viruses and recommendations for vaccine development.
	3	Update of WHO biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines.
	4	WHO recommendations on pandemic (H1N1) 2009 vaccines.
	5	Summary of available potency testing reagents for Pandemic (H1N1) 2009 virus vaccines.
	6	Statement from WHO Global Advisory Committee on Vaccine Safety about the safety profile of pandemic influenza A (H1N1) 2009 vaccines.
2010	1	WHO guidelines for pharmacological management of pandemic (H1N1) 2009 influenza and other influenza viruses.

August 2010, 18,449 laboratory-confirmed deaths from pandemic influenza A (H1N1) 2009 had been recorded *(11)*. However, the actual number of influenza A (H1N1) cases worldwide remains unknown, as most cases were diagnosed clinically and not confirmed in the laboratory.

3.1. *Drug stockpiling during outbreaks of pandemic influenza A (H1N1) 2009*

Table 1 also presents the major timeline for drug stockpiling during the outbreak of pandemic influenza A (H1N1) 2009. As mentioned above, after the outbreak of H5N1, the WHO began to store emergency stocks of antivirals. As the H1N1 virus was susceptible to the drugs oseltamivir and zanamivir just like the H5N1 virus, the WHO started deploying 3 million doses of the drug, stored after 2003, to Mexico and to 71 pre-identified low-income countries immediately after the declaration of pandemic alert Phase 5 on April 29, 2009 *(12)*. Within a month, this rapid-response stockpile had been delivered. Governments also took steps to stockpile antiviral drugs. U.K. government estimated that the UK had enough antiviral drugs for 50% of the population as of 13 June 2009 and over 30 million doses of antiviral treatments available in April 2009 *(13)*. CFDA announced that the China government stored enough antiviral drugs against the outbreak of H1N1 *(14)*. However, even though there was no evidence suggesting an antiviral drug deficiency during the outbreak of H1N1 virus, the specific numbers of each country's emergency stocks of antivirals remained unknown.

3.2. *Vaccine development during outbreaks of pandemic influenza A (H1N1) 2009*

In contrast with the H5N1 virus, vaccines against H1N1 virus for humans are effective. As Table 1 presented, in May 2009, the WHO isolated and sent the wild-type H1N1 virus to vaccine manufacturers that requested it. At the same time, WHO Collaborating Centers for Influenza (WHO CCs), Essential Regulatory Laboratories (ERLs), and other institutions were developing candidate vaccines with coordination by the WHO. In July 2009, the China government announced that vaccination against pandemic H1N1 influenza was first implemented in China *(15)*. U.S. government launched the national influenza 2009 H1N1 vaccination campaign in October 2009. Between September and October 2009, European Union (EU) had approved some vaccines for human use *(16)*.

3.3. *Related policies of drug stockpiling and vaccine development during outbreaks of pandemic influenza A (H1N1) 2009*

After the outbreak of H5N1, many countries have chosen to stockpile antiviral medications in advance against pandemic influenza. Table 3 presents the guidelines for using and stockpiling antivirals against H1N1, proposed by WHO.

WHO also issued some guidelines for using and developing vaccines, as Table 3 shows. Besides the WHO, governments also took some steps. United States Center for Disease Control (USCDC) issued a series of statements including general information and safety information about 2009 H1N1 vaccines soon after the outbreak of H1N1 *(17)*. The China government issued guidelines for development process of vaccine as well *(18)*.

3.4. *Shortcomings of drug stockpiling, vaccine development, and related policies during outbreak of pandemic influenza A (H1N1) 2009*

Compared to drugstockpiling against H5N1, there was a significant improvement. A rapid-response stockpile of antivirals had been prepared in advance, and the stockpile was quickly delivered. As for the development of vaccines, the improvement in both timeliness and results were remarkable. However, some shortcomings should be noted.

Because of the lack of information about the amount of emergency stocks of antivirals, it is hard for people to stock antivirals rationally. A study concluded that approximately half of the prescriptions of Tamiflu during the 2009-10 influenza pandemic went unused in England *(19)* which was a huge waste and an unreasonable disposition of medical resources. Therefore, more attention should be paid to enhance the rationality of drug stockpiling.

4. Drug stockpiling, vaccine development and related policies during outbreaks of influenza A (H7N9)

On March 31, 2013, the National Health and Family Planning Commission (NHFPC) of China (formerly the Ministry of Health) announced three confirmed human cases of influenza A (H7N9). Prior to May 7, 2013, a total of 130 patients in China were confirmed to be infected with the influenza A (H7N9) virus; of these patients, 31 died and 42 recovered *(20)*.

4.1. *Drug stockpiling during outbreaks of influenza A (H7N9)*

Laboratory testing conducted in China have shown that the influenza A (H7N9) viruses are sensitive to anti-influenza drugs. Until now, there is no report of drug shortages. Hong Kong government announced HK well stocked with antiviral drugs with a stock of 18 million doses of Tamiflu and other medicines *(21)*. On April 5, 2013, about one month after the outbreak of H7N9, CFDA approved the production of a new anti-influenza drug (a Peramivir sodium chloride injection) that has proven effective in fighting influenza H7N9 according to existing clinical trials *(22)*, as presented in Table 1.

4.2. *Vaccine development during outbreaks of influenza A (H7N9)*

One month after the outbreak of H7N9, Greffex scientists had created the first comprehensive vaccine for H7N9 avian influenza *(23)*. However, no vaccine for the prevention of influenza A (H7N9) infections has been created by China's scientists. However, as Table 1 shows, viruses have already been isolated and shared with WHO by China government. The NHFPC of China indicated that 6 to 8 months are needed to develop an effective vaccine generally, yet more time may be needed to develop an effective vaccine against a new virus like H7N9. The Ministry of Science and Technology of the People's Republic of China launched research on the H7N9 avian influenza virus *(24)* on April 10, 2013, and the development of vaccine should be completed within seven months.

4.3. *Related policies of drug stockpiling and vaccine development during outbreaks of influenza A (H7N9)*

A statement about vaccine response had been made by WHO on May 2, 2013 *(25)*. USCDC and European centre for disease prevention and control (ECDC) also issued guidelines of using antivirals. However, since the outbreak of H7N9 has been in a relatively short period of time, the existing policies about drugstockpiling and vaccine development to treat influenza A (H7N9) are still limited.

4.4. *Suggestions regarding the drug stockpiling, vaccine development, and related policies against influenza A (H7N9) based on previous experience*

Based on the above information, it is easy to see the response improvement to influenza on both drug stores and vaccine development. From a more than 3 year period to stock enough drugs for H5N1, to deploying a rapid-response stockpile just after the outbreak of H1N1, the world has made huge progress on the drug stockpile. The same kind of progress can be seen in the aspect of vaccine development as an effective vaccine against H5N1 virus is still missing, and the vaccine against H1N1 virus had already been accepted by many countries as an effective control strategy. The aspects of related policies have also taken a huge step. As Table 2 and Table 3 show, the policy document of drugs and vaccines about H5N1 showed up about one year after the outbreak, and the majority of policy documents about H1N1's drugs and vaccines were published in the same year as the outbreak.

Because of the experience handling H5N1 and H1N1, the response to influenza A (H7N9) was timely in terms of both drug stores and vaccine development. The effective new drugs and the isolation of wild type viruses had been accomplished relatively soon after the first outbreak. However, according to the existing experience, people should pay more attention to the rationality of drug stockpiling. Maybe start with disclosing the actual number of antivirals stockpiled to the public. Second, as the influenza occurs in China, the China government should put more emphasis on promoting international cooperation in many aspects such as developing an effective vaccine.

5. Conclusion

Drug stockpiling, vaccine development, and related policies to treat influenza outbreaks have improved markedly. The response was faster and more effective in terms of stockpiling of antivirals and vaccine development. These improvements also suggest advances in related policy planning. These trends also foreshadow better prospects for prevention and control of emerging infectious diseases. However, the rationality of drug stockpiling and international cooperation still need to be enhanced.

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Brief Report

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A new phenoxazine derivative isolated from marine sediment actinomycetes, *Nocardiopsis* sp. 236

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ABSTRACT: During screening of marine actinomycetes for anti-mycobacterial activity, a new phenoxazine derivative (1) was isolated, along with 6-phenazinediol (2), 6-methoxy-1-phenazinol (3), nocardamin (4), and 3-pyridinecarboxylic acid (5), from a culture of *Nocardiopsis* sp. 236 collected from the west Pacific. The chemical structure of 1 was established on the basis of 1D-, 2D-NMR, and HR-Q-TOF MS data. All compounds were evaluated for their anti-mycobacterial activity in vitro, and only compounds 2 and 3 exhibited weak activity.

Keywords: *Nocardiopsis* sp., phenazine, nocardamin, pyridine carboxylic acid

1. Introduction

Marine microorganisms are widely recognized as a rich source of novel natural products (*1*) and numerous novel compounds from marine actinomycetes have been discovered (*2-4*). During screening of antibacterial compounds for activity against *Mycobacterium smegmatis*, strain 236 was identified because of its remarkable activity. Further screening resulted in the isolation of five compounds. This paper describes the isolation, characterization, and antibacterial activity of compounds **1-5** (Figure 1).

2. Materials and Methods

2.1. *General experimental procedures*

Mass spectra were measured using a Bruker BioTOF-Q spectrometer; NMR spectra were measured on Bruker DRX-600 NMR spectrometers with tetramethylsilane (TMS) as an internal standard. Reversed-phase (RP) C18 silica gel for column chromatography (CC) was obtained from Merck and Sephadex LH-20 was obtained from Amersham Biosciences. Silica gel (200-300 mesh) for CC and silica gel GF_{254} for TLC were purchased from Qingdao Marine Chemical Ltd., Qingdao, Shandong, China.

2.2. *Microorganism specimens*

Strain 236 was isolated from sediment collected in the West Pacific Ocean. The strain was identified as *Nocardiopsis* sp. according to its 16s rDNA sequence (accession No. JQ355005) and BLAST search of the NCBI database (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*).

2.3. *Culture and isolation*

The strain was seeded on SYP medium (starch 10%, yeast extract 4%, peptone 2% and agar 2% agar plates, pH 7.2) in an inclined test tube and cultured for 7 d at 28°C to yield seed cultures. Fermentation (10 l) was done on SYP medium for 14 d at 28°C.

The culture was diced and extracted three times with an equal volume of EtOAc/MeOH/AcOH 80:15:5 (*v/v/v*) at room temperature. The organic solutions were collected by filtration and removed under a vacuum at 40°C to obtain the crude extract. This was partitioned between MeOH and petroleum ether (1:1) three times. The MeOH solution was concentrated under a vacuum at 40°C to obtain MeOH extract (5.0 g).

The MeOH extract was subjected to MPLC (140 g RP-18 silica gel; H_2O, MeOH/H_2O 30%, 50%, 70%, and 100% resp., 2 l each) to yield 8 fractions: Fr.A - H. Fr.A (872.8 mg) obtained from 30% MeOH was subjected to Sephadex LH-20 (120 g; MeOH) to obtain Fr.A1 (109.8 mg) and Fr.A2 (134.6 mg). Fr.A1 was purified by CC (SiO_2; chloroform /MeOH 100:1) to yield **3** (7.0 mg). Fr.A2 was further purified by MPLC (40 g RP-18 silica gel; 25% MeOH with 0.5% formic acid) to yield **5** (5 mg). Fr.C (125.2 mg) obtained from 50% MeOH was subjected to CC (30 g RP-18 silica gel; H_2O, MeOH/H_2O 45%, 50%) to obtain Fr.C1 (25.4 mg). Fr.C1 was further subjected to Sephadex

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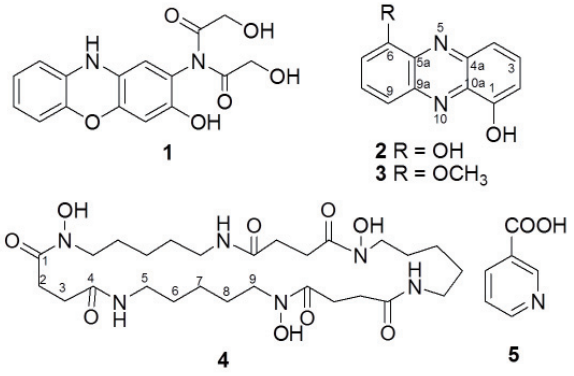

Figure 1. The chemical structures of compounds 1 - 5.

LH-20 (60 g; MeOH) and CC (0.5 g silica gel, chloroform /MeOH 10:1) to yield **1** (1.8 mg). Fr.D (290.8 mg) obtained from 50% MeOH was subjected to Sephadex LH-20 (120 g; MeOH) to obtain Fr.D1-D4. Fr.D1 (49.2 mg) was further purified by CC (0.5 g silica gel, petroleum ether / acetone 30:1) to yield **2** (2.3 mg). Fr.E (400.8 mg) obtained from 50% MeOH was subjected to Sephadex LH-20 (120g; MeOH) to obtain Fr.E1 (15 mg). Fr.E1 was subjected to CC (0.5 g silica gel, petroleum ether/chloroform 30:1) to obtain **4** (3.6 mg).

2.4. *Antibacterial activity*

An antibacterial activity test was performed with *Mycobacterium smegmatis*. The culture was maintained on LB medium (yeast extract 1%, tryptone 0.5%, NaCl 1%, pH 7.2). The antibacterial activity of **1-5** was tested against *Mycobacterium smegmatis* by the disk diffusion assay on agar plates with rifampicin as a positive control.

3. Results and Discussion

3.1. *Elucidation of structures*

Compound **1** was obtained as a yellow amorphous powder and had a positive color reaction with modified Dragendorff's reagent on TLC. The molecular formula of **1** was determined to be $C_{16}H_{14}N_2O_6$ on the basis of HR-Q-TOF MS and NMR data.

The ^{13}C-NMR (DEPT) spectrum of **1** (Table 1) had 16 signals, corresponding to two O-bearing CH_2, six CH (two of them O-bearing), and eight quaternary C-atoms (including two carbonyl groups at δ 172.9 and 173.3). Six olefinic proton signals in the ^{1}H-NMR spectrum (Table 1), including two doublet-doublet and two triplet-doublet signals from δ 7.15 - 7.57 and two singlet protons at δ 6.70 and 8.29, were noted, indicating the presence of a di- and a tetra- substituted benzene ring in the structure. The phenoxazine skeleton of **1** was identified by comparison of the NMR data to those of **3b** and **3d** according to Maskey *et al*. (*5*).

In HMBC, two oxymethylene groups (δ_H 4.17, δ_C 63.0; δ_H 4.43, δ_C 61.9) had long-range coupling to the carbonyls at δ 172.9 and 173.3, respectively. In order to determine the binding of the hydroxyacetyl groups, **1** was allowed to react with acetic anhydride in pyridine at room temperature. After this reaction, the main product was obtained as a colorless powder and

Table 1. ^{1}H- and ^{13}C-NMR data for compounds 1 and 1a. Recorded at 600/150MHz (δ in ppm, *J* in Hz)

Position	**1** (in CD_3OD)			**1a** (in $CDCl_3$)	
	^{1}H	^{13}C	HMBC	^{1}H	^{13}C
1	8.29 (s)	117.4d	C2, C3, C4a, C10a	7.56 (s)	118.3d
2	/	122.8s			126.7s
3	/	147.9s			145.8s
4	6.70 (s)	104.3d	C2, C3, C4a, C10a	7.13 (s)	112.9d
4a	/	120.6s			125.2s
5a	/	152.4s			151.8s
6	7.15 (dd, 7.8, 1.2)	117.8d	C5a, C8, C9a	7.21 (d, 7.8)	125.0d
7	7.25 (td, 7.8, 1.2)	128.4d	C5a, C9	7.33 (t, 7.8)	128.8d
8	7.18 (td, 7.8, 1.2)	124.6d	C6, C9a	7.22 (t, 7.8)	124.1d
9	7.57 (dd, 7.8, 1.2)	126.3d	C7, C5a	7.36 (d, 7.8)	126.8d
9a	/	130.0s			127.3s
10a	/	149.1s			150.8s
1'		172.9s			171.0s
2'	4.17 (s, 2H)	63.0t	C1'	5.20 (AB d, 17.1) 5.02 (AB d, 17.1)	65.5t
1"		173.3s			173.3s
2"	4.43 (s, 2H)	61.9t	C1''	5.10 (AB d, 15.1) 4.83 (AB d, 15.1)	61.7t
-OAC				2.34 (s, 3H),	30.2 q,
-OAC				2.27 (s, 3H),	26.2 q,
-NAC				2.18 (s, 3H),	21.1 q,
-NAC				2.16 (s, 3H)	21.0 q,
					170.9 s
					170.1 s
					168.9 s
					166.4 s

Table 2. ^{1}H- and ^{13}C-NMR data for compounds 2 and 3. Recorded at 600/150MHz (δ in ppm, J in Hz)

Position	**2** (in DMSO-d_6)		**3** (in $CDCl_3$)	
	^{1}H	^{13}C	^{1}H	^{13}C
1	/	152.2s	/	151.5s
2	7.54(dd, 5.0,7.9)	110.5d	7.11(d, 7.8)	109.3d
3	8.38(d, 6.8)	131.2d	7.76(t, 7.8)	131.4d
4	8.69 (dt, 3.7, 7.9)	123.4d	7.95(dd, 0.8,8.8)	120.6d
4a	/	149.9s	/	142.6s
5a	/	137.8s	/	135.6s
6	/	152.2s		155.2s
7	7.54(dd, 5.0, 7.9)	110.5d	7.24(dd, 0.8, 7.8)	106.8d
8	8.38(d, 6.8)	131.2d	7.78(t, 7.8)	130.5d
9	8.69 (dt, 3.7, 7.9)	123.4d	7.83(dd, 0.8, 8.7)	121.0d
9a	/	149.9s		142.0s
10a	/	137.8s		137.6s
6-OCH3	/	/	4.19(s)	56.5q
OH	9.08 (2H, br s)		8.17(1H, br s)	/

designated **1a**. The ESI-MS of **1a** indicated a quasi-molecular ion peak at *m/z* 521.6 $[M + Na]^+$, which suggested the addition of four acetyl groups in **1a**. Both of the hydroxyacetyl groups were determined to be bound to N-C(2) by comparison of the chemical shifts of C(1) and C(4), which were at δ_H 8.29, δ_C 117.4 and δ_H 6.70, δ_C 104.3 in **1** and at δ_H 7.56, δ_C 118.3 and δ_H 7.13, δ_C 112.9 in **1a**, respectively. Thus, the structure of **1** was determined to be 2-hydroxy-N-(3-hydroxy-10H-phenoxazin-2-yl)-N-(2-hydroxyacetyl)acetamide.

Compounds **2** and **3** were obtained as yellow amorphous powder. ESI-MS indicated a quasi-molecular ion peak $[M + H]^+$ at *m/z* 227.3 and 213.3, respectively. These compounds were identified as 6-methoxy-1-phenazinol (**2**) (*6, 7*) and 1,6-phenazinediol (**3**) (*7, 8*) by comparing their ^{1}H and ^{13}C-NMR data (see Table 2) with those previously reported.

Compound **4** was found to be nocardamin by comparing the ^{1}H- and ^{13}C-NMR data to those previously reported (*9, 10*). Both ^{1}H and ^{13}C NMR data (in DMSO-d_6) revealed only seven methylene protons (at δ 1.20(H-7), 1.38(H-8), 1.49(H-6), 2.28(H-2), 2.59(H-3), 3.01(H-9), and 3.47(H-5)) and nine carbons signals (at δ 172.3(C-1), 171.7(C-4), 47.1(C-5), 38.5(C-9), 30.2(C-2), 28.8(C-8), 27.7(C-3), 26.1(C-6), and 23.2(C-7)). The NMR spectra of **4** revealed an N-hydroxy-N-succinylcadaverine unit with a molecular weight corresponded to 200, while ESI-MS analysis of **4** revealed a peak at *m/z* 601.7 $[M + H]^+$. Therefore, **4** consisted of three N-hydroxy-N-succinylcadaverine units that were conjugated iteratively.

Compound **5** was found to be identical to 3-pyridinecarboxylic acid based on the NMR spectra: ^{1}H-NMR (600 MHz, methanol-d_4): δ 9.10 (s, H-2),8.40 (d, 7.8, H-4), 7.56 (dd, 4.8, 7.8, H-5), 8.71 (d, 4.8, H-6); ^{13}C NMR(150 MHz, methanol-d_4): δ 151.0 (C-2), 128.5 (C-3), 138.9(C-4), 124.9(C-5), 153.4 (C-6), 167.6(C-7).

3.2. *Biological study*

The antibacterial efficacy of **1-5** against *M. smegmatis* was studied. Compounds **2** and **3** exhibited activity against *M. smegmatis* with inhibitory zones of 1.0 and 0.8 cm, respectively, at a dose of 20 μg/disk. This result was consistent with the reported data (*6, 7*). All other tested compounds were inactive, whereas the positive control, rifampicin exhibited activity with an inhibitory zone of 2.0 cm (0.1 μg/disk).

Four type of compounds were obtained from the fermentation and culture of *Nocardiopsis* sp. 236, including phenazine, phenoxazine, nocardamin, and pyridine derivatives. As a hydroxamate siderophore, nocardamin was initially isolated as an antibacterial metabolite of a Nocardia strain (*9*). Later, its derivatives were isolated from several actinomycetes (*10, 11*) and are currently the only available therapeutic agent for chronic iron overload and acute iron intoxication. Phenazines and phenoxazines comprise a large group of nitrogen-containing heterocyclic compounds that differ in their chemical and physical properties based on the type and position of functional groups present. More than 100 different phenazine structural derivatives have been identified in nature (*12*). The diverse biological actions of these compounds include cytotoxic, antibacterial, antiparasitic, and antimalarial activities (*13*). The current study obtained 6-methoxy-1-phenazinol (**2**) and 1,6-phenazinediol (**3**) with antibacterial activity against *M. smegmatis*.

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Brief Report

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Effects of Gosha-jinki-gan (Chinese herbal medicine: Niu-Che-Sen-Qi-Wan) on hyperinsulinemia and hypertriglyceridemia in pre-diabetic Zucker fatty rats

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ABSTRACT: The Chinese herbal medicine, Gosha-jinki-gan (GJ) (Niu-Che-Sen-Qi-Wan), has been widely used for treating patients with melalgia, lower back pain, numbness, and diabetic neuropathy. We investigated the effects of GJ on the regulation of serum insulin and triglyceride levels in obese Zucker fatty rats (fa/fa; ZFR). We administrated GJ to 6-week-old ZFR and non-obese lean rats (LR) for 12 weeks. Body weight and serum glucose, insulin, total cholesterol, and triglyceride levels were significantly increased at 18 weeks in ZFR as compared to the LR. GJ treatment in ZFR significantly suppressed elevation in serum glucose, insulin, and triglyceride levels, but no significant differences were observed in body weight and serum cholesterol levels in the ZFR group with GJ treatment compared to the ZFR group without GJ treatment. These results suggest that GJ may improve hyperinsulinemia and hypertriglyceridemia in ZFR and that GJ may be useful for preventing or delaying the onset of diabetes mellitus in a pre-diabetic state.

Keywords: Gosha-jinki-gan, obese rat, hyperinsulinemia, hypertriglyceridemia, pre-diabetic state

1. Introduction

Non-insulin-dependent diabetes mellitus (NIDDM) is a multifactorial disease caused by the interaction of environmental factors and genetic predisposition, leading to two major impairments: insulin resistance and defective β cell function. In the pre-diabetic state that precedes the onset of NIDDM, hyperinsulinemia compensates for insulin resistance (*1*). Hyperglycemia then develops with progressive beta cell dysfunction, resulting in hypertriglyceridemia (*2*). Hypertriglyceridemia is an important risk factor for coronary heart disease, especially in populations with NIDDM. Amelioration of hyperinsulinemia and hypertriglyceridemia in a pre-diabetic state can be significantly beneficial for reducing the incidence of NIDDM by using safer drugs such as Chinese herbal medicine, over a long period.

Gosha-jinki-gan (GJ) (Niu-Che-Sen-Qi-Wan), a traditional Chinese herbal complex of 10 medicinal herbs, has been widely used for treating patients with melalgia, pain in the lower back, numbness and diabetic neuropathy (*3,4*). In addition, Suzuki *et al.* reported that the antinociceptive activity of GJ was significantly greater in diabetic mice than in non-diabetic mice on the basis of nitrous oxide (NO) production (*5,6*). Further, the homeostasis model assessment of insulin resistance (HOMA-R) index of patients with type 2 diabetes showed a significant decrease after GJ treatment (*7*). However, few reports have been published on the effects of GJ on hyperinsulinemia and hypertriglyceridemia in a pre-diabetic state.

Rodent models of diet-induced hyperinsulinemia and hypertriglyceridemia are used to assess the therapeutic efficacy of drugs and nutrients that are likely to affect insulin sensitivity and lipid concentrations in the blood (*8-10*). However, the effects of GJ on ameliorating the metabolic dysregulation of spontaneously obese rats in pre-diabetic states have not been previously reported. Obese Zucker fatty rats (fa/fa; ZFR) are considered a model for pre-diabetes and are characterized by a genetic defect in the leptin receptor (*7*), which results in hyperphagia, hyperinsulinemia, and severe obesity with relatively mild hyperglycemia, hypertriglyceridemia, and hypercholesterolemia (*11*). In the present study, we investigated the effects of GJ on hyperinsulinemia and hypertriglyceridemia in obese ZFR for a period of 12 weeks.

2. Materials and Methods

2.1. *Animals*

6-week-old male lean Zucker (+/+) rats and obese

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Zucker (fa/fa) rats (Japan SLC Inc., Shizuoka, Japan) were used. The rats were maintained on a standard powder diet (MF® diet; Oriental Yeast, Tokyo, Japan) for 1 week. They were allowed free access to rat chow and water and were kept in a room maintained at 22 ± 2°C with a 12-h/12-h light/dark cycle (light cycle began at 8:00 AM). All experimental procedures were conducted according to the Osaka Ohtani University Guidelines for the Care and Use of Laboratory Animals, and the study protocol was approved by the local Animal Ethics Committee.

2.2. *Drugs*

Spray-dried GJ powder was manufactured and provided by Tsumura & Co. Ltd. (Tokyo, Japan). The composition of GJ is as follows: 5 g of Rehmanniae radix (*Rehmannia glutinosa* Liboschitz); 3 g each of Achyranthis radix (*Achyranthes bidentage* Blume), Corni fructus (*Cornus officinalis Sieb.* et Zucc), Dioscoreae rhizoma (*Dioscorea batatas* Decaisne), Plantaginis semen (*Plantago asiatica*), Alismatis rhizoma (*Alisma orientale* Juzep), Hoelen (*Poria cocos* Wolf), and Moutan cortex (*Paeonia suffruticosa* Andrews); and 1 g each of Cinnamomi cortex (*Cinnamomum cassia* Blume) and Aconiti tuber (*Aconitum carmichaelii* Debeaux).

2.3. *Animal treatments and preparation of blood samples*

The lean Zucker +/+ rats (113-136 g) and obese Zucker fa/fa rats (166–196 g) were randomly divided into groups of 6. The lean Zucker +/+ rats in the 2 groups were maintained on standard chow (L+0%GJ) and standard chow containing 3% powdered GJ extract (L+3%GJ). The obese Zucker fa/fa rats in the O+0%GJ group were maintained on standard chow supplementation without GJ, whereas those in the O+1%GJ and O+3%GJ groups were fed standard chow containing 1% and 3% powdered GJ extract, respectively. The rats had access to the chow and tap water *ad libitum*. Body weights of the rats and food intake per cage were measured on a weekly basis. For 12 weeks, fasting blood samples were collected from the jugular vein and centrifuged, and serum aliquots were stored frozen on the same day.

2.4. *Assays to determine serum glucose, insulin, triglyceride, and cholesterol levels*

Serum glucose levels were determined using a commercial assay kit (Glucose CII-Test Wako; Wako Pure Chemical Industries Ltd., Osaka, Japan). Serum triglyceride and cholesterol levels were determined using the commercial lipid assay kits Triglyceride E-Test Wako and Cholesterol E-Test Wako, respectively (Wako Pure Chemical Industries Ltd.). Serum immunoreactive insulin levels were measured using a commercial assay kit (Merodia Insulin Eiken Elisa kit; Mercodia AB Co. Ltd., Uppsala, Sweden).

2.5. *Data analysis*

Experimental data are expressed as mean values with standard deviations (SD). Statistical analysis of the differences between the mean values obtained was performed using Tukey's multiple comparison test and an unpaired Student's *t*-test with a significance level of $p < 0.05$.

3. Results and Discussion

Rodent models of diet-induced hyperinsulinemia and hypertriglyceridemia are used to assess the therapeutic efficacy of drugs and nutrients that are likely to affect insulin sensitivity and lipid concentration in the blood (*8-10*). However, the effects of GJ in ameliorating the metabolic dysregulation of spontaneously obese rats in pre-diabetic states have not been previously reported.

The changes in the body weights of the rats are shown in Figure 1. These changes were significantly greater in the 3 obese-rat groups than in the lean-rat groups ($p < 0.01$). The body weight changes in the O+1%GJ and O+3%GJ rat groups administered GJ were similar to those in the O+0%GJ rat group. The food intake of the 3 obese-rat groups was greater than that in the lean-rat groups (data not shown). In our study, the body weights and intake weights were similar among the 3 groups of obese rats.

Significant changes were detected in serum glucose levels in the O+0%GJ group rats compared to L+0%GJ group rats at week 12 (Figure 2A). Compared with the O+0%GJ group, the O+1%GJ and O+3%GJ rat groups

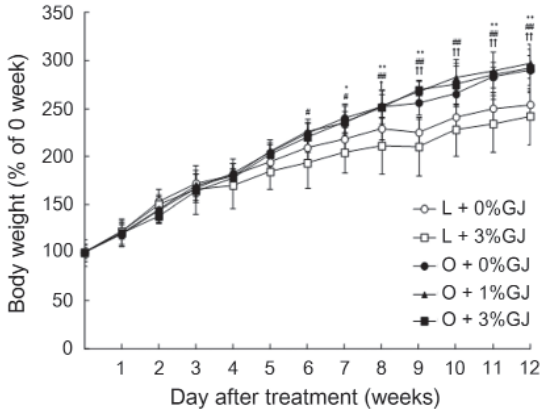

Figure 1. Effects of GJ on body weight in Zucker lean and obese rats. The mean values of O+0%GJ group rats significantly increased compared with that of the L+0%GJ group rats after the 7th week (* $p < 0.05$, ** $p < 0.01$). The mean values of O+1%GJ group rats significantly increased compared with that of the L+0%GJ group rats after the 6th week ($^{\#} p < 0.05$, $^{\#\#} p < 0.01$). The mean values of O+3%GJ group rats significantly increased compared with that of the L+0%GJ group rats after the 8th week ($^{\dagger} p < 0.05$, $^{\dagger\dagger} p < 0.01$). Data represent the mean ± S.D of values in each group ($n = 6$).

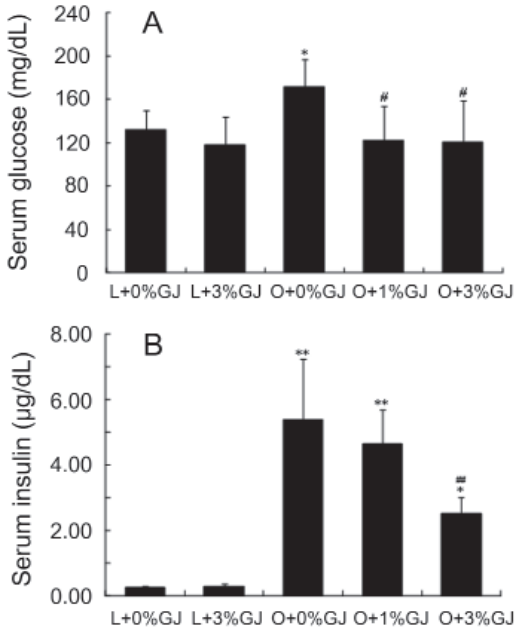

Figure 2. Effects of GJ on serum glucose (A) and insulin levels (B) in Zucker lean and obese rats. (A): The mean value of O+0%GJ group rats significantly increased compared to that of the L+0%GJ group rats (* $p < 0.05$). The mean value of O+3%GJ group rats significantly decreased compared to that of the O+0%GJ group rats ($^{\#}p < 0.05$). **(B):** The mean values of O+0%GJ, O+1%GJ and O+3%GJ group rats significantly increased compared to that of the L+0%GJ group rats (* $p < 0.05$, ** $p < 0.01$). The mean value of O+3%GJ group rats significantly decreased compared to that of the O+0%GJ group rats ($^{\#\#}p < 0.01$). Data represent the mean ± S.D. of values in each group ($n = 6$).

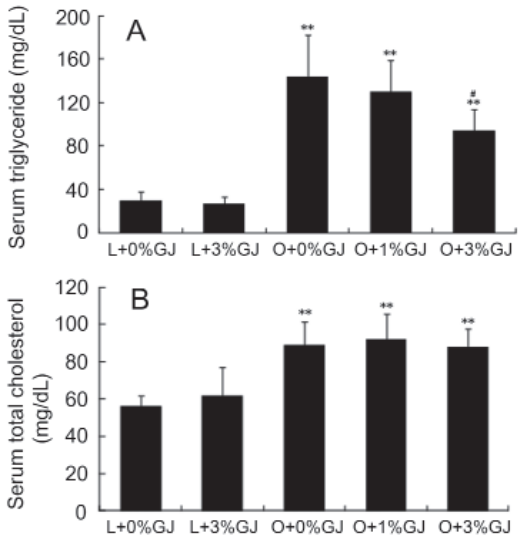

Figure 3. Effects of GJ on serum triglyceride (A) and total cholesterol levels (B) in Zucker lean and obese rats. (A): The mean values of O+0%GJ, O+1%GJ and O+3%GJ group rats significantly increased compared to that of the L+0%GJ group rats (** $p < 0.01$). The mean value of O+3%GJ group rats significantly decreased compared to that of the O+0%GJ group rats ($^{\#}p < 0.05$). **(B):** The mean values of O+0%GJ, O+1%GJ and O+3%GJ group rats significantly increased compared to that of the L+0%GJ group rats (** $p < 0.01$). Data represent the mean ± S.D. of values in each group ($n = 6$).

showed significantly decreased serum glucose levels ($p < 0.05$), similar to the L+0%GJ group rats at week 12. Figure 2B shows the changes in the serum insulin levels. In the O+0%GJ group rats, serum insulin levels were significantly higher than those in the L+0%GJ group rats ($p < 0.01$). The O+3%GJ group rats showed significantly decreased serum insulin levels as compared to the O+0%GJ group rats ($p < 0.01$). These results suggest GJ administration may prevent the development of hyperinsulinemia. We confirmed that GJ did not significantly reduce the serum glucose and insulin levels in lean rats (Figures 2). It has been reported that the nitric oxide pathway may mediate the effect of GJ on insulin action in insulin-sensitive tissues of diabetic model rats (*12*). Cinnamon extract, including cinnamomi cortex, one of the GJ components, has been shown to improve insulin action by enhancing the insulin-signaling pathway in skeletal muscles (*13*). In addition, Qin *et al.* reported that GJ administration improved impaired insulin sensitivity in rats with streptozotocin (STZ)-induced diabetes (*14*). Since GJ is a complex medical preparation in which each ingredient has a different pharmacological action, further studies are required to ascertain the molecular mechanisms underlying the effect of GJ on insulin sensitivity.

Significant increases were detected in the serum triglyceride and cholesterol levels in the 3 obese-rat groups at week 12, compared to the levels in the L+0%GJ group rats (all groups: $p < 0.01$; Figure 3). Serum triglyceride levels in the O+3%GJ group rats were significantly lower than only those in the O+0%GJ group rats ($p < 0.05$; Figure 3A), whereas serum cholesterol levels in the O+1%GJ and O+3%GJ rat groups were not significantly lower than those in the O+0%GJ group rats at week 12 (Figure 3B). Cinnamaldehyde (CA), one of the active components of cinnamon (derived from Cinnamomi cortex), has been reported to reduce plasma triglyceride and nonesterified fatty acid levels when a 40 mg/kg CA-administered group were significantly decreased (*15*). Chemical compounds of the GJ components, such as alisol A 24-acetate (in Alismatis rhizoma), may reduce blood cholesterol levels(*16*). However, this study showed that the administration of GJ did not alter serum cholesterol levels, but did reduce elevated serum triglyceride levels in obese rats.

In conclusion, the data in the present study suggest that GJ may prove useful in the amelioration and/or prevention of hyperinsulinemia and hypertriglyceridemia in a pre-diabetic state. However, further investigation will be necessary to elucidate the molecular mechanisms of GJ in long-term administration.

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Chemopreventive effects of combination of honokiol and magnolol with α-santalol on skin cancer developments

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ABSTRACT: α-Santalol is active component of sandalwood oil and has been shown to have chemopreventive effects against chemically and UVB-induced skin cancer development in mice. α-Santalol is also shown to have skin permeation enhancing effects. Honokiol and magnolol isolated from *Magnolia officinalis* bark extract have also been shown to have chemopreventive effects against chemically and UVB-induced skin cancer in mice. This study was conducted to investigate the combination effects of α-santalol, honokiol and magnolol to study any additive/synergistic effects to lower the doses required for chemoprevention. Pretreatment of combinations of α-santalol with honokiol and magnolol significantly decreased tumor multiplicity upto 75% than control, α-santalol, honokiol and magnolol alone in SKH-1 mice. Combination of α-santalol with honokiol and magnolol also decreased cell viability, proliferation, and enhanced apotosis in comparision to α-santalol, honokiol and magnolol alone in Human epidrmoid carcinoma A431 cells. Overall, the results of present study indicated combinations of α-santalol with honokiol and magnolol could provide chemoprevention of skin cancer at lower doses than given alone.

Keywords: Honokiol, magnolol, chemoprevention, UVB photocarcinogenesis, apoptosis

1. Introduction

Human non melanoma skin cancer including basal cell carcinoma and squamous cell carcinoma is most common type of cancer in the United States for over 1.3 million new cases each year (*1*). Both experimental and epidemiological evidences suggest UVB acts as complete carcinogen in skin cancer (*2,3*). Carcinogenesis induced by UV is a multistep process which involves initiation, promotion and progression by which genetic events accumulated with in a cell leading to dysplastic cellular appearance, deregulated cell growth and finally to carcinoma development (*4*). UVB in mouse models can act as complete carcinogen by initiating and promoting skin cancer.

Chemoprevention involves the administration of natural or synthetic compounds to prevent or reverse the process of carcinogenesis. Cancer chemoprevention by naturally occurring agents, especially the phytochemicals, minerals and vitamins, has shown promising results against various malignancies (*5,6*). It is promising strategy for carcinoma inhibition before development of invasive tumor (*7*). In recent years to inhibit or reverse the multistage process, considerable efforts are focused towards natural occurring compounds (*5,6*). Over 1,000 phytochemicals have shown chemopreventive effects against cancer (*6-9*). Among such phytochemicals, chemopreventive effects of α-santalol, honokiol and magnolol have been investigated against skin cancer and have shown excellent chemopreventive effects.

α-Santalol is active component of sandalwood oil which constitutes about 61% (w/w) of oil. Studies conducted in our laboratory have shown the topical application of 5% (w/v) of α-santalol inhibited skin tumorigenesis against both chemical (*10*) and UVB (*11*) induced animal cancer models. In addition to this, mechanistic studies conducted in human epidermoid carcinoma A431 cells have shown that possible mechanisms involved in chemopreventive effects of α-santalol is by induction of apoptosis through extrinsic and intrinsic pathway and inhibition of cell proliferation through cell cycle arrest at G2/M phase (*7,12,13*).

Honokiol and magnolol are isomers isolated from bark and seed cones of *Magnolia officinalis* which

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are used in traditional Chinese medicine (*14*). Studies conducted recently in our laboratory have shown that honokiol and magnolol have shown chemopreventive effects against skin cancer development (*15,16*). Furthermore, the study conducted in our laboratory have shown that α-santalol acts as a permeation enhancer for certain drug such as 5-flurouracil. This study was conducted to investigate if reduced doses of α-santalol produce similar effects in combination of honokiol and magnolol, may enhance the chemopreventive effects of honokiol and magnolol, and may provide additive/ synergistic effects against UVB-radiation induced skin cancer development in SKH-1 mice and *in vitro* in A431 cells.

2. Materials and Methods

2.1. *Reagents*

α-Santalol was isolated from sandalwood oil and characterized as reported (*18*). Thiazolyl blue tetrazolium bromide (MTT) and other chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell proliferation ELISA kit and In Situ Cell Death Detection Kit were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Vibrant Apoptosis Kit 2 and APO-BrdU TUNEL assay kit were purchased from Molecular Probes (Eugene, OR, USA).

2.2. *Isolation characterization and purity of α-santalol*

α-Santalol (Figure 1a) was isolated from sandalwood oil by distillation under vacuum as described in detail (*18*). On the basis of the NMR spectrum and the boiling point of the distillate, the major component of sandalwood oil is α-santalol. Further, GC-MS analysis, NMR data and mass spectrum of the isolated agent were consistent with the structure of α-santalol. Honokiol (Figure 1b) and magnolol (Figure 1c) were purchased from Nacalai tesque (Kyoto, Japan).

2.3. *UVB exposure source and animals*

UVB light source was four FS-40-T-12-UVB sunlamps (Daavlin, Bryan, OH) emitting 80% radiation within 280-340 nm with a peak at 314 nm. The UVB exposure dose was controlled using two Daavlin Flex Control Integrating 305 Dosimeters. Female SKH-1 hairless mice (five-weeks-old) were purchased from Charles River Laboratories (Wilmington, MA). All animal protocols were approved by the Institutional Animal Care and Use Committee. Mice were housed in the College of Pharmacy, South Dakota State University, animal room facilities (temp. 22 ± 1°C, humidity, 40-60%, light 6.00-18.00 h), and given food and water ad *libitum*. Mice were acclimatized for 2 weeks before starting the experiment.

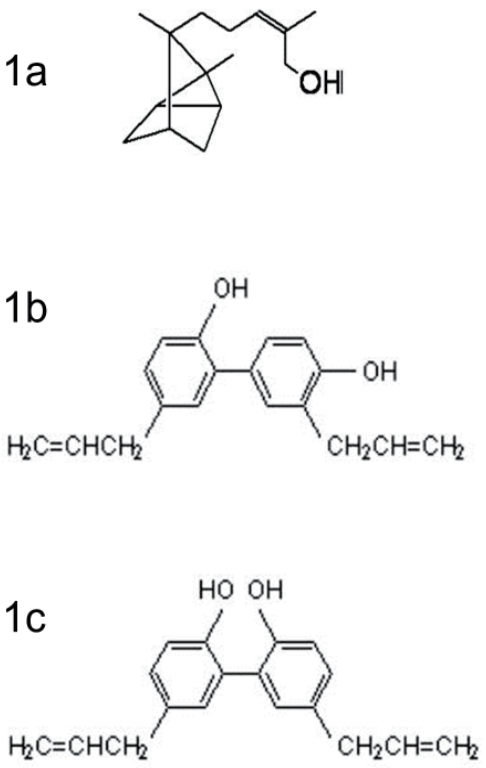

Figure 1. Structures of a) α-santalol, b) honokiol, and c) magnolol.

2.4. *Effects of α-santalol and honokiol on UVB-induced skin cancer development*

Female SKH-1 mice were divided in four groups having 20 mice in each group. Group assignment was as follows: group 1, control (200 µL acetone); group 2, α-santalol (5 mg in 200 µL acetone); group 3, honokiol (30 µg in 200 µL acetone); group 4, α-santalol (5 mg in 100 µL acetone) and honokiol (30 µg in 100 µL acetone). The mice in all groups were irradiated with 30 mJ/cm^2 dose of UVB one hour after the topical application as outlined above. UVB-irradiation was given 5 days a week and the experiment was continued for 30 weeks. Animals were monitored for food and water consumption, and any apparent signs of toxicity such as weight loss or mortality during the entire study period. Skin tumor formation, as evident by an outgrowth > 1 mm in diameter and persisting for ≥ 2 weeks was recorded. Tumor incidence and multiplicity were recorded weekly until the end of the experiment at 30 weeks.

2.5. *Effects of α-santalol and magnolol on UVB-induced skin cancer development*

Female SKH-1 mice were divided in four groups having 20 mice in each group. Group assignment was as follows: group 1, control (200 µL acetone); group 2,

α-santalol (5 mg in 200 µL acetone); group 3, magnolol (30 µg in 200 µL acetone); group 4, α-santalol (5 mg in 100 µL acetone) and magnolol (30 µg in 100 µL acetone). Experimental protocol similar to honokiol was carried out for 30 weeks.

2.6. *Cell culture*

Human epidermoid carcinoma A431 cells were purchased from American Type Culture Collection (Manassas, VA). A431 cells were cultured in DMEM supplemented with 10 % FBS, 100 µg/mL pencillin-streptomycin (Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 95% air and 5% CO_2.

2.7. *MTT assay for cell viability*

Cells (9,000 cells/well) were plated in 96 well plates. After 24 h, cells were treated with different concentrations of control, α-santalol, honokiol, magnolol for 48 h using control as cells treated with growth medium alone. At the end of each treatment, cells were incubated with 10% of MTT stock solution (5 mg/mL) for 4 h. Thereafter medium was aspirated and 150 µL of DMSO (dimethyl sulfoxide) was added to dissolve crystal dye formazan for 1 h. Absorbance was measured at 570 nm with absorbance at 650 nm to correct background for blank (media without cells) using Spectra Max M2 microplate reader.

2.8. *BrdU assay for cell proliferation*

Bromodeoxyuridine incorporation assay is carried out using ELISA kit using manufactures protocol. Briefly 9,000 cells/well were plated in 96 well plate and treated with different concentrations of control, α-santalol, honokiol, magnolol for 48 h. At the end of each treatment period, cells were labeled with BrdU by incubating for 3 h at 37ºC. Then cells were fixed by using FixDenat solution for 30 min followed by incubating fixed cells with anti-BrdU perioxide solution for 90 min. Then cells were rinsed with washing solution and incubated with substrate solution for 20 min. Reaction was stopped using 1M H_2SO_4 and absorbance of samples were measured using microplate reader using 450 nm with absorbance at 690 nm as reference.

2.9. *Apoptosis assay*

Apoptosis was quantified by using Vibrant Apoptosis Kit 2 as per manufactures protocol. Briefly A431 cells (2×10^5) were treated with control, α-santalol, honokiol, magnolol for 48 h. At the end of treatment cells were washed with ice cold PBS and incubated with annexin V labeled with Alexa 488 and PI for 15 min at room temperature. The stained cells were analyzed by FACS using Cell Quest 3.3 software. The early apoptotic cells were stained with Alexa 488 represented in lower right (LR) quadrant that gave green fluorescence, and late apoptotic cells were stained with both Alexa 488 and PI that gave green and red fluorescence represented in upper right (UR) quadrant.

2.10. *Statistical analysis*

ANOVA and Tukey test were performed on sample means using INSTAT software (Graph Pad, Sand Diego, CA). Significance was considered at $p < 0.05$.

3. Results

3.1. *α-Santalol and honokiol combination decreased tumor multiplicity*

The effects of α-santalol, honokiol and combination of α-santalol and honokiol on UVB-induced tumor multiplicity is shown in Figure 2. In the present study we found that topical application of α-santalol and honokiol combination significantly decreased tumor multiplicity compared to control and mice treated with α-santalol and honokiol alone. We found topical application of α-santalol and honokiol combination resulted in strong protection throughout our study where UVB act as complete carcinogen. At the end of 30 weeks experiment, average number of tumors in various groups was found to be 8.3, 5.6, 4.6, and 3. Furthermore, the combination of α-santalol and honokiol not only provided about 75% decrease in number of tumors as compared to control mice but significantly higher decrease than α-santalol and honokiol alone. These results clearly provide the

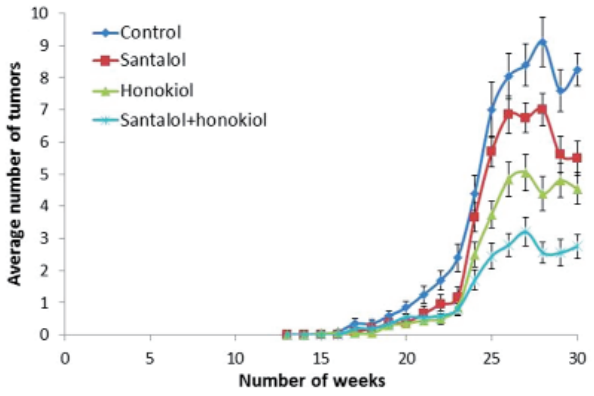

Figure 2. Effects of α-santalol , honokiol and combination of α-santalol and honokiol on tumor multiplicity in SKH-1 mice. Mice were irradiated chronically with UVB, 1 h after drug treatment. Group 1 served as control received acetone and group 2, 3, 4 are α-santalol, honokiol, and combination of α-santalol and honokiol. Combination significantly decreased tumor numbers compared to control and mice treated with individual drugs alone. Each data point represents mean tumor number ± SE.

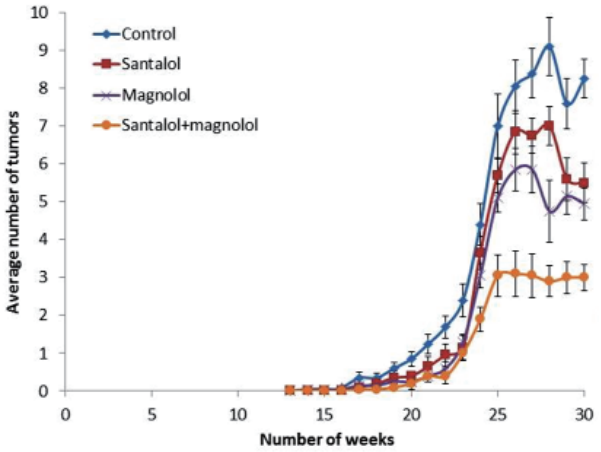

Figure 3. Effects of α-santalol, magnolol, and combination of α-santalol and magnolol on tumor multiplicity. α-Santalol and magnolol combination significantly decreased tumor number compared to control, α-santalol, and magnolol alone. Each data point represents mean tumor number ± SE.

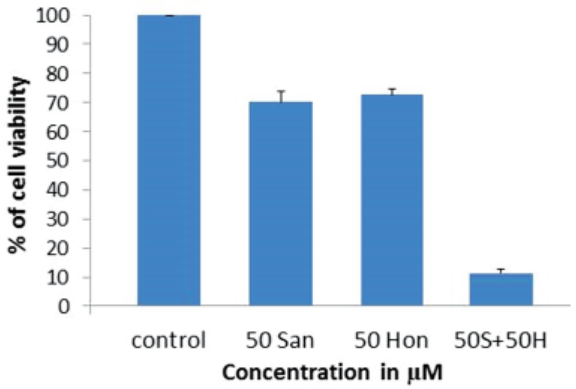

Figure 4. Effects of α-santalol, honokiol, and combination of α-santalol and honokiol on cell viability in A431cells. A431 cells were treated with α-santalol, honokiol, and combination of α-santalol and honokiol for 48 h. By using method as described in materials and methods, MTT assay was performed. Values are expressed as mean ± SE of eight replicates in each experiment in terms of percentage relative to control cells.

evidence that α-santalol significantly enhances the chemopreventive effects of honokiol on UVB-induced cancer development in SKH-1 mice.

3.2. *α-Santalol and magnolol combination decreased tumor multiplicity*

The effects of α-santalol, magnolol and combinations of α-santalol and magnolol on UVB-induced tumor multiplicity are shown in Figure 3. α-Santalol and magnolol significantly decreased the average number tumors when compared with control. A combination of α-santalol and magnolol topical treatment again provided about 75% decrease in the number of tumors per mice. Furthermore, the combination of α-santalol and magnolol provided significantly higher decrease in average number of tumors than both mice treated with α-santalol and magnolol alone. The data again clearly demonstrates the enhancing effects of α-santalol on the chemopreventive effects of magnolol.

3.3. *Effects of α-santalol combination on the effects of honokiol and magnolol on cell viability of A431 cells*

The effects of α-santalol combination on the effects of honokiol and magnolol on cell viability of A431 cells is shown in Figure 4 and 5. α-Santalol and honokiol treatment (50 µm) alone resulted about 30% decrease in the cell viability of A431 cells (Figure 4). However combination of α-santalol (50 µm) with honokiol (50 µm) caused a 90% decrease in cell viability of A431 cells. This combination of α-santalol with honokiol significantly enhances the cytotoxic effects in A431 cells. Similarly, combination of α-santalol (50 µm) with magnolol (100 µm) significantly enhances the cytotoxic effects in A431 cells (Figure 5).

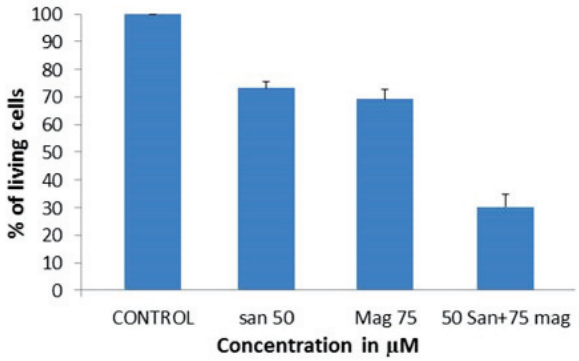

Figure 5. Effects of α-santalol, magnolol, and combination of α-santalol and magnolol on cell viability in A431cells. A431 cells were treated with α-santalol, magnolol, and combination of α-santalol and magnolol for 48 h. By using method as described in materials and methods, MTT assay was performed. Values are expressed as mean ± SE of eight replicates in each experiment in terms of percentage relative to control cells.

3.4. *Effects of α-santalol combination on the effects of honokiol and magnolol on cell proliferation of A431 cells*

Effects of α-santalol combination on the effects of honokiol on cell proliferation of A431 cell is presented in Figure 6. α-Santalol (50 µm) treatment caused a reduction of 20 and 40% respecting cell proliferation of A431 cells. However, a combination of α-santalol (50 µm) with honokiol (50 µm) almost completely stopped the cell proliferation of A431 cells. Although, combination of α-santalol with magnolol significantly enhances the effects on cell proliferation but not completely as with honokiol (Figure 7).

3.5. *Effects of α-santalol combination on the effects of honokiol and magnolol on apoptosis of A431 cells*

The effects of α-santalol combination on the effects

of honokiol and magnolol on apoptosis in A431 cells is shown in Figures 8 and 9. To investigate whether cell death resulted is in response to apoptosis, we determined it by annexin V/PI staining which detects early stages of apoptosis and results were analyzed through FACS. The cells were treated with α-santalol, honokiol, combination of α-santalol and honokiol (50, 50, and 50 + 50 μM) concentrations respectively for 48 h. The results showed that compared to DMSO treated control, apoptotic cells in α-santalol, honokiol, and combination of α-santalol and honokiol were increased. These results suggested that combination of α-santalol and honokiol significantly increased apoptosis compared to cells treated with α-santalol and honokiol alone (Figure 8).

In case of α-santalol and magnolol combination cells were treated with varying concentrations of α-santalol, magnolol, and combination of α-santalol and magnolol (50, 100, and 50 + 100 μM) respectively for 48 h. Similar to α-santalol and honokiol combination, α-santalol and magnolol combination significantly ($p < 0.05$) increased apoptosis (Figure 9).

4. Discussion

Emulsion, paste, or essential oil of sandalwood (SW) has been used for centuries in India for treatment of inflammatory and eruptive skin diseases (*18*). Ayurvedic physicians (traditional medical practitioners in India) treat numerous skin lesions in patients with

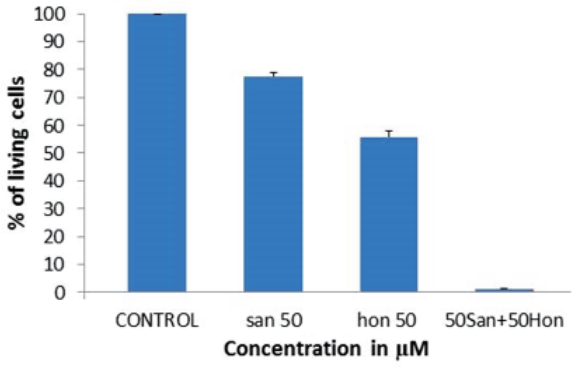

Figure 6. Effects of α-santalol, honokiol, and combination of α-santalol and honokiol on cell proliferation in A431cells. A431 cells were treated with α-santalol, honokiol, and combination of α-santalol and honokiol for 48 h. BrdU assay is performed for determining cellproliferation. Values are expressed as mean ± SE of four replicates in each experiment in terms of percentage relative to control cells.

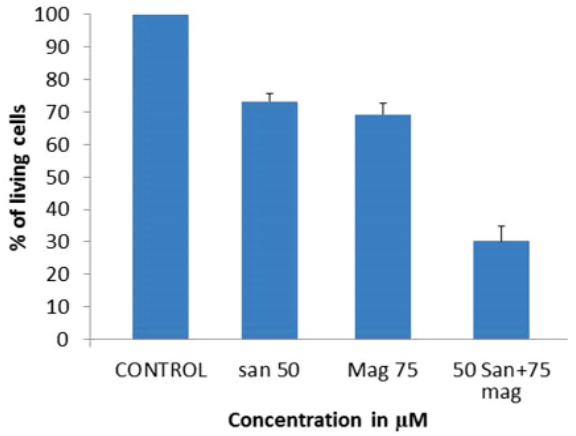

Figure 7. Effects of α-santalol, magnolol, and combination of α-santalol and magnolol on cell proliferation in A431cells. A431 cells were treated with α-santalol, magnolol, combination of α-santalol and magnolol for 48 h. BrdU assay is performed for determining cell proliferation. Values are expressed as mean ± SE of four replicates in each experiment in terms of percentage relative to control cells.

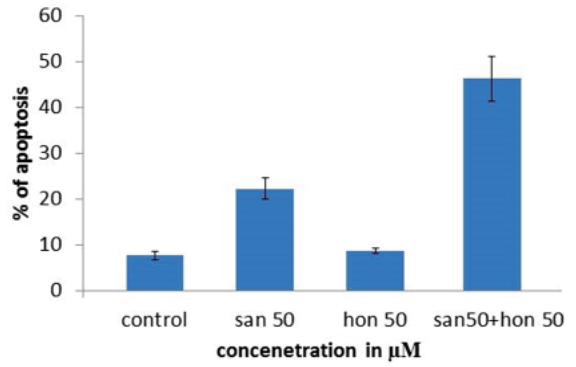

Figure 8. Effects of α-santalol, honokiol, and combination of α-santalol and honokiol on apoptosis in A431cells. A431 cells were treated with α-santalol (san 50) and honokiol (hon 50) at 50 μM and combination of α-santalol and honokiol (san 50 + hon 50) for 48 h. Cells were stained with annexin V/PI by using vibrant kit as described under materials and methods and results were analyzed by flow cytometry. Data represent mean ± SE of three observations. $p < 0.05$ indiicates statistical significance in drug treated groups.

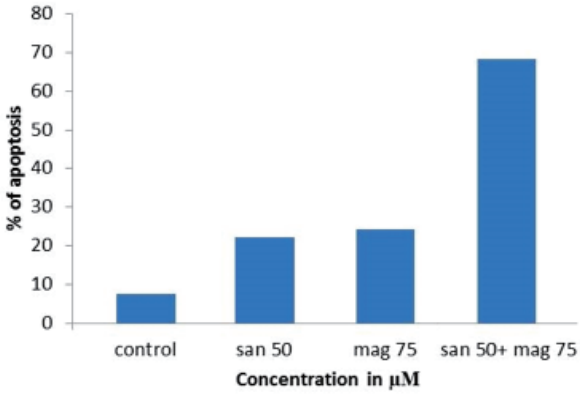

Figure 9. Effects of α-santalol, magnolol, and combination of α-santalol and magnolol on apoptosis in A431cells. A431 cells were treated with α-santalol (san 50) and magnolol at 100 μM (mag 100) and combination of α-santalol and magnolol (san 50 + mag 100) for 48 h. Cells were stained with annexin V/PI by using vibrant kit as described under materials and methods and results were analyzed by flow cytometry. Data represent mean ± SE of three observations. $p < 0.05$ indicates statistical significance in drug treated groups.

SW oil (*18*). The essential oil of SW is distilled from the small chips and billets cut out of the heartwood of SW. The oil is extremely viscid, of a light yellow color, and possesses a characteristic pleasant odor. The major constituent of oil is santalol, a mixture of two isomers, α- and β-santalol. Other constitutes of oil are aldehydes, ketones, isovaleric aldehyde, santanone, esters, and free acids (*19*). SW oil inhibits the replication of Herpes simplex viruses-1 and 2 *in vitro* (*19*). SW oil treatment (100 µL, 5% in acetone, twice a week, topically) significantly decreased papilloma incidence by 67% and multiplicity by 96% in 7, 12-dimethyl benzanthracene (DMBA) –initiated and 12.0-tetradecahoye phorbol-13-acetate (TPA)-promoted CD-1 mice. SW oil treatment also decreased TPA-induced epidermal ODC activity in CD-1 mice (*20*). SW oil treatment decreased papilloma incidence and multiplicity in a time- and concentration-dependent manner in CD-1 mice (*20*). The pretreatment with 5% SW oil 1 h before DMBA and TPA treatment provided a maximum reduction in papilloma incidence and multiplicity (*21*). Our laboratory isolated α-santalol from SW oil by distillation and characterized it by NMR and GC-MS. NMR and GC-MS indicated that α-santalol is a major component (~61%) of SW oil.

α-Santalol (5% topical) inhibited skin papilloma development during the promotion phase of DMBA and TPA protocol in CD-1 and SENCAR strains of mice. Induction of epidermal ODC activity and DNA synthesis are some of the prominent effects of TPA treatment on skin. As expected, α-santalol treatment (5% topical) significantly decreased ($p < 0.05$) TPA-induced ODC activity and incorporation of 3H-thymidine in DNA in the skin of CD-1 and SENCAR strains of mice. The effects of α-santalol on skin papilloma incidence and multiplicity are very similar to the effects of SW oil as reported earlier from our laboratory (*20,21*). α-Santalol has a pleasant fragrance, does not produce any stain, and appears to be nontoxic at concentrations used in our study. The effectiveness of α-santalol as chemopreventive agents appears to be very promising in skin cancer control.

Since UV radiations are the major cause of skin cancer in humans the effects of α-santalol on ultraviolet B (UVB) radiation-induced skin tumor development and UVB-caused increase in epidermal ornithine decarboxylase (ODC) activity in female hairless SKH-1 mice were studied (*10*). For the tumor studies, 180 mice were divided into three groups of 60 mice each and each group was divided into two subgroups of 30 mice. The first subgroup served as control and was treated topically on the dorsal skin with acetone. The second subgroup served as experimental and was treated topically on the dorsal skin with α-santalol (5%, w/v in acetone). The tumorigenesis in the first group was initiated with UVB radiation and promoted with TPA; in the second group it was initiated with DMBA and promoted with UVB radiation; and in the third group it was both initiated and promoted with UVB radiation. UVB radiation was at a dose of 180 mJ/cm^2. Topical application of α-santalol significantly ($p < 0.05$) decreased tumor incidence and multiplicity in all the three protocols, suggesting chemopreventive efficiency against UVB radiation-induced skin cancer. α-Santalol also significantly inhibited UVB-induced epidermal ODC activity. These findings suggest that α-santalol could be a potential chemopreventive agent against UVB-induced skin tumor development.

Honokiol and magnolol isolated from the bark of *Magnolia officinalis* have been shown to decrease chemically-induced skin cancer development in mice (*22*). We investigated the chemopreventive effects of honokiol and magnolol on UVB-induced skin cancer development in mice, a model more relevant to human. Both honokiol and magnolol provided a significant protection against UVB-induced skin cancer development in mice (*15,16*). Furthermore ,mechanistic studies conducted *in vivo* in SKH-1 mice and in A431 cells showed that honokiol and magnolol inhibited skin carcinogenesis by inducing apoptosis and inhibiting cell proliferation by causing cell cycle arrest (*16,17*).

Since α-santalol, honokiol and magnolol are isolated from plants and are relatively expensive, α-santalol, honokiol and magnolol have a great potential as chemopreventive agent for the skin cancer development. We investigated the effects of α-santalol pretreatment on the effects of honokiol and magnolol on UVB-induced skin cancer development in mice to evaluate if pretreatment with α-santalol enhances the effects of honokiol and magnolol.

The present study, for the first time demonstrated chemopreventive effects of topical applications of α-santalol and honokiol and α-santalol and magnolol combinations on skin tumor development in SKH-1 mice and in A431 cells. Pretreatment of combinations of α-santalol with honokiol and magnolol significantly decreased tumor multiplicity upto 75% than control, α-santalol, honokiol and magnolol alone. Overall, the results of present study indicated combinations of α-santalol with honokiol and magnolol at lower doses than alone provides protrction against UVB-induced skin tumorigenesis in SKH-1 mice possibly by inducing apoptosis.

Acknowledgements

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Original Article

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Evaluation of *in vitro* mutagenicity and genotoxicity of magnetite nanoparticles

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ABSTRACT: For successful application of nanomaterials in bioscience, it is essential to understand the biological fate and potential toxicity of nanoparticles. The aim of this study is to evaluate the genetic safety of magnetite nanoparticles (MNPs) (Fe_3O_4) in order to provide their diverse applications in life sciences, such as drug development, protein detection, and gene delivery. Concentrations of 10 ppm, 30 ppm, and 70 ppm (10-70 µg/mL) of the MNPs of 8.0 ± 2.0 nm were used. Characterization of MNPs was done with transmission electron microscopy (TEM), X-Ray Diffractometry (XRD) and a vibrating sample magnetometer (VSM). The MNPs mutagenic potential was evaluated using the Salmonella Ames test with Salmonella strains TA100, TA2638, TA102, and TA98 in the presence and the absence of metabolic activation with S9-liver extract. Genetic mutations at the chromosomal level and extent of DNA damage using the alkaline Comet assay were applied to peripheral blood lymphocytes and HEK-293 cell lines respectively. There were significant changes in the results of the Salmonella mutagenicity test at the 70 ppm concentration of MNPs which might reflect their mutagenic activity at higher concentrations. Cytogenetic evaluation revealed the absence of genetic mutations at the chromosomal level. The extent of DNA damage quantified by Comet assay and the mutagenicity study using Ames test were significantly correlated for the MNPs. Our results indicated that magnetite nanoparticles with the defined physicochemical properties caused apparent toxicity at higher concentrations of 30 ppm and 70 ppm without chromosomal abnormalities under the experimental conditions of this study.

Keywords: Magnetite nanoparticles, mutagenicity, genotoxicity, *in vitro* assays

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1. Introduction

Nanomaterial safety is becoming an increasingly debatable issue that has intensified over the past several years. The small size and particular shape, large surface area and surface activity, which make nanomaterials attractive in many applications, may contribute to their toxicological profile. Regarding their safety assessment nanomaterials cannot be treated in the same manner as chemical compounds. Therefore, the establishment of principles and test procedures to ensure the safe use of nanomaterials in the marketplace is urgently required. Magnetite nanoparticles (MNPs) are used with the intent to be utilized in bioscience for targeted delivery applications because they offer benefits such as separation and gathering of materials of interest in the presence of a magnetic force (*1*). Accordingly, for such a purpose, sufficient data regarding the toxicity and biological fate of the MNPs should be collected. In this study synthesized MNPs with an average size of 8.0 ± 2.0 nm were evaluated for their genotoxic effect to study their potential chronic toxicity, however the same particles were patented to be used as a single dose treatment for iron deficiency anemia and did not show any apparent toxicity on experimental animals during *in vivo* acute, sub-acute and chronic toxicity testing (*2*). Experience with non-nano substances taught us that mechanisms of genotoxic effects could be diverse. Application of standard genotoxic methods to nanomaterials and the interpretation of results are of highest consideration. Thus a practical approach is the use of a battery of standard genotoxicity testing methods covering wide ranges of mechanisms (*3*). In the presented work a comparative study for number of *in vitro* mutagenicity and genotoxicity investigations were performed including a bacterial reverse mutation test (Ames test), Single Cell Gel Electrophoresis assay (SCGE; comet assay), and standard karyotyping detection of chromosomal aberrations. Several studies on different types of synthesized MNPs have already proven their biocompatibility at the cellular level (*4,5*),

yet detection of gene mutation and DNA damage at the molecular level are still to be investigated.

Gentotoxicity studies on nanomaterials concluded that the particle size and charges, concentration, coating surfactant aggregation and surface properties of the nanoparticles, have profound influence on interpreting the genotoxicity testing. Small particle size (high surface area/mass) with high absorption capacity causes coating of the particles with proteins and nutrients from the culture media. This could obscure the essential nutrients for cell division in *in vitro* genotoxicity tests and influence cell proliferation leading to false positive results (*6*). In other cases, particles surface energy enhances catalytic activities leading to production of genotoxic reactive oxygen species. In the presented study, we tried to provide some characteristics of the chemical composition and physiochemical properties of the synthesized MNPs to draw a valid conclusion about their potential genetic side effects in comparison to other studies. Our aim is to help at improving our understanding of the underlying toxic mechanisms of MNPs. Such findings will have practical consequences in the risk assessment processes as well as the biomedical applications of these substances at the nanomaterials level.

2. Materials and Methods

2.1. *Synthesis of biocompatible magnetite nanospheres*

Magnetite nanospheres of 8.0 ± 2.0 nm were synthesized according to Jakubovic 1994 (*7*). In this work, water-soluble magnetite nanocrystals have been prepared *via* a co-precipitation reaction where 0.54 g of anhydrous ferric chloride ($FeCl_3$, sigma-Aldrich, 99.99%) were dissolved in deionized water (Milli-Q Ultrapure Water System, Millipore) at room temperature. Zero point six gram sodium carbonate (Na_2CO_3, BioXtra, ≥ 99.0% Sigma-Aldrich) powder dissolved in deionized water is added to the $FeCl_3$ solution with continued stirring for 10 min , the solution turned viscous with a brown color. Directly 0.12 g ascorbic acid (99.0%, Sigma-Aldrich) as a reducing agent and a biocompatible capping material was added with vigorous stirring for 15 min, and the color of solution turned black. The solution was autoclaved at 120°C for 4 h then washed with deionized water three times to remove the excess of the non-reacted precursors. The synthesized magnetite nanoparticles (MNPs) were characterized by Transmission Electron Microscope (TEM, Tecnai G20, FEI), X-Ray Diffractometry (X'Pert Pro, PanAlytical) and a Vibrating Sample Magnetometer (VSM, Lakeshore 7400).

2.2. *Toxicological evaluation of the synthesized MNPs in biological systems in vitro*

Salmonella typhimurium TA100, TA2638, TA102, and TA98 bacterial strains were purchased from Bonnie Kuenstler, Discovery Partners International Inc., San Diego, CA, USA and Trinova Biochem GmbH, Gießen, Germany. HEK-293 human embryonic kidney fibroblasts were obtained from the American Type Culture Collection (ATCC, CRL 1573). One million HEK-293 cells/mL were cultured in Eagle's MEM supplemented with 10% fetal bovine serum and L-glutamine (200mM). Cells were counted and treated with different concentrations of MNPs and the positive controls then incubated in the dark for either 1 h or 30 min at 37°C and 5% CO_2.

In this work four different strains of the bacteria *S. typhimurium* were used. They differ in characteristic mutations in the *hisG* or *hisD* gene. Strains TA2638 and TA102 have a genotype mutation in hisG428 while TA100 and TA 98 showed a mutation in HisG46 and His D3052 respectively. TA98 has a frame shift mutation, and all other strains show a base pair point mutation. Additionally, these strains have some other mutations that make them more susceptible to potential mutagens, using *S. typhimurium* mutants with a *rfa* – gene to increase the uptake of hydrophobic substances. The *uvrB* gene which is involved in DNA repair, guarantees the mutation would not be corrected by DNA repair machinery. Strains harbor the pKM101 plasmid that codes for ampicillin resistance to further improve the sensitivity of the assay.

Blood samples were collected in sterile heparinized vacutainer tubes and processed for Human Peripheral Lymphocytes (HPL) separation according to Singh *et al*., (*8*). The lymphocyte layer seen as a buffy coat was aspirated and washed with PBS and then incubated overnight in RPMI growth medium containing 0.001% phytohemagglutinin. Equal numbers of cells were seeded in 24 well plates for testing each experimental condition. Cells were treated with different concentrations of MNPs and positive controls. Tested compounds were incubated with the cells in the dark for either 1 h or 30 min at 37°C and 5% CO_2.

2.3. *Ames mutagenicity test*

The Salmonella mutagenicity assay was performed according to Maron *et al*., 1983 (*9*), using *S. typhimurium* strains TA100, TA2638, TA102, and TA98. Benzo[a]pyrene; 0.1 mg/plate (Sigma-Aldrich) was used as a positive control for activity of cytochromes P450. Other positive controls were used including sodium azide, 1.5 µg/plate; 2-aminoanthracene (2-AA) 10.0 µg/plate (Trinova Biochem, Germany), and cyclophosphamide, 0.2 mg/plate (Sigma-Aldrich). Positive controls were dissolved in deionized distilled water (DDW) or dimethylsulfoxide (DMSO). Bacterial cells were inoculated on NB-NaCl medium containing 75 µg/mL ampicillin and 90 µM histidine

and cultured for 15 h at 37°C with continuous agitation at 150 rpm until the cell density became 1-2 × 10^5/mL. Concentrations of 10 ppm, 30 ppm, and 70 ppm MNPs diluted in DDW were tested. DMSO was used as a reference negative control. All positive and negative controls as well as the MNPs concentrations were incubated at 37°C for 45 min with the bacterial cells in presence and absence of a 0.5 mL metabolic mixture containing subcellular fractions from male rat livers S9 liver extract (5% (v/v) in the metabolic mixture), (Sigma-Aldrich Chemie GmbH) using NADPH and G-6-P as cofactors (Sigma-Aldrich). The reaction mixture was then mixed with 2 mL top agar supplemented with 7 mM histidine (Sigma-Aldrich) and 5 mM biotin (Sigma-Aldrich) and then poured onto minimal glucose agar plates lacking histidine and allowed to harden. Agar plates containing treated bacterial strains were incubated at 37°C for 48 h, and the number of reverting colonies in the positive controls and the different dilutions of tested MNPs were counted and compared to those in the negative control.

2.4. *In vitro comet assay in HEK-293 and HPL cells*

Human embryonic kidney cells (HEK-293) and HPL cells from blood samples were used for the *in vitro* Comet assay investigation. A suspension of 10^6 cells prepared in 1 mL MEM fresh medium were incubated with the positive control compound (cyclophosphamide monohydrate) and the different dilutions of MNPs (10, 30, and 70 ppm MNPs diluted in DDW) at 37°C for 30 min and 1 h in the dark and in serum depleted medium in order to minimize DNA repair mechanisms. Cells incubated with PBS were used as a negative control. At the end of each incubation time, cells were washed twice with 1 mL PBS. Centrifugation was in the dark and cold at 1,400 rpm for 3 min. Viability of treated cells was determined using the trypan blue exclusion method and monitored at 75% with reference to the PBS treated cells in order to exclude cytotoxic effects in the interpretation of the assay results (*10,11*). Treated cells were finally suspended in 200 µL of 1.2% low melting point agarose (LMPA) at 37°C. Duplicate slides were prepared for each group of treated cells. One hundred µL volumes of cell suspensions in LMPA were pipetted on each slide pre-coated with 0.6% normal melting point agarose (NMPA). Slides were covered with a suitable cover slip, and gels were allowed to solidify on an ice-cold surface for 15 min. Cells were incubated in the dark for 1 h at 4°C in lysis buffer (2 M NaCl, 100 mM EDTA, 10 mM Trizma base pH 10, freshly added 1% Triton X-100, and 10% DMSO). Slides were then washed three times with distilled water. Subsequently, the slides were incubated in an alkaline electrophoresis buffer (1 mM EDTA, 300 mM NaOH at pH 13) in an electrophoresis chamber (Thistle Scientific) for 30 min to allow the DNA to unwind and then subjected to electrophoresis in the same buffer, for 30 min at 25 V and 300 mA. All electrophoresis steps were carried out in dark conditions followed by proper washing in neutralizing buffer (0.4 M Tris, pH 7.5) and finally staining with 0.001% 20 µg/mL ethidium bromide solution diluted in water before being scored.

For scoring the comet results, duplicate slides were counted for each dose and positive control groups. A total number of 100 cells in each slide were examined using a fluorescent microscope (Carl Zeiss, Axiostar plus, 37081) connected to an image analysis system (CometImager, metasystems GmbH). Quantification of DNA damage was assessed as a percentage of damaged DNA migrating to the tail.

2.5. *In vitro Karyotyping for HPL*

Duplicate whole blood cultures from healthy female donors were set up using a 0.5 mL peripheral blood sample in RMPI medium supplemented with 20% FBS, containing (10 µg/mL) phytohemagglutinin in order to stimulate mitotic division. Treatment with each MNP dilution took place for 3 h or 24 h before harvesting the metaphase chromosomes. The mitotic spindle fiber inhibitor colcimid (0.2 µg/mL) was added 18 min prior to cell harvesting which was performed 72 h after culture initiation. Cells were incubated with 10 mL of hypotonic solution (0.56% KCl) followed by fixation in a solution of methanol: acetic acid (3:1, v/v). Replicate slides of metaphases were prepared from each culture representing different drugs or concentrations of MNPs. Slides were incubated overnight at 40°C in phosphate buffered saline (3.4 g/L K_2HPO_4, PH 6.8) at 56ºC for 10 min followed by Giemsa banding of Metaphase chromosomes for 10 min. One hundred metaphase cells from each treatment group were analyzed for chromosomal damage. All slides were coded and screened in random order.

Aberrations were identified according to Savage, 1976 (*12*) as chromosome and chromatid type damage. Displaced and un-displaced fragments separated by a non-staining region equal to or greater than the width of the chromatid were scored as deletions. Non-staining regions of less than the chromatid width were scored as gaps. Prior to mutational analysis and Karyotyping the mitotic index (MI) for each culture was estimated based upon 500 lymphocytes.

2.6. *Statistical analysis*

For each of the applied assays, data were expressed as mean ± SE from three independent experiments using Graphpad Prism (San Diego, CA, USA). Data were subjected to statistical analysis by Tukey Multiple comparison ANOVA test with 95% confidence interval levels. Treatment groups were considered significantly different from the control group at a value of $p < 0.05$.

3. Results

3.1. *Magnetite nanospheres characterization*

In the current study, the co-precipitation method for preparation of magnetite nanocrystals was adapted to obtain uniform, a nearly spherical well-dispersed aqueous solution with perfect magnetite properties. Ascorbic acid was used to protect the trivalent ion from reduction giving better biocompatibility character in hydrated aqueous solution. The TEM images of the synthesized MNPs show an average size of 8.0 ± 2.0 nm with spherical shape (Figure 1). The formation of pure magnetite nanoparticles was confirmed by XRD analysis where only the magnetite phase pattern appeared with a cubic crystal structure (ICCD card: 04-0025683, High score plus) (Figure 2). Measurement of magnetic properties was done using VSM on an un-oriented, random assembly of particles at room temperature; a hysteresis loop was generated from which the intrinsic coercivity (Hc) remnant magnetization (Mr) and saturation magnetization (Ms) were calculated. The M(H) curve or hysteresis loop for the magnetite sample measured under 15,000 Oe at room temperature is displayed in Figure 3. The saturation magnetization of the product is 5.2 emu/g and is much smaller than that (68.7 emu/g) of magnetite nanoparticles sized about 70 nm prepared through a hydrothermal method without any surfactant (*13*). In addition, the coercivity of synthesized magnetite approaches zero Oe indicating that the obtained magnetite nanoparticles are super paramagnetic.

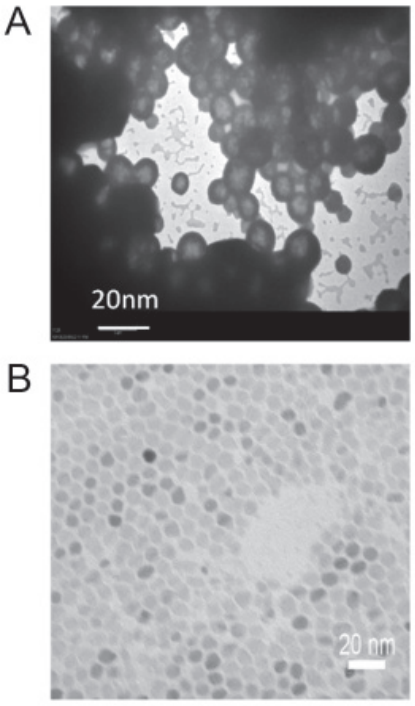

Figure 1. High power (A) and low power (B) TEM images of the MNPs capped with ascorbic acid showing that these particles have spherical shape with average size of 8.0 ± 2.0.

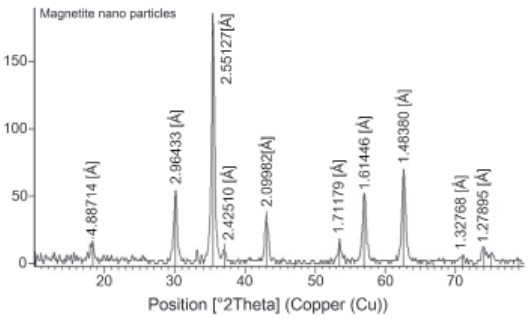

Figure 2. X-ray diffractometry (XRD) analysis of the magnetite phase pattern with cubic crystal structure.

3.2. *MNPs mutagenic effect*

The mutagenic effect of various MNP concentrations exerted on *Salmonella* strains with/without metabolic activation (S9 liver extract) were compared to negative control materials (water and DMSO) and positive control mutagenic agents benzo[a]pyrene, cyclophosphamide monohydrate, sodium azide, and 2AA. Results showed insignificant mutagenic activity ($p > 0.05$) for the MNPs at 10 ppm and 30 ppm concentration when incubated with all types of *Salmonella* strains in presence and absence of metabolic activation. On the other hand, 70 ppm of MNPs showed mutagenic activity only after metabolic activation, with TA100 ($p < 0.05$) compared to the negative control (Figure 4). Tested substances can reverse and correct *Salmonella* strains mutations to the wild type. It is known that TA100 and TA2638 strains may not detect certain oxidizing mutagens, cross-linking agents and hydrazines. Such substances may be detected by *S. typhimurium* TA102 which have an AT base pair at the primary reversion site.

3.3. *MNPs DNA damage*

The Comet assay was performed using the respective MNP concentrations after incubation with HEK 293 and

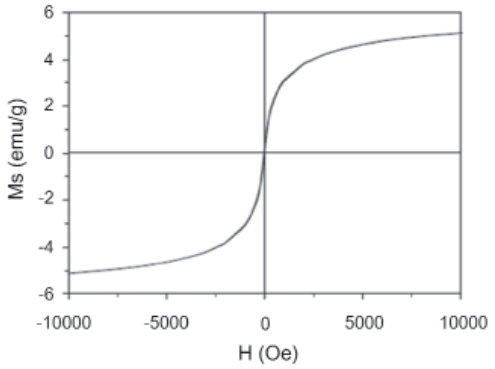

Figure 3. Hysteresis loop measurements of Fe_3O_4 nanoparticles capped with ascorbic acid proving its magnetic field properties.

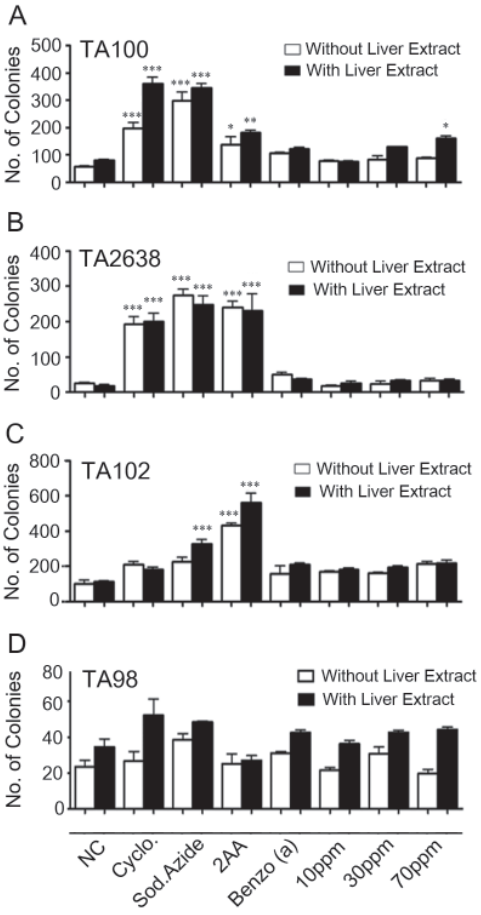

Figure 4. Mutagenic effect of MNPs in presence and absence of S9 liver extract. Results are reported as N° of revertant colonies with strains TA100 (A), TA2638(B), TA102 (C), and TA98 (D). Cyclophosphamide, sodium azide, 2-AA, and benzo (a) pyrene represent the positive controls. Results show the mean value + SE of three independent experiments. Significantly different from the control group at value of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

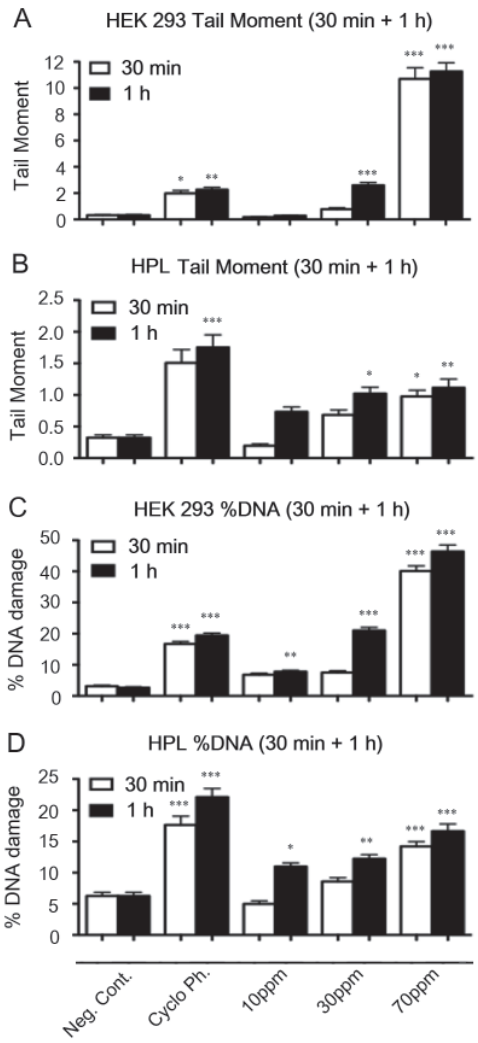

Figure 5. Genotoxic effect of MNPs after 30 and 60 min on HEK-293 cells (A, C) and HPL (B, D). Results are reported as tail moment and %DNA damage, respectively. Cyclophosphamide represents the positive control. Results show the mean value + SE of three independent experiments. Significantly different from the control group at value of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

HPL cells for 30 min and 1h in comparison to a positive control; Cyclophosphamide monohydrate. The tail moment and % DNA damage data were expressed as mean ± SE pooled from three independent experiments. No significant difference in tail moment measurements at MNP concentrations of 10 ppm and 30 ppm after 30 min were observed with HEK 293 cells nor HPL cells compared to the negative control ($p > 0.05$ *vs*. control), while a statistically significant increase in the tail moment was noticed at the maximum concentration of 70 ppm ($p < 0.01$). Exposure time up to 1 h showed a significant increase in the tail moment at 30 ppm and 70 ppm of MNPs with both types of cells (Figures 5A and 5B).

Genotoxicity level was measured as %DNA damage of HPL and HEK 293 cells after incubation with the three MNP concentrations. %DNA damage results showed an insignificant increase in genotoxicity at lower MNP concentrations (10 ppm and 30 ppm) at 30 min incubation. Significant genotoxicity was detected at all MNP concentrations after 1 h incubation with both types of cells (Figures 5C and 5D, Figure 6).

3.4. *Effect of MNPs on mitotic index and chromosomal structure*

An *in vitro* cytogenetic test was carried out by treating human lymphocyte cultures with MNP concentrations using cyclophosphamide monohydrate as a positive control for both short term (3 h) and long term (24 h) incubation. Results at the short incubation time, revealed an absence of chromosome and chromatid types of mutation upon treating blood lymphocytes (HPL) with any of the MNP concentrations. Evaluation of the MNPs influence on the mitotic index proved that the mean percentages of mitotic indices after treatment with higher concentrations (30 ppm and 70 ppm) of MNPs for 3 h showed an insignificant decrease in mitotic index with the 30 ppm ($p > 0.05$) relative to a highly significant drop of metaphase count at 70 ppm ($p > 0.01$) in comparison to the control cells. However, at

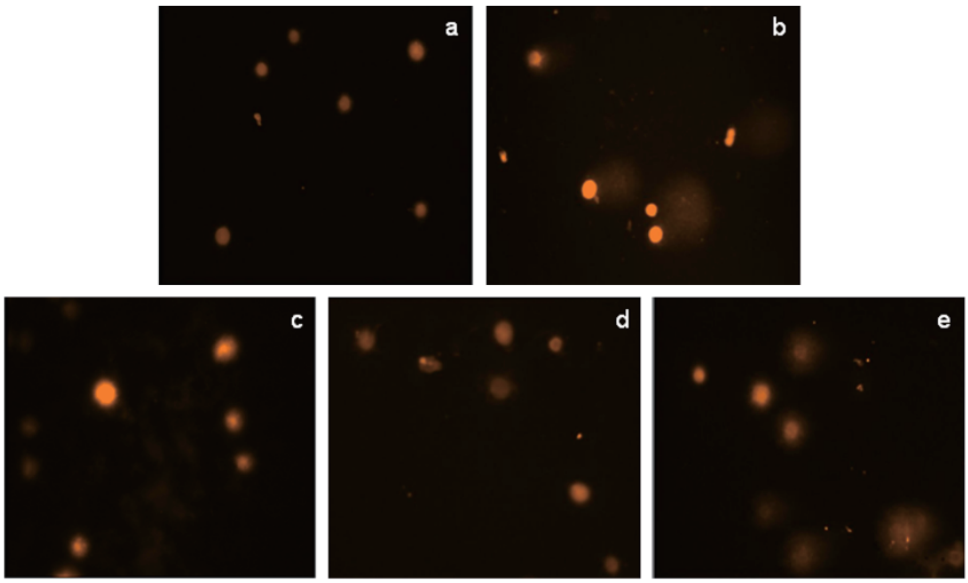

Figure 6. Comet images of HEK293 cells treated with PBS (a), cyclophosphamide (b), MNP concentrations of 10 ppm (c), 30 ppm (d), and 70 ppm (e).

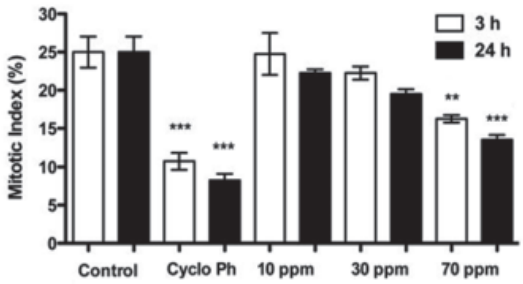

Figure 7. Mitotic index of human peripheral blood lymphocytes treated with different concentrations of MNPs at 3 h and 24 h. Results represented as % of the normal control. Cyclophosphamide represents the positive control. Results show the mean value + SE of three independent experiments. Significantly different from the control group at value of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

24 h results were significantly different for the 30 ppm ($p < 0.05$) and very highly significant in case of the 70 ppm ($p < 0.001$) compared to the non-treated control cells (Figure 7). Treatment of blood lymphocytes with (100 µg/mL) of cyclophosphamide for 3 h resulted in structural chromosomal aberrations in the form of breaks and gaps of both the chromatid and chromosome types with reference to the non-treated cells (Figure 8). However, extending the MNP incubation period to 24 h showed hypoploidy for several chromosome numbers in all examined metaphases. Accordingly, results of the mitotic index reflected severe suppression upon treatment with the MNPs both at 3 h and 24 h incubation times as indicated by extremely a significant decrease in mitotic indices in comparison to the untreated cells ($p < 0.001$) at both incubation periods.

4. Discussion

The present findings proved that MNPs had concentration dependent and a reproducible pattern suggesting a mutagenic effect of synthesized MNPs with the *S. typhimurium* strains used under the present experimental conditions. Results obtained with the Ames test and Comet assay recorded high consistency. The intensity of DNA strand breaks measured as tail moment and/or %DNA damage were well correlated and were greater at 1 h incubation than at 30 min incubation. Maximum DNA migration was observed at 70 ppm MNP concentration after 1 h incubation. Detection of DNA migration after 1 h supports reported publications on the optimal detection time in Comet assay being less than 3 h for DNA damage observation. Otherwise, false negative results could be obtained as a result of DNA repair initiation (*14*). In comparison to our study, Sun *et al*. reported the effect of magnetite Fe_3O_4 particles with a size range (8-20 nm) using a 10-fold increase in concentration (0.1-4.6 mg/mL) as compared to ours (*5*). The Sun *et al*., study on 3T3 monkey cells indicated the absence of a cytotoxic effect but didn't confirm the lack of a genotoxic effect. Our results also showed no cytotoxic effect with high cell viability (more than 75%) at lower MNP concentrations but evidenced a genotoxic effect. Thus it could be concluded that the MNP concentrations used, although not cytotoxic were significantly genotoxic especially at 70 ppm concentration. Comparison of our results to those of Sun *et al*. is difficult due to dissimilarity in the nanoparticles surface charge and composition characterization that could lead to different biological responses; however both cytotoxicity and genotoxicity should be conducted to confirm nanoparticle biocompatibility.

Results of the standard karyotyping revealed suppression of the mitotic index after incubation with the higher MNP concentrations (30 and 70 ppm).

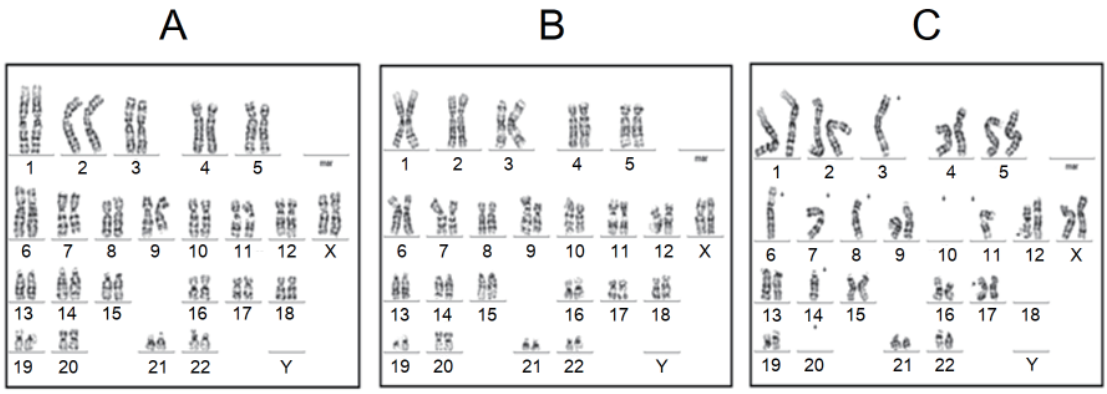

Figure 8. G-banded human Karyotypes of normal control cells (A), MNP treated cells (B), and cyclophosphamide (C), showing chromosomal breaks and fragments.

These concentrations of MNP induce mitotic cell arrest followed by cell death. This could be explained on the basis of extension in interphase due to inhibition of DNA synthesis and an increase in G1 phase duration (*15*). On the other hand, it might also be traced back to inhibition of some metabolic events necessary for the normal sequence of mitosis (*16*), or inhibition of protein synthesis (*17*).

In the present study, in spite of the fact that MNPs showed neither chromatid nor chromosome types of mutations, they could still be considered as clastogenic agents. In support of this, data obtained from the Comet assay as well as Ames test revealed that higher concentrations of MNPs exhibited significant genotoxic and mutagenic effects as compared to non-treated cells. Discrepancies in the results of the assays used might rely in one part on the test sensitivity as certain types of mutations such as sister chromatid exchange cannot be detected except with the application of more sensitive molecular techniques such as multiplex fluorescent in situ hybridization (mFISH) (*18*) which we intend to undertake in future studies.

For most nanoparticles it is unknown whether they directly interact with DNA as association of gold nanoparticles with the major groove of DNA (*19*) or indirectly through an inflammation mediated oxidative stress effect. Recognition of the oxidative DNA strand break in terms of FPG (formamidopyrimidine glycosylate) sensitive sites which cleaves DNA sites at the oxidized 8-oxoguanine purines has been reported in bronchoalveolar lavage cells from rats instillated with TiO2 (*20*). In another study, iron nanoparticles were reported to be redox reactive with a pro-inflammation and pro-oxidative effect on various cell models (*21,22*). Since the mechanism underlying the interaction of DNA with iron nanoparticles has not been investigated yet, their elucidation can be demanding. Future studies on the exact interaction mechanism of iron nanoparticles with DNA are under investigation in our laboratories.

In conclusion, this work highlights that *in vitro* cytotoxicty testing of nanomaterials does not imply safety and biocompatibility as has been reported in previous studies. We rather suggest an urgent demand for applying a battery of genotoxicity testing to assess the genotoxic hazard of nanoparticles. Information on the full characterization of the chemical composition and physiochemical properties of MNPs is important to elucidate the uptake potential in individual cell lines and how the particles will interact electrostatically with DNA and proteins in biological fluids to elucidate the underlying mechanism. Recognition of the genotoxic mechanism will improve the possibility of optimal choice of applied genotoxicity tests.

Future recommendations are directed towards investigation of the mutagenicity and genotoxicity of MNPs in *in vivo* model systems. This is due to the general belief that the positive results at the *in vitro* level are too extreme to be taken as an indication in terms of human exposure. The reason for that is pharmacokinetics, metabolism, tissue and species specificity may all play an important role in the *in vivo* genotoxicity and clastogenicity assays of different shapes, sizes, and chemical compositions of nanomaterials (*23*).

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Commentary

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The first inhibitor-based fluorescent imaging probe for aminopeptidase N

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ABSTRACT: Höltke and his co-workers firstly reported the synthesis and characterization of an inhibitor-based fluorescent imaging probe for aminopeptidase N. This fluorescent probe demonstrated high binding affinity to APN and could specifically bind to APN high expressed cells, thus revealed the distribution of APN. As a milestone, this outstanding work provided a useful tool to understand APN pathophysiology.

Keywords: Aminopeptidase N, affinity-based, fluorescent probe, cell imaging

Aminopeptidase N (APN/CD13) is a zinc-dependent exopeptidase positioned on the surface of diversified cells, such as the brush border membranes of kidney, synaptic membranes in the central nervous system, mucosal cell of the small intestine, as well as the fibroblasts, monocytes and myeloid progenitors (*1-3*).It is up-regulated on a number of carcinoma cells formediating the angiogenesis and metastasis of the tumor (*4*,*5*). Moreover, APN participates a criticalrole in cancer stem cells since it has become a diagnostic or prognostic biomarker for cancers (*6*). As a result, it is meaningful to image the distribution of APN *in cellulo*. Among the current imaging strategies, fluorescent imaging approach reflects the distinctive advantage, such as reasonable sensitivity, high spatiotemporal resolution and noninvasive feature to be employed in real-time examination (*7*,*8*).

Our group have reported a ratiometric fluorescent probe that can image APN activity in living cells (*9*). However, after being hydrolyzed by APN, the obtained fluorophores went into the whole cells, so it failed to capture the accurate location of APN. CNGRC (Cys-Asn–Gly–Arg-Cys) is a tumor homing peptide that can specifically bind to APN in tumor vasculature (*10*). Although numerous CNGRC based imaging probes has been documented throughout the literature, the binding affinity of CNGRC to APN was moderate (*11*). It's well known that inhibitors often have appropriate binding affinity to their enzymatic target, therefore the connection of a inhibitor with a fluorophore at the appropriate position, which does not affect the inhibitor-target interaction, may acquire an efficient and specific probe. The first inhibitor-based fluorescent APN probe, bestatin-linker-fluorescein, was synthesized by the Green baum lab (*12*). However, they did not report the imaging result of this probe on cells.

The first successful example for APN cell imaging was recently reported by Höltke and his colleagues (*13*). As depicted in Figure 1, this imaging probe (**Cy5.5-23**) includes three portions *i*) the inhibitor moiety that can tightly bind to APN; *ii*) a fluorescent reporter; and *iii*) a short polyethylene glycol (PEG) spacer that can reduce the hindrance of interaction between APN and fluorophore. Compared to the lead inhibitor, **Cy5.5-23** revealed even better inhibitory activity to APN. Additionally, **Cy5.5-23** displayed low cytotoxicity, which is essential for an imaging probe. Cell binding assays disclosed that **Cy5.5-23** could bind to APN-positive BT-549 cells but not to APN-negative BT-20 cells, and this binding could be significantly reversed by 100-fold excess of bestatin (a positive APN inhibitor).

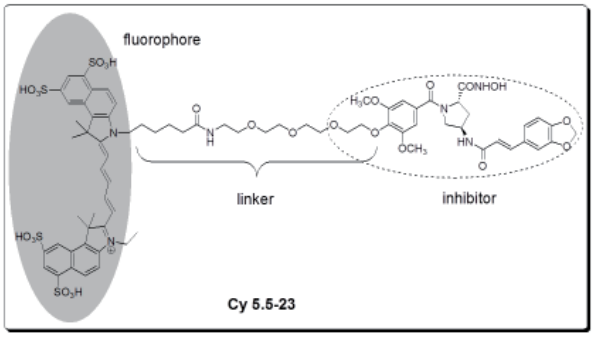

Figure 1. The structure of probe Cy 5.5-23.

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Based on the above-mentioned superiorities, **Cy5.5-23** provides a significant breakthrough for surveying the distribution of APN. Furthermore, considering that the near-infrared light emitted by Cy5.5 has exceptional deep tissue penetration capability, **Cy5.5-23** may be facilitated to image APN distribution in tissues and even in living animals.

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Commentary

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Rare disease patients in China anticipate the sunlight of legislation

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ABSTRACT: It is estimated that there are over ten million rare disease patients in China currently. Due to a lack of effective drugs and reimbursement regulations for medical expenses the diseases bring most patients enormous physical suffering and psychological despair. Past experience in other countries such as the United States, Japan, and the European Union have shown that legislation is the critical step to improve the miserable situation of rare disease patients. Laws and regulations for rare diseases in these countries prescribe a series of incentives for research and development of orphan drugs which turn out to obviously allow these drugs to flourish. Legislation has also established a drug reimbursement system to reduce the medical burden of the patients. These measures effectively protect the rights and interests of patients with rare diseases. In China, legislation for rare diseases has begun to attract the attention of authorities. It is anticipated that relevant laws and regulations will be established as early as possible to provide safeguards for rare disease patients in China.

Keywords: Rare disease, orphan drugs, laws and regulations, medical reimbursement

A rare disease is referred to as any disease that affects an extremely small percentage of the population. The World Health Organization (WHO) defines a disease as a rare disease when its incidence ranges approximately from 0.65-1‰ in the whole population. In different countries, identification standards of a rare disease varies based on their specific legislation. For example, a rare disease is identified in the United States (US), Japan, and Australia when it afflicts less than 200,000 (approx. 0.75‰ of the population), 50,000 (approx. 0.4‰ of the population), and 2,000 (approx. 0.1‰ of the population) people, respectively (*1*). In China, the definition of a rare disease is not officially established due to a lag in legislation. Expert consensus indicates that a rare disease could be identified in China when the incidence of the disease in adults or neonates is less than 1 in 500,000 and 1 in 10,000, respectively (*2*). Although each specific disease affects a limited number of patients because of its rarity, the total number of patients with rare diseases represents a striking proportion of the total population because it is estimated that there are 5,000-7,000 distinct rare diseases worldwide (*3*). In the US and European Union (EU), it is estimated that 30 million people suffer from rare diseases in each of these regions (*4,5*). In China, this figure may be over 10 million according to the definition of rare diseases by WHO and the number of cases of rare diseases patients in other countries. These statistical data suggest that the overall incidence of rare diseases is far from rare either in China or in other regions of the world.

Missed or delayed diagnosis, shortage of effective drugs, and the high cost of currently available drugs are three features in treatment of rare diseases. In China, the latter two are particularly prominent since lack of incentive measures for the development of so called 'orphan drugs' and absence of drug reimbursement for the high price tag of these medicines. Thus far, no orphan drugs have been successfully developed and marketed by the domestic pharmaceutical companies in China. Patients with rare diseases are faced with the situation that they are nearly dependent on imported drugs. However, there are two hindrances that limit the availability of these orphan drugs to the patients. First, it takes a long time to obtain imported drug licenses in China. The result is that orphan drugs cannot be approved for marketing in China in a timely fashion. Thus, rare disease patients have difficulty getting suitable treatments in a short time period although the effective drugs have been approved and used in clinics in other countries. The delayed treatment leads to irreversible pathological changes and thus causes lifelong suffering for patients. Second, the government has not established a specific national healthcare system for rare disease patients, which leads to poor affordability of treatment costs for general families. The complicated research process, high expenses for pre-clinical and clinical trials, and the small targeted population decide the high prices

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of successfully developed orphan drugs. Since 80% of rare diseases have genetic origins and 50% occur in childhood, most of these patients must receive therapies for a lifetime (*6*). Without strong support from the drug reimbursement system, most patients feel powerless and frustrated in obtaining effective but expensive treatments. In brief, rare diseases bring patients in China substantial physical suffering and psychological despair due to the lack of therapeutic hope and the absence of practical support for everyday life. Since these kinds of diseases require a significant amount of labor for the patient's care, they also cause a heavy burden on other family members, both financially and mentally. The helpless state of patients with rare diseases in China should be of concern to the entire society.

Legislation has proven to be the critical step to improve the miserable situation of rare disease patients. In fact, the development of orphan drugs for treatment of rare diseases had not been the focus of the pharmaceutical companies in the world for a long time due to worries of small market demand for a certain kind of disease and expensive research and development costs. Owing to the relentless work of patient and parent organizations, the neglected status of orphan drug development has attracted the attention of public health authorities and policy makers in recent decades. Since 1983 when the first law on rare disease in the world, *i.e.* Orphan Drug Act, was enacted in the US, thus far over 30 countries and regions such as Japan, the EU, Australia, and South Korea have passed laws and regulations to stimulate research and development of orphan drugs and help build a health care system for rare disease patients. Legislation has provided incentives for orphan drug development such as, new drug exclusivity, tax credits, subsidies for research, fast government evaluation, and drug reimbursement to a variable extent (Table 1) (*7*). With the aid of legislation, the number of approved orphan drugs has substantially increased. In the US, since the mid-1990s there has been a near tripling in the annual number of orphan drug designations for drugs in development, from 57 in 1996 to 165 in 2008 (*8*). In the 29 years (1983-2011) since the Orphan Drug Act was established, a total of 398 orphan drugs have been approved (*8*). On the contrary, only less than 10 orphan drugs were marketed in the US for a long time period before the law was enacted. Orphan drugs also represent a growing proportion of Food and Drug Administration (FDA) approvals, accounting for 30% in the most recent five-year period. In 2011, 35 new molecular entities were approved, in which 11 are for patients with rare

Table 1. Outline of laws and regulations for rare diseases in different countries

Items	US	Japan	Australia	EU	South Korea
Laws or regulations	Orphan Drug Act	Pharmaceutical Affairs Law (Article 77-2)	Orphan Drug Policy	Regulation(EC) No 141/2000	Orphan Drugs Guideline
Issue year	1983	1993	1998	2000	2003
Population size	305,000,000	127,000,000	21,000,000	500,000,000	48,000,000
Disease identification	< 7.5 in 10,000	< 4 in 10,000	< 1.1 in 10,000	< 5 in 10,000	< 4 in 10,000
Application range	Drug, biological products, medical device, medical foods, parenteral nutrition	Drug, biological products, medical device	Drug, biological products (including vaccine and in vivo diagnostic reagents)	Drug, biological products (including vaccine and in vivo diagnostic reagents)	Drug, biological products, medical device
New drug exclusivity	7 years	10 years	5 years	10 years	6 years
Tax credit	Yes; 50% of clinical trial expense	Yes; 6% of total drug development expense and 10% of corporate tax (Maximum)	No	Encourage member countries to offer tax favors	No
Subsidy for research	Yes; clinical trials	Yes; the entire process	No	Yes	No
Technical support for preparing application documents	Yes	Yes	No	Yes	No
Fast review program	Yes	Yes	Yes	Yes	No
Re-review after marketing	No	Yes	Yes (every 12 months)	Yes (6 years after marketing)	Unknown
Medical reimbursements	National health insurance, commercial health insurance	National health insurance plus 10% discount of drug price	Life Saving Drugs Program	Unique in each member country	Reimbursing two-thirds of medical expense

Data are from China Rare Disease Manual (*http://www.chinararedisease.cn*).

diseases such as the genetic defect of congenital factor XIII deficiency, several cancers, and scorpion poisoning (*9*). The flourishing of development of orphan drugs is largely ascribed to the stimulation effect of the laws and regulations that have been established in the orphan disease area.

It is worth noting that the laws and regulations that have been issued prescribe terms on the assurance or reimbursement of medical expenses for rare diseases. In the US, rare disease patients are not only covered by the national basic medical care system, they can also be accepted as insured by commercial health insurance. In Japan, the National Health Insurance System covers everyone who lives in this country and will reimburse 70% of medical costs in general cases. For rare disease patients, another 10% discount of drug prices is offered. In South Korea, the insurance system undertakes two-thirds of medical expenses for the patients. These measures provide powerful financial security for rare disease patients to obtain essential health care. In addition, they also offer economic security for pharmaceutical industries to cover costs and profits from the product.

Legislation on rare diseases has begun to attract the attention of government in China. In the National People's Congress (NPC) and Chinese People's Political Consultative Conference (CPPCC) of 2009, several NPC and CPPCC delegates proposed that legislation on rare diseases in China should be established as early as possible (*10,11*). Their suggestions include *i*) establishing an authentic and scientific definition of rare diseases; *ii*) constructing a reasonable drug reimbursement system; *iii*) simplifying the registration process for imported orphan drugs; *iv*) promoting research and development of orphan drugs through intensified policy support; *v*) building-up national health service institutes for rare disease patients. Although these proposals have been admitted by the Legislative Affairs Commission of the Standing Committee of the National People's Congress, it may take quite a long time (3-10 years) for the whole process from the motion to the final legislation. Fortunately, some districts in China have introduced policies that could reduce the medical care burdens of rare disease patients. In Shanghai, twelve kinds of rare diseases such as, phenylketonuria, maple syrup urine disease, and tyrosinemia have been covered by the city medical insurance (*12*). In addition, the Medical Mutual-Aid Foundation for Hospitalized Children, which is affiliated with the Shanghai Branch of the Red Cross Society of China, has announced that drugs against four rare diseases including: Gaucher's disease, Fabry's disease, Mucopolysaccharidosis, and Pompe's disease are covered by the mutual-aid funds for hospitalized children in Shanghai (*12*). According to this policy, one child with a rare disease could receive a maximum of 200,000 RMB per school year as reimbursement for treatment costs. The experiences of these districts will provide beneficial lessons for the final foundation of nationwide laws and regulations on rare diseases.

Rare disease patients are vulnerable groups in current China. They are suffering from a series of difficulties including disease treatments, attending school, and surviving for daily life. Parents could not take care of these children for a lifetime, but the laws and regulations can. Since every individual has the possibility to develop a rare disease, the final enacted legislation will be a safeguard for the whole society. This is the hope of rare disease patients and the direction of legislation in the future.

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