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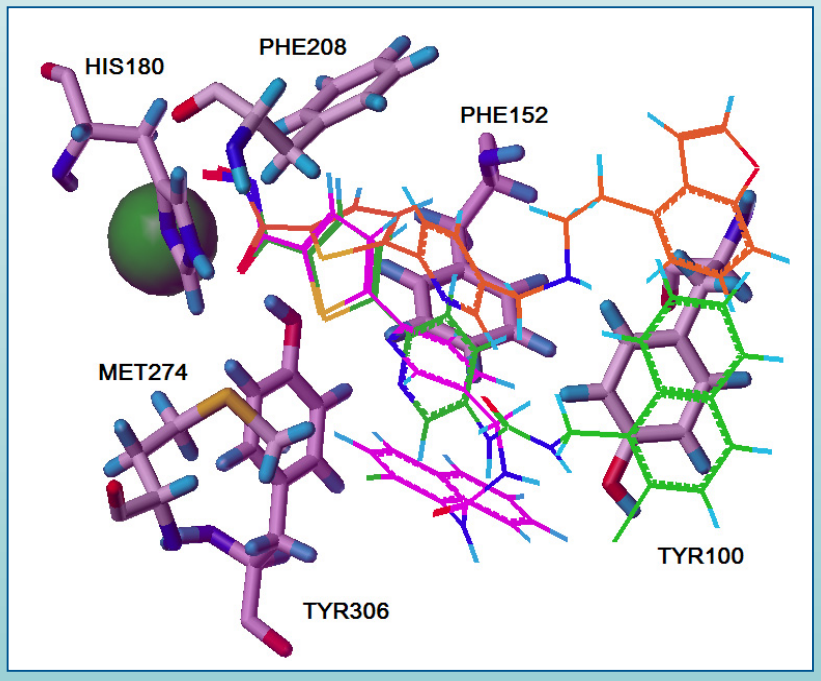

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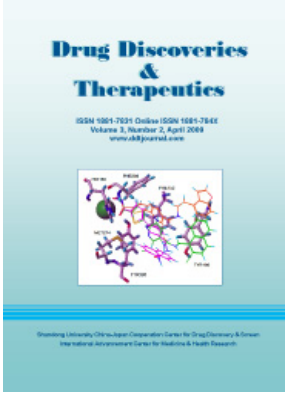
Drug Discoveries & Therapeutics



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Editorial and Head Office

Wei TANG, MD PhD
Executive Editor
Drug Discoveries & Therapeutics

TSUIN-IKIZAKA 410,
2-17-5 Hongo, Bunkyo-ku,
Tokyo 113-0033, Japan.
Tel: 03-5840-9697
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Drug Discoveries & Therapeutics

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Review

From chemotherapy to signal therapy (1909-2009): A century pioneered by Paul Ehrlich

Hiroshi Maruta*

NPO "NF CURE Japan", Melbourne, Australia.

ABSTRACT: Paul Ehrlich (1854-1915), a German microbiologist who was awarded a 1908 Nobel Prize in Physiology/Medicine for his pioneer work on the antibody production, pioneered the modern chemotherapy by discovering his magic bullet for syphilis, called "606" or "Salvarsan" in 1909 with a Japanese young scientist, Sahachiro Hata (1873-1938) from "Denken" (Institute for Infectious Diseases, now called IMS for Institute for Medical Sciences) in Tokyo. His magic bullet was used to eradicate syphilis for more than a half century until a more safe and effective antibiotic called "Penicillin" was introduced to this world towards the end of WWII by Howard Florey (1898-1968).

Celebrating this year the 100th anniversary of his discovery, this brief review will discuss how Ehrlich, now known as the Father of Chemotherapy, managed to design the first effective therapeutic for this then formidable sexually transmitted disease, which is equivalent to AIDS, HIV-infection, in the present century, and how so many new chemotherapeutics have been successfully developed during the past 100 years for other formidable diseases such as cancers and AIDS by his followers (microbe hunters and oncogene hunters) such as Alexander Fleming (1881-1955), Hamao Umezawa (1914-1986) and Brian Druker, culminating in the first signal therapeutics of cancers such as "Gleevec" that block the oncogenic signaling, around the turn of this century.

Keywords: Paul Ehrlich, chemotherapy, signal therapy

1. Ehrlich's Magic Bullet

More than a century ago there were three giants in modern medicine who fought against infectious diseases in Europe. Louis Pasteur in France, and Robert Koch (1843-1910) and Paul Ehrlich in Germany. Pasteur developed a vaccine against rabies, and Koch discovered a bacteria which causes TB (tuberculosis), and Ehrlich developed the first therapeutic for syphilis. These three giants are the major figures in the best-selling book "Microbe Hunters" published by Paul de Kruif in 1926 (*1*). Ehrlich was the anchor of 14 microbe hunters in this book. He developed not only an effective antiserum against diphtheria with Emil von Behring (1854-1917), but also pioneered the chemotherapy by developing the first modern chemical medicine, "Salvarsan" for syphilis (*2*). His scientific life was featured in 1940 MGM film "Dr. Ehrlich's Magic Bullet" which I have treasured since my youth (Figure 1).

He was born on March 14, 1854, between Jewish parents in Silesia then a part of Germany, but now in the territory of Poland. In terms of science, the most influential member of his family was his elder cousin Carl Weigert who introduced the young Ehrlich to histochemistry, staining of tissue specimens by aniline dyes. Through this histochemistry, Ehrlich learned that each chemical shows a specific affinity for a certain tissue or bacteria. Since then he became obsessed with dye-staining for the rest of his life. Soon he developed a new concept, no reaction without binding. Based on

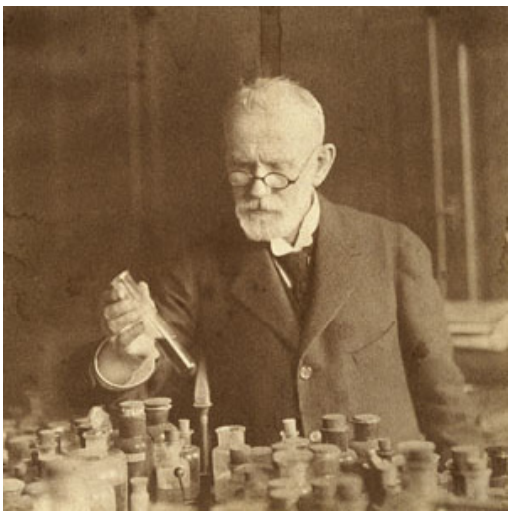

Figure 1. Dr. Paul Ehrlich (1854-1915). Father of Chemotherapy discovering "Salvarsan".

**Address correspondence to:*
Dr. Hiroshi Maruta, NPO "NF CURE Japan", Melbourne, Australia.
e-mail: maruta19420@hotmail.com

the specific binding of a given dye to a certain tissue or bacteria, he began cherishing an idea that if a given dye having a specific binding site for a certain pathogen is linked to a toxic side chain, this chimeric dye could kill this pathogen selectively without harming any tissues of our body. He called such a dye "magic bullet", and making such a dye became his life dream.

Shortly after he developed a new staining method for identifying TB bacillus for Robert Koch, he realized that he was contracted with TB himself, and spent a few years with his beloved wife Hedwig in Southern Europe and Egypt for TB therapy. Around 1892, he joined a newly founded Koch's Institute of Infectious Diseases in Berlin, and helped his old friend Emil von Behring develop the effective antiserum from houses against diphtheria. In 1899, a few years after their great success in anti-diphtheria serum therapy, Ehrlich was awarded a new institute in Frankfurt, and later the 1908 Nobel Prize in Medicine, while Behring was given another institute in Marburg, and the 1901 Nobel Prize in Medicine.

At Ehrlich Institute in Frankfurt, he started developing the magic bullet from aniline dyes for sleeping sickness and syphilis. With a young Japanese scientist Kiyoshi Shiga (1871-1957), Ehrlich discovered "Trypan Red" as an effective therapeutic for sleeping sickness caused by a trypanosome in 1903. Then his team started focusing their effort on syphilis which is caused by a bacterium called spirochete. In this team, two young scientists played the major role. The German organic chemist Alfred Bertheim (1879-1914) who synthesized a series of aniline dye-arsenate compounds, and the Japanese microbiologist Sahachiro Hata who assayed the anti-syphilis potential of each dye-arsenate compound, using animal models. In 1909, they discovered that their 606th compound turned out to be the most potent anti-syphilis drug in animal models. It was a hydroxyl aniline-arsenate compound, later called "Salvarsan" which was mass-produced by the pharmaceutical company "Hoechst" in Frankfurt for clinical trials and therapy worldwide. The dye selectively binds the pathogen "Spirochete" and the arsenate kills this parasite.

2. Beyond "Salvarsan"

After the death of Paul Ehrlich, the father of chemotherapy, several scientists followed his foot steps. One of them was Gerhard Domagk (1895-1964), a German pathologist and bacteriologist who developed "Sulfonamidochrysoidine" (KI-730), the first commercially available antibacterial drug (the brand name "Prontosil Red"), for which he received the 1939 Nobel Prize in Medicine (*3*).

Domagk studied medicine at the University of Kiel, but volunteered to serve as a soldier in World War I, where he was wounded in December 1914, working the rest of the war as medic. After the war, he started working at the University of Greifswald, where he studied infections caused by bacteria. In 1925, he followed his professor Walter Gross to the University of Muenster, and became professor there. He also started working at the Bayer laboratories at Wuppertal, as the director of Bayer's Institute of Pathology and Bacteriology, where he continued the studies of Josef Klarer and Fritz Mietzsch, based on works by Paul Ehrlich, to use dyes, products of IG Farben, as antibiotics. He found the sulfonamide "Prontosil" to be effective against streptococcus, and treated his own daughter with it, saving her the amputation of an arm.

In 1939, Domagk was announced to receive the Nobel Prize in Medicine for this discovery. However, he was forced by the Nazi regime to refuse the prize and was arrested by the Gestapo for a week. Sulfonamides became a revolutionary weapon at the time, surpassing phage therapy, but were later replaced by "Penicillin", which showed both better effects and fewer side effects (sulfonamides can cause kidney stones and changes in bone marrow). Domagk's work on sulfonamides eventually led to the development of the anti-TB drugs thiosemicarbazone and isoniazid, which helped to curb the epidemic of TB which swept Europe after World War II. Eventually, after the war, in 1947, Domagk was able to receive his 1939 Nobel Prize.

3. The first natural antibiotics

After Domagk's work, the majority of anti-bacterial drugs were developed from natural (bacterial/fungal) products called antibiotics. One of the first effective antibiotics was "Penicillin" which was discovered by Alexander Fleming (1881-1955) and further developed by Howard Florey (Figure 2) and Ernst Chain (1906-1979). These three scientists shared the 1945 Nobel Prize in Medicine (*4*).

Figure 2. Dr. Howard Florey (1898-1968). Developing "Penicillin" (Australian $50 Note).

In 1928, Fleming was working in London on the bacteria "Staphylococcus", and noticed a bacteria-free circle around a blue fungus colony which was a contaminant in this bacterial plate. During his further study, he found that an extract from this mold kills the bacteria, and he called this substance "Penicillin". However, he did not expect this antibiotic to be developed as the major therapeutic for bacterial infection later.

It was Howard Florey, an Australian scientist working at Oxford University, who realized the great potential of "Penicillin" and developed it for clinical application during the WWII. In 1939, when the war broke in Europe, he started focusing his effort on the mass-production of "Penicillin" by fermentation of this blue fungus, in an attempt to treat so many wounded British soldiers during the war. Ernst Chain, a Jewish-German scientist born in Berlin, who left Nazi Germany for England, joined Florey's team at Oxford for the mass-production and purification of "Penicillin", in collaboration with three American companies. This "miracle drug" eventually would replace both "Salvarsan" and "Prontosil Red", because of its less side-effects and more potency against syphilis and other bacterial infection in general after the end of WWII.

The great success of Penicillin was followed by a flood of new anti-bacterial antibiotics, such as Streptomycin (1943), Chloramphenicol (1945), Tetracyclin (1947), *etc*. during 1940s. However, no anti-viral antibiotics were discovered, and instead a few vaccines such as anti-polio (1955) were developed by Jonas Salk (1914-1995), the founder of Salk Institute in San Diego (*5*).

4. Anti-cancer antibiotics: DNA/MT poisons

A success by a series of anti-bacterial antibiotics would be followed by the discovery of several anti-cancer antibiotics in 1950s-1960s. One of them was Mitomycin C, which was discovered in 1955 by Toju Hata (1908-2004) of Kitasato Institute, a son of Sahachiro Hata. Hamao Umezawa of Tokyo University, who discovered the anti-bacterial antibiotic Kanamycin in 1956, also discovered another anti-cancer antibiotic called "Bleomycin" in 1965 (*5*). These anti-cancer antibiotics as well as chemically synthesized anti-cancer drugs such as Cisplatin (1978) and 5FU (1950s) are so-called DNA/RNA poisons which block DNA/RNA synthesis. Another series of anti-cancer antibiotics such as Vinblastin (1958) and Taxol (1977) are so-called MT (microtubule) poisons which block spindle formation during cell division. These conventional DNA/MT poisons are effective to inhibit the growth of fast-glowing cancers such as leukemias, but not slow-growing tumors such as NF (neurofibromatosis) as well as melanomas, gliomas, pancreatic and lung cancers, and are quite toxic even for normal fast-growing cells such as bone marrow, hair follicle, and digestive duct cells, causing a series of side effects such as suppression of immune system, hair loss and intestinal inflammation.

5. Signal therapeutics (STs) as new anti-cancer drugs

These serious side effects could be avoided or minimized if anti-cancer drugs are developed on the basis of blocking the very cause of cancers, instead of DNA/MT inhibition which would affect the growth of both normal and cancer cells. These conventional anti-cancer drugs (DNA/MT poisons) were screened by their ability to inhibit the growth of Ehrlich's ascites tumor (a mammary gland tumor in mice developed by Paul Ehrlich) and other cancer models in which the genetic cause of carcinogenesis is totally unknown. In 1976, however, a group of oncogene hunters led by Mike Bishop and Harold Varmus at UCSF revealed for the first time that an oncogene (vSRC) in chicken retrovirus (oncogenic RNA virus) called Rouse sarcoma virus is a mutant of a normal cellular (proto-onco) gene (cSRC). This mutation causes an abnormal (constitutive) activation of the Tyr-kinase SRC which phosphorylates Tyr residues of its target proteins. In other words if one can develop a specific inhibitor for vSRC, one could treat cancers caused by vSRC, without affecting the normal cell growth. This discovery opened the entirely new avenue to both understanding of carcinogenesis and therapy of cancers. For this discovery both Bishop and Varmus shared the 1989 Nobel Prize in Physiology/Medicine. I should point it out that a Japanese virologist Hidesaburo Hanafusa (1928-2009) at Rockefeller University contributed greatly to the epoch-making discovery of the first oncogene SRC, and was awarded the 1982 Lasker Award.

Since then more than 100 oncogenes such as RAS and ABL and more than 50 tumor suppressor genes such as p53, RB, NF1 and NF2 were hunted down (cloned). It is now generally accepted that cancers (malignant tumors) are caused by either gain-of-function mutation of proto-oncogenes or loss-of-function of tumor suppressor genes or by a combination of some of these mutations. Interestingly, the majority of these proto-oncogene or tumor suppressor gene products are signal transducers which control the growth of normal cells, positively or negatively, respectively. Among these oncogene products Tyr-kinases such as SRC, ABL and ErbB1 (EGF receptor) kept drawing the first attention from a new generation of anti-cancer developers (so-called "signal therapeutic hunters").

So far the most successful signal therapeutic (ST) was a Tyr-kinase inhibitor called STI-571 or "Gleevec" which was created in 1996 by Novartis' team and further developed by Brian Druker (Figure 3) of OHSU, Oregon Health & Science University (*6*). Gleevec inhibits selectively three Tyr-kinases, ABL, PDGFR

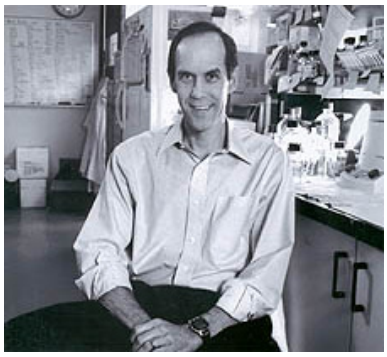

Figure 3. Dr. Brian Druker at OHSU. Developing "Gleevec" for CML & GIST.

(PDGF receptor) and KIT. Gleevec turned out to be the most effective in the treatment of at least two rare cancers CML (Chronic myelogenous leukemia) and GIST (gastrointestinal stromal tumor). CML is caused by an accidental fusion of two genes, ABL and BCR, by a chromosomal translocation that abnormally activates the Tyr-kinase ABL. GIST is caused by abnormal activation of another Tyr-kinase KIT. Since Gleevec inhibits both ABL and KIT, it was approved by FDA in 2001 as the first ST for the treatment of cancers, namely CML and GIST. Unlike the conventional anti-cancer drugs such as DNA/MT poisons, Gleevec causes no serious side effects such as immuno-suppression and hair loss.

6. Future perspective for anti-cancer STs

Unfortunately, however, Gleevec can be used only for these very rare cancers which represent less than 0.1% of all human cancers. The remaining vast majority of cancers should be treated by a much more general ST which would block the major oncogenic signal transducer(s) essential for the growth of majority of cancers such as breast, prostate, colon, lung, ovarian, cervical, and pancreatic cancers as well as melanomas, gliomas, MM (multi-myeloma), and NF tumors. We and others found recently that the kinase PAK1, a Rac/CDC42-dependent Ser/Thr-kinase, is essential for the growth of these cancers/tumors which represent more than 70% of all human cancers, but not for the normal cell growth (*7,8*). Furthermore, this kinase is required for both metastasis of cancers and angiogenesis (blood vessel formation) which is essential for the growth of solid tumors (*8*). In other words anti-PAK1 drugs (synthetic chemicals or natural products) would be a much more general ST, a magic bullet, that would be useful for the treatment of these PAK1-dependent solid cancers/tumors in the future.

During past several years we have identified and developed a series of anti-PAK1 drugs such as FK228 and Bio 30. Among them FK228, a ring peptide antibitotic developed by a Japanese pharmaceutical company called "Asteras" (formerly "Fujisawa") in 1994, is the most potent. However, it is still in clinical trials (phase 2) for only CTCL (cutaneous T-cell lymphoma), and is not available on the market as yet (*7,8*). Bio 30 is a water-miscible CAPE-based extract of NZ (New Zealand) propolis which is inexpensively available on the market (*8,9*). CAPE (caffeic acid phenethyl ester) is its major anti-cancer/anti-PAK1 ingredient and works synergestically with a few other anti-cancer ingredients of Bio 30 (*8,9*), and originally found in propolis sample from Israel in 1988 by Dezider Grunberger of Columbia University (*10*). NZ propolis is the richest in CAPE (6-7% of dry weight). Propolis is a honey bee product which has been used as an antibiotic for the treatment of various infection and inflammation, and preparation of mummies since the ancient Egypt for several thousand years. Thus, propolis is a unique ST for cancers and NF as well as several other diseases including bacterial/viral infection such as AIDS, inflammation such as arthritis and asthma, and neurodegenerative diseases such as Alzheimer (AD) and Huntington's (HD), epilepsy, and malaria (*8,9*). It causes no side effect.

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Original Article

QSAR studies of histone deacetylase (HDAC) inhibitors by CoMFA, CoMSIA, and molecular docking

Lei Zhang, Hao Fang, Huawei Zhu, Qiang Wang, Wenfang Xu*

Department of Medicinal Chemistry, School of Pharmacy, Shandong University, Ji'nan, Shandong, China.

ABSTRACT: In order to develop highly potent antitumor agents, three-dimensional quantitative structure-activity relationship (3-D QSAR) studies were conducted using a series of thienyl-based hydroxamic acids. Comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) methods were applied to provide the structural information for further chemical modification and optimization. ClogP was applied as an additional descriptor in the CoMFA analysis to study the effects of lipophilic parameters on the activity of these compounds, and it did improve the statistical significance of the model. Two molecules were designed based on the 3-D QSAR analysis, their activity values were predicted by the generated model, and their binding mode was elucidated by a docking approach compared to molecules in the dataset.

Keywords: HDAC, QSAR, CoMFA, CoMSIA, docking

1. Introduction

Histone deacetylases (HDACs) are considered to be one of the most interesting and promising targets for the treatment of cancer. More and more scientists pay close attention to them for their intensive correlation with the pathogenesis of cancer. So far, at least 18 HDAC subtypes exist as subdivided into four classes: class I proteins (HDACs 1, 2, 3, and 8), are homologous to the yeast Rpd3 deacetylase; class II enzymes (HDACs 4, 5, 6, 7, 9, and 10), are related to the yeast Hda1 deacetylase; class III (Sirtuins 1-7) are yeast Sir2 homologs, and class IV (HDAC11) has homology to both class I and class II enzymes. It needs to be noted that class I, II, and IV HDACs are all zinc-dependent hydrolases (*1-3*).

It is widely believed that alterations in the balance between histone deacetylases (HDACs) and histone acetylase (HATs) play an important role in tumorigenesis. Histone deacetylases and histone acetylase are enzymes responsible for deacetylating and acetylating the amino-terminal tails of histones, respectively. These chromatin changes help regulate transcription and many other nuclear events. Non-histone proteins (such as oncosupressor p53) and a few cytoplasmic proteins are also accommodated by HDACs/HATs (*4*). Studies on the molecular pathogenesis of acute myeloid leukemias have shown that the aberrant recruitment of HDACs has a significant role in leukemogenesis. Leukemia-associated fusion proteins (such as promyelocytic leukemia-retinonic acid receptor and acute myloid leukemia 1 -ETO) recruit HDACs to repress the transcription of genes involved in differentiation and impair the function of p53 (*5*). HDACs can decrease the half-life of several substrates by exposing the lysine residue for ubiquitylation, and also affect protein location, DNA binding, protein-protein interactions (such as the association of the mainly nuclear DNA-damage-response protein Ku70 with the pro-apoptotic protein BAX) (*6-9*).

Histone deacetylase inhibitors (HDACi) exert cell-type-specific effects including apoptosis, cell-cycle arrest and differentiation. In leukemias, HDACi include the expression of members of the tumor-necrosis factor-related apoptosis-inducing ligand and FAS death receptor pathways. This induction is responsible for the pro-apoptotic efforts of HDACi (*10-13*). So far several kinds of HDACi have been studied in clinical trials, and a case in point is that suberoyl anilide hydroxamic acid (SAHA) was approved by the FDA for once-daily oral treatment of advanced cutaneous T-cell lymphoma (CTCL) in 2006.

HDACi can be subdivided into 5 structural categories: short chain fatty acids (such as butyrate and phenylbutyrate) (*14-17*), hydroxamic acids (such as trichostatin A and SAHA) (*18-24*), epoxyketone-containing cyclic tetrapeptides (such as trapoxin B and HC-toxin) (*25,26*), epoxyketone-containing cyclic tetrapeptides (such as CHAP53, apicidin) (*27-30*), and

**Address correspondence to:*
Dr. Wenfang Xu, Department of Medicinal Chemistry, School of Pharmacy, Shandong University, No. 44, West Culture Road, Ji'nan 250012, Shandong, China.
e-mail: xuwenf@sdu.edu.cn

amides (such as MS-275) (Figure 1) (*31*).

The hydroxamic acids have three structural motifs, including a zinc binding group (ZBG), a linker and an external motif, the so-called "surface recognition motif" (Figure 2) (*32*).

To obtain more potent HDACis as anti-proliferative agents, Price and coworkers synthesized a series of thienyl-based hydroxamic acids which have excellent potency in the HDAC assay (*33*). To rationalize the observed variance in inhibitory activity, to propose a possible mechanism of antitumor activity and to guide the synthesis of additional compounds, comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) were employed to derive three-dimensional quantitative structure-activity relationship (3-D QSAR) models. CoMFA and CoMSIA methods have been the most powerful tools in the 3-D QSAR approach and are used in understanding the mechanism of interactions between various receptors and ligands. Based on the information derived by 3-D QSAR study, two molecules were designed, and their pIC_{50} values were predicted by the generated models.

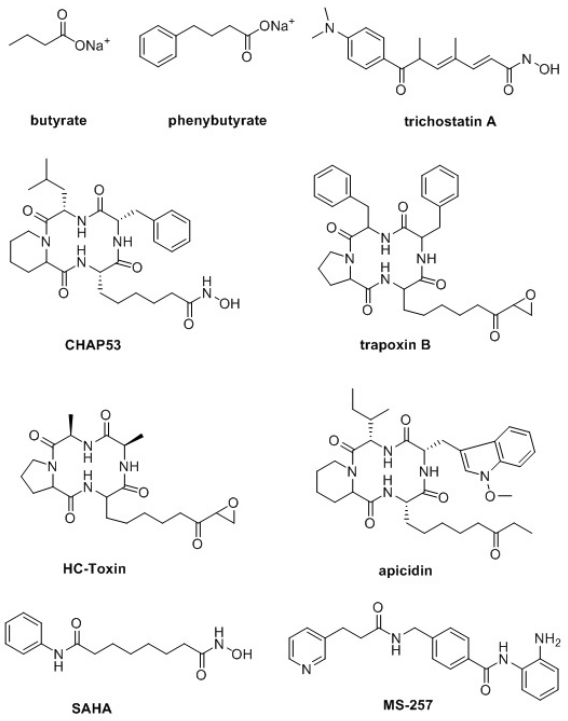


Figure 1. Structure of HDACi molecules from different categories.

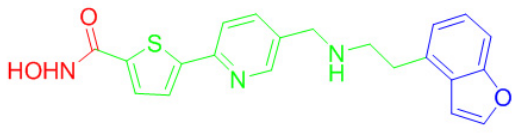


Figure 2. The general structure of hydroxamic acid HDACi. The red part is ZBG, the green part is linker, and the blue part is the surface recognition motif.

As 3-D QSAR is only a ligand based approach, a docking method was used to validate the results. The compounds in the dataset were tested against nuclear extracts which contain a mixture of HDACs, so it is unclear which subtype plays a predominant role, and moreover, only the crystal structure of human HDAC8 is available at the moment. Therefore the *holo*-form crystal structure of HDAC8 (*34*) was used to study the binding mode of molecules in the dataset and the designed molecules.

2. Materials and Methods

2.1. *Data set*

Thirty-five molecules selected for the present study were taken from the published work of Price and coworkers (*33*). The structure and activity data of the compounds belonging to various chemical classes are given in Table 1. The 3-D QSAR models were generated using a training set of 28 molecules. The predictive ability of the resulting models was evaluated using a test set of 7 molecules.

2.2. *Molecular modeling*

The docking studies were performed using Sybyl 7.0 (Tripos Inc., St. Louis, MO, USA) software running on a DELL Precision 390 workstation and the remaining computational studies were performed using the Sybyl 7.3 (Tripos Inc.) program running on a DELL Precision 360 workstation. The molecular structures were built based on the bioactive conformation of compound **5u** which was generated by a docking procedure. Energy minimization was performed using Powell optimization in the presence of the Tripos force field with a convergence criterion of 0.05 kcal/mol•Å and then assigned with the Gasteiger-Hückel charges.

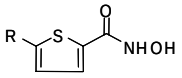
2.3. *Alignment rules*

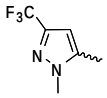
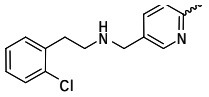
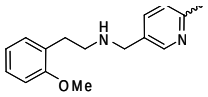
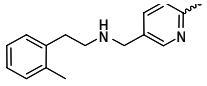
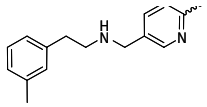
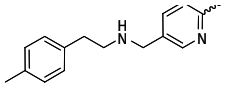
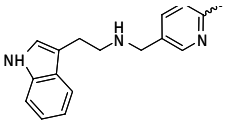
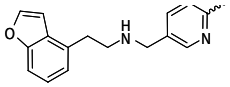
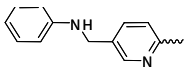
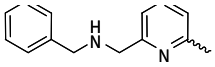
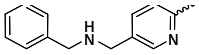
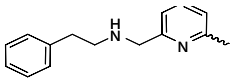
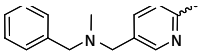
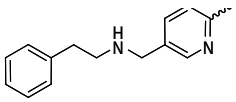
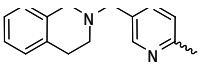
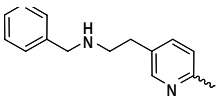
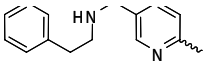
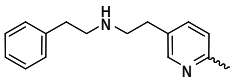
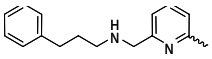
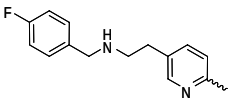
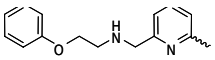
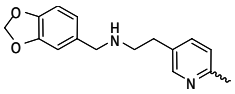
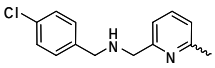
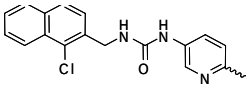
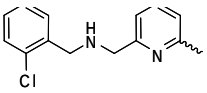
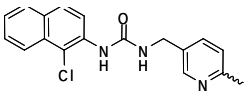
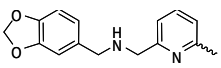
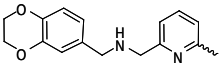
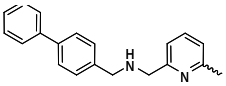
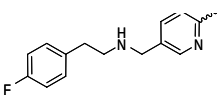
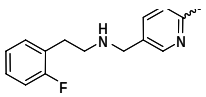
There are several moleclar alignment rules, such as atom based, structure based, docking based, field based, pharmacophore based approaches and so on. Wang and Zhu in our lab have reported the application of the above methods (*35,36*). Herein molecular superimposition was carried out by using the relatively simple atom fit method, and a favorable alignment was derived. The reference structure for RMSD fitting was shown in Figure 3.

2.4. *CoMFA and CoMSIA analysis*

CoMFA steric and electrostatic fields were separately calculated using the sp^3 carbon probe atom with a *van der Waals* radius of 2.0Å and +1 charge. The energies were truncated to ± 30 kcal/mol, and the electrostatic

Table 1. Structures and bioactivity data of the compounds in the data set



Compounds	R	HDACa (IC_{50}, μM)	Compounds	R	HDACa (IC_{50}, μM)
ADS10038		0.750	**5o**		0.006
3a		2.500	**5p**		0.017
3b		0.900	**5q**		0.008
3c		0.243	**5r**		0.009
3d		1.130	***5s**		0.014
***3e**		1.260	**5t**		0.008
3f		0.186	**5u**		0.004
5a		0.016	**7a**		0.359
5b		0.016	**7b**		0.581
5c		0.035	***9**		0.080
***5d**		0.030	**13a**		0.009
5e		0.012	**13b**		0.031
5f		0.018	**13c**		0.013
5g		0.011	***13d**		0.009
5h		0.009	**#z1**		-
***5i**		0.007	**#z2**		-
5j		0.008			
5k		0.011			
5l		0.007			
5m		0.009			
***5n**		0.012			

* Molecules in test set; # molecules designed. The data are referring to Price *et al*., Bioorg Med Chem Lett. 2007; 17:363-369.

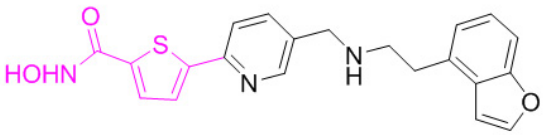


Figure 3. Structure of the reference molecule 5u. The magenta part was used for atomic alignment.

contributions were ignored at the lattice intersection with maximal steric interactions. The CoMFA fields generated were automatically scaled by the CoMFA-STD method.

The CoMSIA method involves a common probe atom and similarity indices calculated at regularly spaced grid intervals. The same grid constructed for the CoMFA fields was used for the CoMSIA calculation. CoMSIA can derive hydrophobic, H-bond donor and acceptor fields in addition to CoMFA steric and electrostatic fields. The distance dependence between the grid point and each atom was determined by Guassian function through the similarity indices calculated at all grid points, and a default value of 0.3 was used as an attenuation factor.

2.5. *Partial least square (PLS) analysis*

The predictive values of models were evaluated by leave-one-out (LOO) cross validation method, and an optimal number of components obtained from each calculation were used to generate the final model without cross validation. The result from a cross validation analysis was depicted as r^2_{cv} which is defined as

$$r^2_{cv} = 1 - \text{PRESS}/\sum(\text{Y-Y}_{mean})^2$$

where

$$\text{PRESS} = \sum(\text{Y-Y}_{pred})^2$$

2.6. *Test set validation*

It is widely considered that more than 0.5 of $q^2(r^2_{cv})$ value is a necessary condition for a predictive QSAR model. Many methods are established to validate the predictive ability of the generated model, such as Tropsha's validation criteria (*37*,*38*). We used a relatively simple method to investigate the robustness of the models derived by CoMFA and CoMSIA methods. The linear correlation coefficient R^2 between the bioactivity and the predicted activity of the test set molecules was used to evaluate the predictive ability of the derived model.

2.7. *Docking analysis*

The crystal structure of HDAC8 was obtained from the protein data bank (PDB entry: 1t64 (1.90Å)). Compound **5u** and the design molecules were docked to the active site of HDAC8. Since there was no water in the active site, before docking, all water molecules were removed from the crystal structure, hydrogen atoms were added, and Amber charge was loaded. The docking studies were performed using the FlexX module in Sybyl 7.0, and the maximum number of poses per ligand was set to 90. One of the symmetrical subunits formed by chain A was selected as the protein for the docking study to save computational time. Cscore was applied to evaluate the docking results. The active site was defined as 6.5Å radius circles around the ligand TSN386 (trichostatin A) and other parameters were set as default.

3. Results and Discussion

3.1. *CoMFA*

A good alignment of the dataset is essential for a predictive 3D-QSAR model. The docking method was first applied to align all the molecules, but too many flexible bonds made it difficult to align the molecules in the dataset to the same position. Therefore only the structure of the most bioactive compound **5u** was derived by docking, the rest of the molecules were constructed using **5u** as a reference, then the alignment was carried out by atomic fit (Figure 4).

Table 2 summarizes the PLS results of the CoMFA and CoMSIA analysis. It is obvious that application

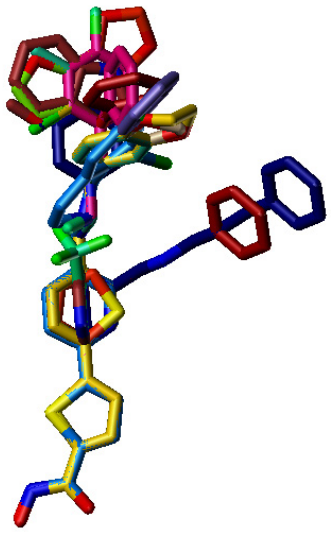


Figure 4. Alignment of the molecules in the data set.

Table 2. Summary of PLS results

Parameters	CoMFA	CoMFA (ClogP)	CoMSIA
r^2_{cv}	0.848	0.850	0.917
ONC	2	6	8
r^2	0.922	0.985	0.991
SEE	0.232	0.110	0.090
F	147.482	234.360	262.346
Steric	0.691	0.558	0.190
Electrostatic	0.309	0.347	-
Hydrophobic	-	-	0.580
Donor	-	-	0.231
ClogP	-	0.068	-

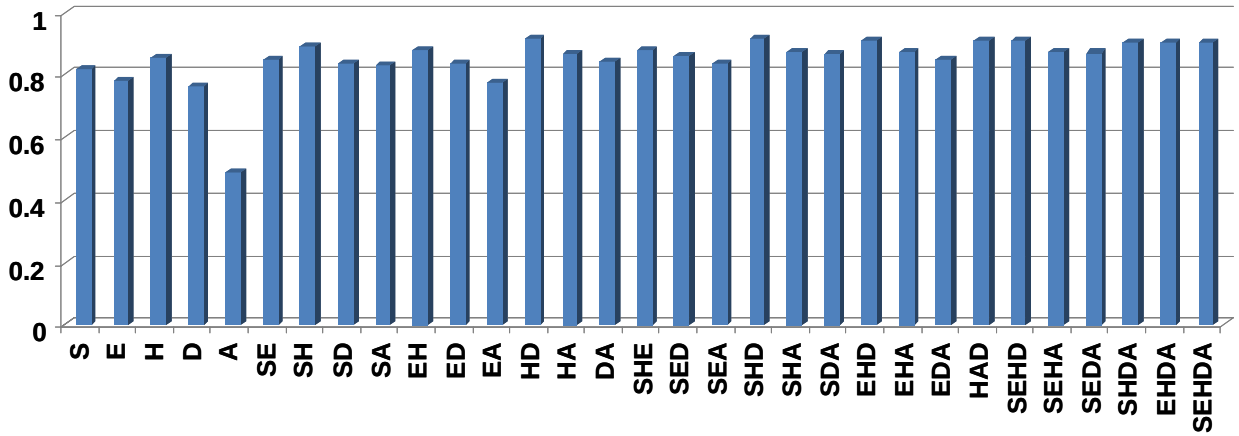


Figure 5. Results of all the possible CoMSIA field combinations. S, steric; E, electrostatic; H, hydrophobic; D, H-bond donor; A, H-bond acceptor.

of ClogP improved the statistical significance of the CoMFA model. All the statistical parameters showed that the model derived considering ClogP is more robust. Therefore we can conclude that the hydrophobic property of the molecules plays an important role in their activity.

3.2. *CoMSIA*

CoMSIA analysis was performed using steric, electrostatic, hydrophobic, H-bond donor and acceptor descriptors. The five different fields can form various combinations to study the role of each field. Herein r^2_{cv} values of the 31 combinations were derived by using the SAMPLS method (Figure 5). Among these combinations hydrophobic field has the highest r^2_{cv} in the single field analysis (r^2_{cv} = 0.851), steric has the second rank (r^2_{cv} = 0.819). The combination of hydrophobic and H-bond donor has the highest r^2_{cv} value in the two field analysis (0.912). The addition of the steric field ascended the r^2_{cv} value to 0.916 which is the highest r^2_{cv} in these combinations. Therefore, steric, hydrophobic and H-bond donor fields were used to generate the final CoMSIA model.

As shown in Table 3, the CoMSIA model is more statistically significant than the two CoMFA models.

3.3. *Validation of 3-D QSAR models*

The significance and utility of 3-D QSAR models was validated by predicting the activity of test molecules which were not included in model development. Compounds **3e**, **5d**, **5i**, **5n**, **5s**, **9**, and **13d** were selected as a test set to verify the robust and predictive ability of the derived models. The CoMFA model generated considering ClogP in addition to electrostatic and steric fields has a higher R^2 (Figure 6). It is also evidence that the hydrophobic property of the molecules is essential for their enzyme inhibitory activity. The CoMSIA model is the most statistically significant for the training set itself, but to the test set, its predictive ability is the poorest. This is because the CoMSIA method is more sensitive to the structural diversity, and the alignment of the dataset is not perfect. Table 3 shows details of predictive properties of the three models.

Table 3. Actual activity, ClogP and predicted activity of the data set

Compounds	pIC_{50}	ClogP	CoMFA	CoMFA (ClogP)	CoMSIA
13a	8.05	2.527	7.841	7.943	7.931
13b	7.51	2.446	7.746	7.623	7.556
13c	7.89	2.670	7.844	7.955	7.944
*13d	8.05	2.492	7.826	7.953	7.902
3a	5.60	2.178	6.177	5.638	5.680
3b	6.05	2.310	6.320	6.096	5.893
3c	6.61	1.062	6.508	6.644	6.585
3d	6.89	0.852	6.469	6.800	6.910
*3e	5.90	0.852	6.209	6.266	6.704
3f	6.73	0.111	6.372	6.672	6.963
5a	7.80	2.009	7.532	7.821	7.835
5b	7.80	1.588	7.898	7.857	7.862
5c	7.46	2.664	7.879	7.507	7.486
*5d	7.52	2.949	7.754	7.300	7.266
5e	7.92	2.527	7.995	8.095	7.991
5f	7.74	2.906	7.818	7.586	7.704
5g	7.96	2.395	7.900	8.036	7.933
5h	8.05	2.301	7.961	7.915	8.020
*5i	8.15	2.301	7.893	7.721	7.873
5j	8.10	1.553	8.071	8.030	7.974
5k	7.96	1.512	8.104	7.971	7.929
5l	8.15	3.476	8.134	8.260	8.206
5m	8.05	2.670	7.999	8.017	8.004
*5n	7.92	2.670	7.911	7.923	8.102
5o	8.22	3.240	8.020	7.951	8.343
5p	7.77	2.446	8.031	7.846	7.822
5q	8.10	2.976	8.130	8.163	8.188
5r	8.05	3.026	8.027	7.994	8.064
*5s	7.85	3.026	8.007	8.002	8.022
5t	8.10	2.517	8.071	8.133	8.065
5u	8.40	3.087	8.169	8.319	8.209
7a	6.44	1.588	6.312	6.397	6.447
7b	6.24	2.906	6.146	6.198	6.242
*9	7.10	3.645	7.720	7.291	7.609
ADS	6.12	1.688	6.284	6.202	6.172

* Molecules in test set.

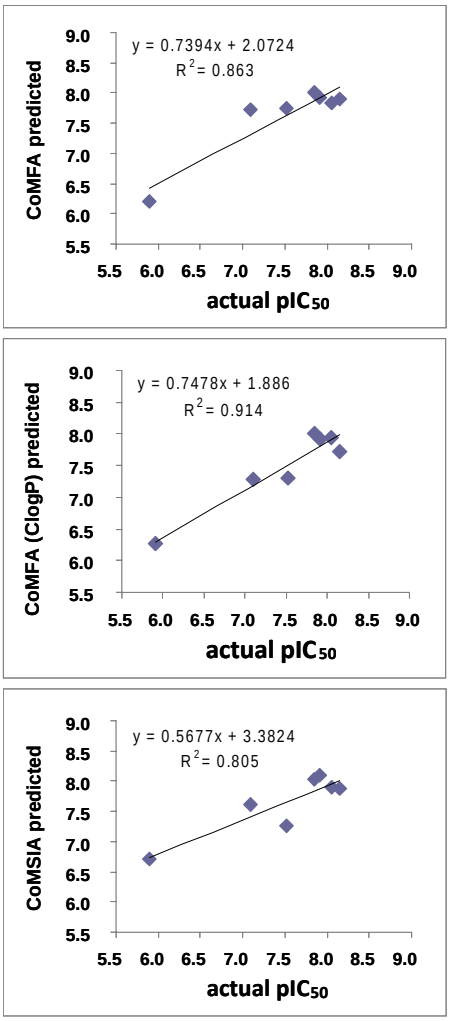


Figure 6. Scatterplot of actual pIC_{50} versus predicted pIC_{50} of the test set molecules.

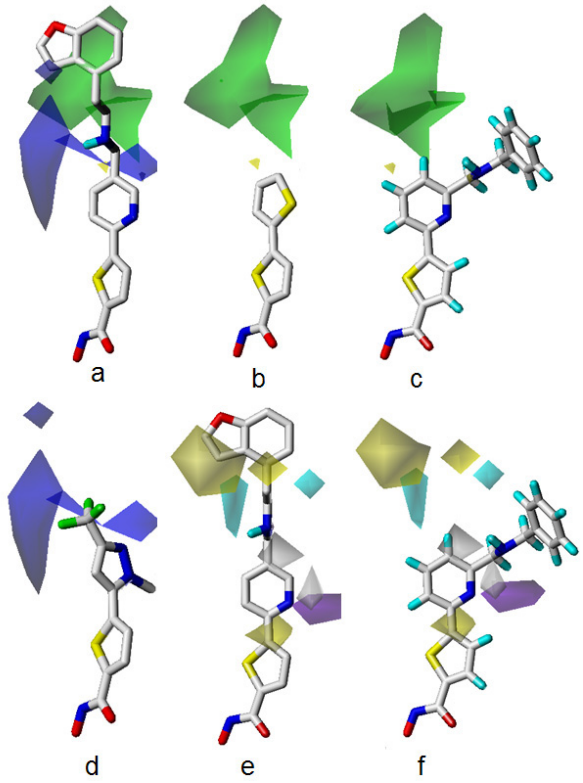


Figure 7. Contour maps of the QSAR analysis. a, CoMFA steric fields and electrostatic fields around **5u**; b and c, CoMFA steric fields around **3a** and **7a**; d, electrostatic fields around **ADS** (color code: steric favored, green; positive charge favored, blue); e and f, CoMSIA hydrophobic and H-bond donor fields around **5u** and **7a** (color code: hydrophobic favored, yellow; hydrophobic disfavored, white; H-bond donor favored, cyan; H-bond donor disfavored, purple).

3.4. *3-D QSAR contour maps*

CoMFA and CoMSIA contour maps were generated to visualize the field distribution of the 3-D QSAR models. Figure 7a shows the steric contour maps around molecule **5u**. The evidence that the high steric tolerance region (the big green contour) is located at the position of the externa motif indicates that the larger substituent herein is essential for high activity. For example, the aromatic ring of molecule **5o**, **5t**, and **5u** is very bulky in this region, and all of them have high enzyme inhibiting activity (pIC_{50} = 8.22, 8.10, and 8.40, respectively). Compounds in class 3 are less active than those in other classes (mean pIC_{50} = 6.14), because their corresponding parts are too small to fit in this region (Figure 7b). Because the phenyl rings of compound **7a** and **7b** stretch to somewhere else (Figure 7c) is a reason for their low activity (pIC_{50} = 6.44, 6.24). The blue contours in Figure 7a describe a region where a positive charged group enhances activity. The big blue region is around the -NH_2- group in the linker suggesting that increasing positive charges in this area can improve activity. Most molecules have this feature but because molecules in class 3 cannot reach into this region it is believed to be another cause of their low activity, one negative charge taking the fluorine atom of **ADS** also is considered to be an important reason for its low activity (Figure 7d). The steric fields of CoMSIA provide similar information as CoMFA, so they will not be described in detail here. CoMSIA hydrophobic field has the most contribution to the model, it is further evidence that the hydrophobic properties of the molecules plays an important role in the activity. In the hydrophobic contour plots (Figure 7e), the favorable yellow bulk overlapping the aromatic ring means improving the hydrophobic property of this part is essential for high enzyme inhibiting activity. The unfavorable white regions located around the -NH- group in the linker indicates hydrophilicity is required in this region. The H-bond donor favorable contours (cyan) located at the hydrogen atom region of the -NH_2- group indicates that increasing H-bond donors in this part will improve inhibitory activity. Lack of occupation of the hydrophobic region (the phenyl ring) and the adjacent H-bond donor unfavorable purple region (the -NH_2- group) are other reasons for the low activity of **7a** and **7b** (Figure 7f).

Two carbamido groups containing hydroxamic acids were designed based on the information given by the above analysis. Their predicted pIC_{50} values are 6.916 and 7.217, respectively.

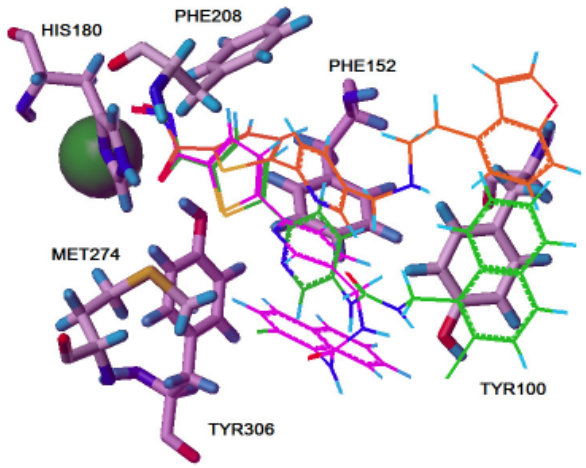


Figure 8. Docking of the designed molecules z1, z2, and the most potent compound 5u into the active site of HDAC8. The orange molecule is **5u**, the green one is **z1**, and the magenta one is **z2**. The green ball is zinc.

3.5. *Docking analysis*

The predictive activities of the designed molecules are not as high as expected. Therefore molecular docking was used to further clarify the binding mode of the molecules and to determine whether it is necessary to synthesize the designed molecules.

The crystal structure of HDAC8 reveals a channel formed by several residues, the core residues are labeled in Figure 8 (TYR100, PHE152, HIS180, PHE208, MET274, and TYR306). In the end of the channel, there is a zinc ion for binding with the hydroxamic acid part (bidentate chelation). A significant π-π stacking interaction can be found in the aromatic ring of the external motif of **z1** and **5u** with the phenyl ring of TYR100. The linker locating the channel has hydrophobic and π-π stacking interactions with the amino acids forming a hydrophobic pocket. The -NH- group in the linker of **z1** and **z2** has H-bond interactions with the hydroxyl group of TYR100. This suggests that a strong chelating interaction could stabilize the compound in the active site, and that hydrophobic and H-bond binding would also enhance the interaction between the molecule and the protein.

The total docking scores of **5u**, **z1**, and **z2** are -31.532, -39.420, and -41.461, respectively. The binding modes and the docking scores both emphasize the necessity of the synthesis of the two designed compounds, and their synthetic work is under way.

4. Conclusions

Two 3-D QSAR methods, CoMFA and CoMSIA, were applied to a series of thienyl-based hydroxamic acids as HDAC inhibitors. ClogP was considered in the CoMFA study, and it improved the robustness and predictive ability of the generated model. To study the effect of the five fields in CoMSIA, r^2_{cv} values of 31 combinations were derived using the SAMPLS method. The steric, hydrophobic and H-bond donor fields proved to be the most important. The contour maps of both methods explained the influence of substitutions on HDACs activity. Two molecules were designed based on the above models, and their activities were predicted. Although the predicted activities are not very high, the binding mode clarified by a docking approach showed they are worth synthesizing. Their bioactivities will also be a validation of the generated model.

Acknowledgments

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Original Article

Comparative analysis of free radical scavenging potential of several fruit peel extracts by *in vitro* methods

Hamendra Singh Parmar*, Anand Kar

Thyroid Research Unit, School of Life Sciences, Devi Ahilya University, Indore, India.

ABSTRACT: We studied the radical scavenging potential of several fruit peel extracts using various standard chemical and biochemical *in vitro* methods. Peel extracts of *C. sinensis* (CS), *P. granatum* (PG), *M. paradisiaca* (MP), *C. vulgaris* (CV), *C. melo* (CM), *M. indica* (MI), and *C. papaya* (CP) were used in the present study and butylated hydroxyl anisole (BHA) was used as a standard. Marked 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was observed with 25 µg/mL of CS and MP and 50 µg/mL of PG, while all the studied doses of CP were found to be pro-oxidative. β-Carotene bleaching revealed a higher singlet oxygen scavenging potential of all the peel extracts except MI. High NO-radical scavenging activity was observed at 25 µg/mL of PG and MP. Inhibition test of H_2O_2-induced LPO in erythrocytes or in liver tissue showed that all the peels tested were effective on peroxy-radicals at one or other doses. The present study revealed the radical scavenging activity of the test peel extracts in a manner that was dose- and radical/method-specific. Therefore, evaluation of the efficacy of herbal extracts should be carried out using different methods and not merely a single *in vitro* method.

Keywords: NO-radical scavenging, β-carotene bleaching, free radicals, peroxidation, *in vitro* study

1. Introduction

Dietary antioxidants including polyphenolic compounds, vitamin C, and carotenoids are believed to act as effective supplements in the prevention of several oxidative stress-related diseases (*1,2*). Various epidemiological studies have established an inverse correlation between the intake of fruits and the occurrence of health-related problems such as cardiovascular diseases, cancer, diabetes, and aging (*3-5*). In addition, fruit peels are gradually emerging as a potential source of antioxidants that possess a rich amount of flavonoids, polyphenolics, ascorbic acid, dietary fibers, and dopamine (*3,6,7*). Recently, the current authors reported various biological/pharmacological properties of several fruit peel extracts, and their antiperoxidative properties were found to be one of the major mechanisms protecting against various diseases including diabetes, cardiovascular problems, and thyroid abnormalities (*8-14*). However, no detailed study of radicals scavenged by the fruit peel extracts of *Citrus sinensis*, *Punica granatum*, *Musa paradisiaca*, *Mangifera indica*, *Citrullus vulgaris*, *Cucumis melo*, and *Carica papaya* has been conducted to date. Therefore, the present study attempted to determine the radical scavenging potential of these peel extracts using various recommended *in vitro* methods (*3,9,15*).

2. Materials and Methods

2.1. *Materials*

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich, St. Louis, MO, USA; while β-carotene, Greiss reagent, sodium nitroprusside, butylated hydroxyl anisole (BHA) and thio-barbituric acid (TBA) were supplied by Hi Media Laboratories Ltd., Mumbai, India. Malondialdehyde (MDA), sodium dodecyl sulphate (SDS), and acetic acid, and all other reagents were purchased from E-Merck (India) Ltd., Mumbai, India.

2.2. *Plant materials*

Sweet orange (*C. sinensis*, CS), pomegranate (*P. granatum*, PG), banana (*M. paradisiaca*, MP), mango (*M. indica*, MI), watermelon (*C. vulgaris*, CV), melon (*C. melo*, CM), and papaya (*C. papaya*, CP) were purchased from a local market in Indore, India. They were identified by the departmental taxonomist and voucher specimens (CS-16/05, PG-11/14, MP-11/07, MI-16/04, CV-14/04, CM-17/04, and CP-19/02,

**Address correspondence to:*
Mr. Hamendra Singh Parmar, Thyroid Research Unit, School of Life Sciences, Devi Ahilya University, Indore-452001, India.
e-mail: hamendrasingh999@yahoo.co.in

respectively) were deposited in the School of Life Sciences, D.A. University, Indore, India.

2.3. *Preparation of fruit peel extracts*

Fruit skins were removed mechanically and usable peels were air-dried in the shade and then ground into a fine powder. The dried powder of *C. sinensis* and *M. paradisiaca* was extracted in 10 volumes of cold water and stored at 4°C for three days. The extract was centrifuged at 1,500 × g for 10 min and the supernatant was dried at 40°C and stored until use (*7*). In contrast, methanolic extraction was utilized for *P. granatum*, *M. indica*, *C. vulgaris*, *C. melo*, and *C. papaya* given its use by other works (*16*). In brief, 100 g of powdered peel were collected and extracted with 600 mL of methyl alcohol (100%) at 30°C; the solution was stirred continuously with a magnetic stirrer for 4 h and then filtered (*16*). The filtrate was then dried and stored until use.

2.4. *Sample preparation for in vitro assays*

For the analysis of radical scavenging activity (RSA), β-carotene bleaching assay, and NO-radical scavenging assay, the aliquots of 25, 50, and 100 µg/mL concentrations of the peel extracts were prepared in methanol and used, while the actual concentrations of the peel extracts at 0.25, 0.50, 1.0, and 2.0 µg/mL in reactions mixtures were used in H_2O_2-induced lipid peroxidation (LPO) study in erythrocytes and liver tissues (*9*).

2.5. *Radical scavenging potential*

A few methods are available for total antioxidant determination and each has its limitations (*15,17*). Several *in vitro* methods are thought to be nonspecific (*18*), while others are more specific for the determination of antioxidant potential, particularly in fruits and peels (*3,9,15*). Therefore, different methods were used for each peel to understand trends in radical specificity in terms of scavenging activity as described below.

2.6. *Radical scavenging activity (RSA) using the DPPH method*

DPPH is a synthetic, relatively stable nitrogen radical and this assay system is based on electron transfer, where an antioxidant compound reduces the oxidant by donating an electron, resulting in a change of color and subsequent change in absorbance (*19,20*).

Different concentrations of the peel extracts (25, 50, and 100 µg/mL) were prepared in separate test tubes. The volume of each was adjusted to 100 µL by adding methanol. Five milliliters of a 0.1 mM ethanolic solution of DPPH were added to all. Tubes were then allowed to stand at 27°C for 20 min. Control tubes had all of the components mentioned above except for the peel extract. Methanol was used for baseline correction. Changes in the absorbance of the samples were measured at 517 nm. RSA was calculated as the percent inhibition [%RSA = 100 × (control OD – sample OD) / control OD] as described earlier (*3*). BHA was used as a standard antioxidant and antiperoxidative activity was expressed in % in relation to BHA.

2.7. *Antioxidant assay using β-carotene*

Singlet oxygen produced during photosensitized oxidations can cause cellular damage by reacting with DNA and proteins or by inducing lipid peroxidation (*21*), and β-carotene commonly protects against photosensitized tissue injury by scavenging free radicals or quenching singlet oxygen. Because of this, β-carotene is often used clinically to prevent photosensitized tissue damage in humans with erythropoietic porphyria (*22*). Because of the significance of singlet oxygen radicals in biological systems, a β-carotene bleaching assay is considered an elegant model in which to evaluate the singlet oxygen scavenging potential of various natural products (*19,21*).

β-Carotene (0.2 mg) dissolved in 0.2 mL of chloroform and linoleic acid (20 mg) was mixed with 200 mg of Tween-40 (polyoxyethylene sorbitan monopalmitate). Chloroform was removed at 40°C under a vacuum. The resulting mixture was diluted with 10 mL of distilled water, to which 40 mL of oxygenated water was added. Aliquots of this emulsion (4 mL) were mixed with 0.2 mL of the extract at the concentrations of 25, 50, and 100 µg/mL. A control was prepared by adding 0.2 mL of ethanol instead of the extract. The reaction mixture was initially incubated at 50°C for 5 min and absorbance was determined at 470 nm at the starting time ($t = 0$). Measurement of absorbance continued for 180 min at an interval of 15 min. A mixture prepared as above without β-carotene served as the blank.

The antioxidative activity (AA) of each peel extract was evaluated in terms of potential in bleaching the fixed quantity of β-carotene: $AA = 100 \times [1 - (A_0 - A_t) / (A_0^0 - A_t^0)]$, where A_0 and A_0^0 are the absorbance values measured at the start of incubation for the test sample and control, respectively, and A_t and A_t^0 are those measured in the test sample and control, respectively, after incubation for 180 min (*23*).

2.8. *NO scavenging activity*

NO radicals are known to be involved in cytotoxicity and can also interact with superoxide anions to result in the formation of peroxynitrite ($ONOO^-$), which is the most reactive nitrogen species (RNS) (*24*).

This assay is based on the principle that scavengers of nitric oxide compete with oxygen, leading to a reduced production of nitrite. Samples at various

concentrations (0.5 mL each) were diluted with 0.5 mL of 10 mM sodium nitroprusside solution and incubated at 25°C for 150 min. At the end of incubation, 1 mL of Greiss reagent was added to each sample and absorbance was determined at 542 nm. The nitrite concentration was calculated in relation to the absorbance of a standard solution of sodium nitrite. Results were expressed as percentage nitrite produced with respect to the control, which was devoid of peel extract (*25*).

2.9. *In vitro LPO estimation in red blood cells (RBCs) and in liver slices*

Reactive oxygen species (ROS) are known to be involved in the pathogenesis of many diseases, including thyroid and heart problems, diabetes, and cancer (*4*), so a crucial step in this study was to evaluate the ROS scavenging potential of the test peel extracts. In fact, H_2O_2-induced lipid peroxidation is believed to be the result of ROS formation. Therefore, RBCs and chopped liver tissues were found to be a suitable biochemical *in vitro* model in which to evaluate the antiperoxidative potential of herbal extracts (*26-29*). Erythrocytes are considered suitable because they contain high concentration of polyunsaturated fatty acids (PUFA), ferrous ion, and molecular oxygen, which make them vulnerable to oxidative stress (*27*), while the liver is considered suitable as it is a major drug target site (*30*).

About 50 mL of rat blood were collected in 2% citrated vials after the animals were decapitated. Erythrocytes were washed thrice with 0.1 M phosphate buffered saline (PBS, pH 7.4) and the volume was adjusted to 5% with PBS (pH 7.4). LPO in the erythrocytes was determined after the addition of 2 mL of 5% RBCs and 2 mM sodium azide to a test tube (*9,26,28*). The erythrocyte suspension was exposed to a 100 mM H_2O_2 solution and different concentrations of the extracts (0.25, 0.5, 1.0, and 2.0 µg/mL of CS/PG/MP/MI/CV/CM/CP). The total volume of each tube was brought to 4 mL with 0.1 M PBS (pH 7.4). Samples were incubated at 37°C for 1 h followed by the addition of 2 mL of 28% TCA solution. The solution was then centrifuged at 1,000 × g for 5 min. One mL of 1% TBA was added to 4 mL of supernatant and this was then placed in a boiling water bath for 1 h, cooled, and absorbance was measured at 532 nm. A control sample was prepared without the H_2O_2 solution, while the blank was prepared without tissue. In both of these tubes the volume was adjusted by 0.1 M PBS, pH 7.4. For liver slices, a similar method was used in which 200 mg of the tissues were incubated with 2 mL PBS and different concentrations of the extracts along with 100 mM H_2O_2. Simultaneously a standard curve for malondialdehyde (MDA) was determined based on its different concentrations (1-10 nM prepared in 0.1 M PBS). A blank was prepared without the MDA solution.

2.10. *Determination of polyphenols*

Total polyphenolic contents of the test peel extracts were estimated following the protocol of Leontowicz *et al*. (*3*). In brief, 0.125 mL of each peel extract of known concentration (100 mg/mL) were diluted with 0.5 mL distilled water and then 0.125 mL of Folin-Ciocalteu reagent were added to the mixture and incubated at room temperature for 6 min. After incubation, 1.25 mL of 7% sodium carbonate were added to the mixture. The final volume was adjusted to 3.0 mL with distilled water and incubated at room temperature for 90 min. The absorbance was measured against the prepared blank at 765 nm in comparison to a standard of known concentrations of gallic acid. The mean of three readings was used, and results were expressed in mg gallic acid equivalent/100 g dry weight of the extract. The coefficient of determination was $R^2 = 0.9748$.

2.11. *Determination of total flavonoids*

Total flavonoids were determined colorimetrically by following the protocol of Leontowicz *et al*. (*3*). An amount of the peel extracts (0.25 mL) in 100 mg/mL concentration was diluted with 1.25 mL of distilled water. Then, 75 µL of 5% sodium nitrite ($NaNO_2$) solution were added to the mixture followed by 150 µL of 10% aluminium chloride ($AlCl_3 \cdot 6H_2O$) solution. After incubation for 5 min, 0.5 mL of 1 M NaOH were added. The total volume was brought to 2.5 mL with distilled water. Finally, the absorbance was measured with respect to the prepared blank at 510 nm in comparison to standards prepared similarly with known concentrations of quercetin. The mean of three readings was used, and results were expressed in mg quercetin equivalent/100 g dry weight of the peel extract. The coefficient of determination was $R^2 = 0.9008$.

2.12. *Determination of ascorbic acid content*

Ascorbic acid content was measured by a modified method of Omaye *et al*. (*31*). One mL of the sample including ascorbic acid was mixed with 1 mL of ice cold 10% metaphosphoric acid and 2 mM EDTA for deproteinization and stabilization of ascorbic acid. Six hundred µL of the 50 mM citrate/acetate buffer (pH 3.5) were added to the mixture, followed by 0.3 mL of 2,6-dichlorophenolindophenol (DCPIP) solution (0.1 mg/mL); the solution was then vortexed. One min later, the absorbance of the mixture was measured at 520 nm. Standard ascorbic acids were dissolved in 5% metaphosphoric acid at 2.5, 5, 10, 20, and 50 µg/mL. A standard curve of ascorbic acid was determined using the assay method described above. The coefficient of determination was $R^2 = 0.9095$.

2.13. *Statistical analysis*

Data are expressed as mean ± SEM and were analyzed by the analysis of variance (ANOVA) followed by a post hoc Newman-Keuls Multiple Comparison Test using a trial version of Prism 4 software for Windows (GraphPad Software, Inc., La Jolla, CA, USA) (*8*).

3. Results

Although peel extracts with more than 50% free radical scavenging activity were considered to be effective, almost all peels were found to be effective according to one or the other method.

3.1. *DPPH radical scavenging activity*

The DPPH radical scavenging assay revealed that the 25 µg/mL concentration of CS and MP showed a marked inhibition in DPPH radicals (95.65 and 61.37%, respectively), while PG showed the highest radical scavenging potential at 50 µg/mL (152.07%) (Table 1). In addition, a marked inhibition was also observed with a 25 and 100 µg/mL concentration of PG. Interestingly, all the studied doses of CP were found to be pro-oxidative, as evidenced by increased DPPH absorbance, whereas MI, CV, and CM showed less inhibition in the DPPH radical scavenging assay.

3.2. *β-Carotene bleaching activity*

Results of β-carotene bleaching revealed the highest activity at 50 µg/mL of CV (165.19%), while all the studied doses of MI and 100 µg/mL of CS were found to be not as effective since their inhibition was less than 50% (Table 2).

3.3. *NO-radical scavenging activity*

A NO radical scavenging assay revealed the highest scavenging potential at 25 µg/mL of PG (52.06%) and almost similar inhibition by 25 µg/mL of MP (51.38%) (Table 3). The remaining test peel extracts at all the studied doses were found to be less effective.

3.4. *Inhibition of H_2O_2-induced LPO in erythrocytes*

With respect to the inhibition of H_2O_2-induced LPO in erythrocytes, all the test peels were found to be effective at one or other doses (Table 4). The highest inhibition was observed with 0.25 µg/mL of MP (147.31%).

3.5. *Inhibition of H_2O_2-induced LPO in liver tissue*

H_2O_2-induced LPO in chopped liver tissue was inhibited by all the peel extracts at one or other doses. The maximum activity was observed at 0.5 µg/mL of MP (Table 5).

3.6. *Total amounts of the deduced radical scavenging compounds in peel extracts*

As shown in Table 6, the highest amount of total polyphenolic and flavonoid compounds were present in PG (1,799 mg gallic acid equivalent/100 g dry weight of peel extract and 741.71 mg quercetin equivalent/100 g dry weight of the extract, respectively), while the highest amount of ascorbic acid was present in CV (879.12 mg/kg dry weight of peel extract).

Table 1. Radical scavenging activity in various fruit peel extracts observed with a DPPH assay system

Peel extracts[1]	Radical scavenging activity (%)[2]		
	25 µg/mL	50 µg/mL	100 µg/mL
	95.65 ± 1.56[a]	41.95 ± 4.83[a]	9.86 ± 0.85[a]
	116.25 ± 8.52[b]	152.07 ± 3.30[b]	90.41 ± 4.62[b]
	61.37 ± 4.30[c]	8.67 ± 0.72[c]	7.03 ± 1.16[c]
	12.61 ± 2.01[d]	17.96 ± 1.69[c]	11.33 ± 1.34[c]
	18.02 ± 2.54[d]	11.95 ± 2.23[c]	7.14 ± 1.72[c]
	7.19 ± 2.18[d]	19.31 ± 3.05[c]	3.83 ± 0.48[c]
	102.52 ± 1.75[e] ↑	105.72 ± 1.40[d] ↑	115.12 ± 3.31[d] ↑

[1] CS, *C. sinensis*; PG, *P. granatum*; MP, *M. paradisiaca*; MI, *M. indica*; CV, *C. vulgaris*; CM, *C. melo*; CP, *C. papaya*; [2] Data are expressed in % inhibition in comparison to that of BHA (mean ± SE of five measurements). Means in columns without letters in common differ significantly ($P \leq 0.05$). ↑ indicates an increase in radicals.

Table 2. Radical scavenging activity in various fruit peel extracts observed with a β-carotene bleaching assay system

Peel extracts[1]	Radical scavenging activity (%)[2]		
	25 µg/mL	50 µg/mL	100 µg/mL
CS	67.68 ± 1.50[a]	57.52 ± 9.47[a]	37.95 ± 2.08[a]
PG	62.91 ± 4.14[a]	59.25 ± 2.81[a]	120.21 ± 1.69[b]
MP	105.01 ± 3.40[b]	121.48 ± 2.38[b]	99.72 ± 7.99[c]
MI	12.61 ± 2.01[c]	17.96 ± 1.69[c]	11.33 ± 1.34[d]
CV	133.86 ± 7.48[d]	165.19 ± 4.82[d]	159.36 ± 7.91[e]
CM	122.79 ± 4.45[d]	123.95 ± 12.11[e]	149.53 ± 11.92[e]
CP	147.49 ± 5.52[e]	145.69 ± 4.57[f]	148.39 ± 4.42[e]

[1] CS, *C. sinensis*; PG, *P. granatum*; MP, *M. paradisiaca*; MI, *M. indica*; CV, *C. vulgaris*; CM, *C. melo*; CP, *C. papaya*; [2] Data are expressed in % inhibition in comparison to that of BHA (mean ± SE of five measurements). Means in columns without letters in common differ significantly ($P \leq 0.05$).

Table 3. NO radical scavenging (% inhibition) by various fruit peel extracts

Peel extracts[1]	NO radical scavenging (% inhibition)[2]		
	25 µg/mL	50 µg/mL	100 µg/mL
CS	33.47 ± 1.81[a]	31.38 ± 1.84[a]	8.10 ± 1.87[a]
PG	52.06 ± 3.62[b]	9.90 ± 0.36[b]	19.13 ± 2.50[b]
MP	51.38 ± 1.37[b]	12.43 ± 1.24[b]	5.32 ± 1.17[c]
MI	17.04 ± 1.00[c]	17.11 ± 1.20[b]	13.00 ± 1.25[d]
CV	31.38 ± 1.84[d]	8.49 ± 0.68[c]	3.63 ± 0.89[e]
CM	12.21 ± 1.83[e]	20.31 ± 2.50[d]	15.42 ± 3.37[d]
CP	24.46 ± 2.46[f]	38.98 ± 3.84[e]	21.07 ± 2.44[f]

[1] CS, *C. sinensis*; PG, *P. granatum*; MP, *M. paradisiaca*; MI, *M. indica*; CV, *C. vulgaris*; CM, *C. melo*; CP, *C. papaya*; [2] Values are the mean ± SE of five measurements. Means in columns without letters in common differ significantly ($P \leq 0.05$).

Table 4. Inhibition of H_2O_2-induced LPO in erythrocytes by various fruit peels

Peel extracts[1]	Inhibition (%)[2]			
	0.25 µg/mL	0.5 µg/mL	1.0 µg/mL	2.0 µg/mL
CS	72.31 ± 4.42[a]	119.67 ± 6.26[a]	94.68 ± 2.76[a]	76.60 ± 3.87[a]
PG	107.12 ± 5.77[b]	140.33 ± 4.74[b]	109.98 ± 6.10[a]	73.09 ± 2.33[a]
MP	147.31 ± 8.31[c]	89.95 ± 4.01[c]	99.43 ± 4.25[a]	35.76 ± 5.25[b]
MI	86.87 ± 6.58[d]	95.12 ± 6.46[c]	81.84 ± 5.50[a]	82.48 ± 1.54[c]
CV	111.18 ± 10.66[d]	102.33 ± 9.06[c]	100.54 ± 6.58[a]	85.99 ± 4.09[c]
CM	89.45 ± 6.84[d]	24.49 ± 8.37[d]	55.65 ± 3.12[b]	12.27 ± 1.87[d]
CP	45.41 ± 8.05[e]	82.62 ± 5.10[e]	40.67 ± 7.79[b]	46.09 ± 4.34[e]

[1] CS, *C. sinensis*; PG, *P. granatum*; MP, *M. paradisiaca*; MI, *M. indica*; CV, *C. vulgaris*; CM, *C. melo*; CP, *C. papaya*; [2] Data are expressed in % inhibition in comparison to that of BHA (mean ± SE of five measurements). Means in columns without letters in common differ significantly ($P \leq 0.05$).

Table 5. Inhibition of H_2O_2-induced LPO in liver slices by various fruit peels

Peel extracts[1]	Inhibition (%)[2]			
	0.25 µg/mL	0.5 µg/mL	1.0 µg/mL	2.0 µg/mL
CS	65.84 ± 7.75[a]	123.12 ± 11.74[a]	63.76 ± 4.43[a]	20.87 ± 0.92[a]
PG	44.12 ± 5.77[a]	72.02 ± 2.12[b]	121.78 ± 3.07[b]	99.99 ± 6.84[b]
MP	44.49 ± 6.88[a]	160.19 ± 7.01[c]	51.56 ± 2.97[c]	99.64 ± 2.43[b]
MI	65.35 ± 5.58[a]	77.96 ± 3.42[d]	90.94 ± 5.99[d]	89.11 ± 1.77[b]
CV	131.00 ± 3.96[b]	104.19 ± 4.49[e]	79.33 ± 1.99[e]	55.08 ± 3.87[c]
CM	24.89 ± 6.53[c]	107.91 ± 7.13[e]	74.01 ± 2.55[e]	81.74 ± 2.73[d]
CP	111.78 ± 7.86[d]	57.89 ± 2.45[f]	60.42 ± 1.09[f]	72.62 ± 7.30[d]

[1] CS, *C. sinensis*; PG, *P. granatum*; MP, *M. paradisiaca*; MI, *M. indica*; CV, *C. vulgaris*; CM, *C. melo*; CP, *C. papaya*; [2] Data are expressed in % inhibition in comparison to that of BHA (mean ± SE of five measurements). Means in columns without letters in common differ significantly ($P \leq 0.05$).

Table 6. Total amounts of radical scavenging compounds deduced from peel extracts

Peel extracts[1]	Polyphenols[2]	Flavonoids[3]	Ascorbic acid[4]
CS	1,407 ± 4.15	311.80 ± 6.17	780.30 ± 11.3
PG	1,799 ± 7.29	741.71 ± 8.08	733.40 ± 11.32
MP	89 ± 2.12	47.85 ± 3.75	756.45 ± 10.89
MI	1,161 ± 6.88	558.09 ± 7.28	811.17 ± 11.12
CV	1,147 ± 4.94	105.23 ± 5.0	879.12 ± 13.21
CM	1,139 ± 4.77	136.47 ± 5.19	826.15 ± 11.89
CP	272 ± 2.03	49.70 ± 4.09	810.19 ± 12.88

[1] CS, *C. sinensis*; PG, *P. granatum*; MP, *M. paradisiaca*; MI, *M. indica*; CV, *C. vulgaris*; CM, *C. melo*; CP, *C. papaya*; [2] Values are presented as mg gallic acid equivalent/100 gm dry weight of peel extract (mean ± SE); [3] Values are presented as mg quercetin equivalent/100 g dry weight of the extract (mean ± SE); [4] Values are presented as mg/100 g of dry weight of the extract (mean ± SE).

4. Discussion

This study has shown that different fruit peel extracts exhibit varying degrees of free radical scavenging/ antiperoxidative potential depending on their concentrations and on the type of assay system/radicals used. In particular, the DPPH assay method revealed a different trend, where, unlike other test peels, CP extract was found to be pro-oxidative. In fact, marked inhibition (more than 50%) of DPPH radicals was observed only with CS, PG, and MP peel extracts. These findings are similar to those for several other fruit peels reported previously (*3,7,23,32*). Like CP, toxic or pro-oxidative effects of several other herbal extracts have also been reported (*32-34*).

DPPH radicals are believed to act as a suitable model compound for free radicals originating in lipids and have been used in several studies of other natural products (*35-37*). The present study revealed that the test peel extracts, and particularly CS, PG, and MP, contain a considerable amount of compounds scavenging for nitrogen radicals and that these compounds might be mediated *via* direct electron donation and subsequently reduce DPPH radicals. However, only CP appeared to be negatively involved in the redox reaction, as it increased the color intensity/ absorbance of the solution, suggesting the presence of several compounds similar to DPPH radicals or with an absorption maxima (517 nm) like that of DPPH. The other three test peels (MI, CV, and CM) did not substantially quench DPPH radicals. Therefore, these peels may not scavenge nitrogen radicals or may not work *via* direct electron donation.

In the present study, the β-carotene bleaching assay showed that all of the test peel extracts except MI exhibited marked antiperoxidative or radical scavenging activity, suggesting their potential for scavenging singlet oxygen evolved in the reaction (*19*). Similar findings have been obtained by other authors using the β-carotene bleaching assay (*38,39*). However, only limited studies of peel extracts have been conducted (*3,23,32*). The present findings, therefore, indicate that most of the test peels have the potential to inhibit singlet oxygen produced during photosensitized oxidations (*21*).

When the NO radical scavenging activity of the experimental peels was evaluated, results revealed that only 25 µg/mL of PG and MP peel extracts are able to reduce NO radicals substantially. However, CS, CV, and CP peel extracts did not substantially inhibit NO radical generation, which further tallied with the results

of DPPH scavenging, *i.e.* that these peels may not work *via* nitrogen radical inhibition. These results are also in accordance with earlier reports on fruit peels (*3,32*) and on other natural products (*24,25,39*).

In the present study, H_2O_2-induced lipid peroxidation in RBCs and in liver slices was inhibited by all of the test peel extracts at one or other concentrations, suggesting their potential to scavenge peroxyradicals or other reactive oxygen species (ROS) induced by H_2O_2 (*19*) as reported by several earlier works (*24,28,40*). Similar findings were also obtained for several of the test peel extracts by using RBCs (*9*) and other herbal extracts using liver slices (*40*).

Reviewing all of these findings suggest that the antiperoxidative activity of test peel extracts tends to be method-specific, as was thought earlier (*18*). This could be due to the differences in the mechanism of action(s) and the radical specific scavenging activity of the peel extracts studied (*19,24*). The radical scavenging activity of the test peel extracts might have been mediated by the presence of total polyphenolic components, flavonoids, and ascorbic acid, as posited by earlier works (*3,7,41*). This possibility is further supported by the high content of total phenolic compounds, total flavonoids, and ascorbic acid (Table 6). Interestingly, the present study found that the test peels have a dose-specific radical scavenging potential, which is also in accordance with earlier reports on fruit peel extracts based on *in vivo* studies (*8-14*). This is explained by the fact that polyphenolic compounds and/or flavonoids present in herbal extracts usually lead to various polymerization reactions and changes in chemical nature and spatial conformations that can modify the reactivity of molecules in a concentration-dependent manner (*41-43*). In fact, naturally occuring polyphenolic compounds are known to exhibit dual behavior in a dose-dependent manner as their antiperoxidative and/or pro-oxidative nature depends upon their dose (*8-16,41,44-47*). In conclusion, the present findings clearly suggest that the test peel extracts have a varying degree of radical scavenging potential for different radicals in a dose-specific manner. Therefore, evaluation of the efficacy of herbal extracts should be carried out using different methods and not merely a single *in vitro* method. Further study of this point may lead to the development of specific antioxidant formulations for different peel extracts in accordance with the radicals to be scavenged.

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Original Article

In vitro radical scavanging activities of *Chrysaora quinquecirrha* nematocyst venom

Elumalai Balamurugan, Venugopal Padmanabhan Menon*

Department of Biochemistry & Biotechnology, Annamalai University, Tamil Nadu, India.

ABSTRACT: The venom of *Chrysaora quinquecirrha* (sea nettle) contains several toxins that have bioactivity in mammals. In our study we aimed to extract proteins from *Chrysaora quinquecirrha* and to test the antioxidant potential of both crude protein and purified fractions. Proteins extracted from sea nettle nematocyst venom were purified through Sephadex G-100 column chromatography. The molecular weight of purified proteins was determined by gel filtration and SDS-PAGE and was found to be 105, 65, and 9 kDa for Frc-1, Frc-2, Frc-3, respectively. The *in vitro* antioxidant potential of *Chrysaora quinquecirrha* was evaluated in different systems *viz*. radical scavenging activity by DPPH reduction, superoxide radical scavenging activity in PMS/NADH-NBT system, hydroxyl radical by Fe^{3+}-Ascorbate-EDTA-H_2O_2 system and nitric oxide (NO) radical scavenging activity in sodium nitroprusside/ Greiss reagent system. Frc-3 displayed the maximal antioxidant activity and found to have different levels of antioxidant properties in the models tested. In scavenging hydroxyl radicals, its activity was intense (IC_{50} = 50.8 µg/mL) while in scavenging NO radical, it was moderate (IC_{50} = 381.4 µg/mL).

Keywords: Antioxidant peptide, *Chrysaora quinquecirrha*, DPPH radical, superoxide radical, hydroxyl radical, nitric oxide radical

1. Introduction

Oxidative-free radicals are byproducts of the normal reactions within our body. These reactions include the generation of calories, the degradation of lipids, the catecholamine response under stress, and the inflammatory processes (*1*). If the balance between oxidative-free radical production and eradication is maintained, the harmful effects of free radicals would be minimized in the body. However, if the unwanted free radicals are not eradicated efficiently, oxidative stress would occur. Oxidative stress, caused by reactive oxygen or free radicals, has been shown to be associated with the progression of many diseases including cancer, heart disease, and depression, among others (*2-4*). An antioxidant, which can quench reactive free radicals, can prevent the oxidation of other molecules and may, therefore, have health-promoting effects in the prevention of degenerative diseases (*5*). The interest in antioxidants has been increasing because of their high capacity in scavenging free radicals related to various diseases (*6*). Various antioxidant compounds are identified in many natural sources including some protein compounds. Proteins of jellyfish (*7*) and protein hydrolysates from different sources, such as milk protein (*8*), maize zein (*9*), egg-yolk (*10*), porcine proteins (*11*), yellow stripe trevally (*12*), yellowfin sole frame (*13*), mackerel (*14*), have been found to possess antioxidant activity.

The jellyfish, *Chrysaora quinquecirrha*, a cnidarian of the class Scyphozoa, the order, Semaeostomeae and the family Pelagiidae is distributed widely in the Bay of Bengal, India. The *Chrysaora quinquecirrha* has two types of tentacles, fishing and mesenteric tentacles. These tentacles have been shown to contain many types of stinking organelles or nematocyst (*15*). It has been reported that venom from the fishing tentacles nematocyst is considerably more lethal than that of mesenteric tentacles nematocyst (*16*). Research on *Chrysaora quinquecirrha* has indicated the presence of numerous compounds, including various amines and large molecules such as proteins or peptides (*16*) as its components. Jellyfish nematocyst has been venom found to possess myotoxic (*17*), neurotoxic (*18*), apoptotic (*19*), cytotoxic (*20*), cardiotoxic (*21*) and antimicrobial activity (*22*). Regarding jellyfish derived peptides, to the best of our knowledge there are only a few reports dealing with their antioxidant properties (*7*). In this study we isolated two proteins and a peptide from fishing tentacles nematocyst of *Chrysaora quinquecirrha* and demonstrated its antioxidant potential.

**Address correspondence to:*
Dr. Venugopal P. Menon, Department of Biochemistry & Biotechnology, Annamalai University, Annamalainagar -608 002, Tamil Nadu, India.
e-mail: biocmr@sify.com

2. Materials and Methods

2.1. *Chemicals*

Sephadex G-100, 2-deoxy ribose, 1,1-diphenyl-2-picryl-hydrazil (DPPH), ethylene diamine tetra acetic acid (EDTA), hydrogen peroxide (H_2O_2), nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), sodium nitroprusside and trolox were purchased from M/s. Sigma Chemical Co., St. Louis, MO, USA. Protein molecular weight maker was purchased from Genei (India). All other chemicals and solvents were of analytical grade and were obtained from Himedia chemicals, Mumbai, India.

2.2. *Specimen collection and protein purification*

Nettles were collected during the summer months in the Bay of Bengal near Pazhayar, Tamilnadu, India. The fishing tentacles were excised manually *in vivo* and nematocyst were isolated by the method described by Rice and Powell (*23*). The nematocyst mass was suspended in phosphate buffered saline and observed under microscope to confirm the presence of nematocyst. When required, approximately, 1 mg of freeze dried nematocysts were resuspended in 10 mL of double distilled water, and then subjected to 8 × 30 sec sonication cycles at 4°C with 1 min rest between cycles. The venom preparation was aliquoted, lyophilized and stored at -20°C. Crude proteins extracted from nematocyst were purified by Sephadex G-100 gel filtration and 3 mL fractions were collected and their absorbance, at 280 nm was determined by an ultraviolet detector. Protein concentration was measured by the method of Lowry *et al.* (*24*).

The molecular weight of the proteins were estimated by Sephadex G-200 gel filtration chromatography according to the method of Andrews (*25*) by using the parameter $K_{av} = (V_e - V_o) / (V_t - V_o)$. A standard curve was determined by chromatographing phospholipase, bovine serum albumin, ovalbumin, carbonic anhydrase, soyabean trypsin inhibitor and lysozyme calculating K_{av} for each, and plotting native MW *vs.* K_{av}. Reducing SDS-PAGE analysis was conducted (*26*) on crude nematocyst extracts and chromatography fractions using a Bio-Rad Mini-PROTEAN II electrophoresis system. Samples containing low amounts of protein were concentrated by TCA precipitation prior to analysis. Protein samples were separated on 10-15% polyacrylamide gels and protein bands were visualized with Coomassie brilliant blue R-250 (*27*).

2.3. *Radical scavenging assays*

The antioxidant activity of the proteins were determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blios (*28*). A measurement of superoxide anion scavenging activity of proteins was performed based on the method described by Nishimiki *et al.* (*29*). Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the test compound (proteins) for hydroxyl radical generated by Fe^{3+}-Ascorbate-EDTA-H_2O_2 system (Fenton reaction) according to the method described by Kunchandy and Rao (*30*). The nitric oxide radical inhibition activities of proteins were measured by the method described by Garrat (*31*). All the tests were performed for six times.

2.4. *Statistical analysis*

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) by using statistical package of social science (SPSS) version 10.0 for windows. The values are mean ± SD for six experiments in each group. *P* values < 0.05 were considered as level of significance.

3. Results

Nematocyst preparation yielded cleanest preparation with no visible ruptured nematocyst (Figure 1). Three major protein peaks appeared in the elution of Sephadex G-100 (Figure 2). The eluted volume of the first peak was 48 mL (Frc-1) and the eluted volume of the second

Figure 1. Light microscopic views of the nematocysts prepared suspensions of *Chrysaora quinquecirrha* species.

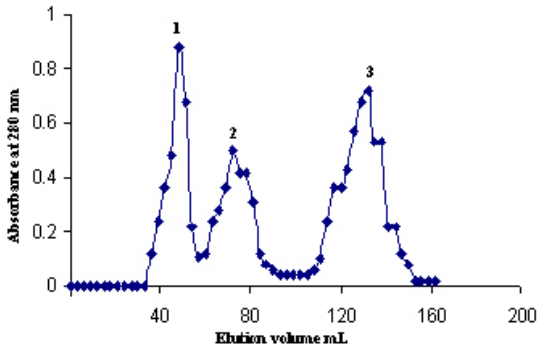

Figure 2. Chromatogram of crude nematocyst venom by Sephadex G-100 size-exclusion chromatography. Protein concentration was monitored at 280 nm. Three major protein peaks were obtained at the flow rate of 25 mL/h.

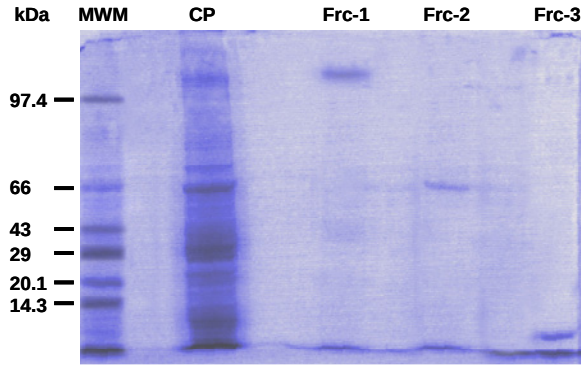

Figure 3. SDS-PAGE analysis of *Chrysaora quinquecirrha* nematocyst proteins purified by gel filtration. Protein bands were stained with Coomasie Blue. *Lane* 1, molecular weight marker (MWM); *lane* 2, crude protein; *lanes* 3, 4, and 5, Frc-1, 2, and 3, respectively.

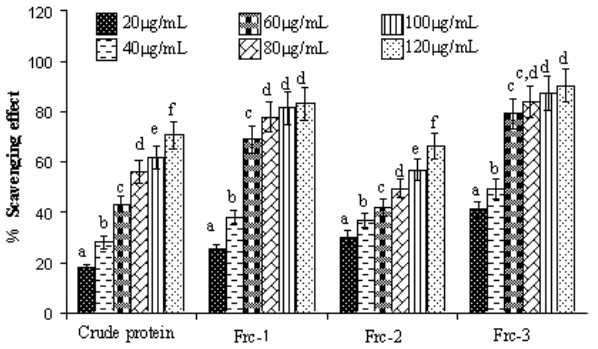

Figure 4. Scavenging effect of protein samples on DPPH radical. The superscripts a-f in the figure represents that the difference between the samples is less than 0.05 ($P < 0.05$) which is statistically significant.

Table 1. IC_{50} values of protein samples

Assays	Crude protein (µg/mL)	Frc-1 (µg/mL)	Frc-2 (µg/mL)	Frc-3 (µg/mL)
DPPH radical scavenging assay	78.4 ± 1.80	59.7 ± 1.53	82.0 ± 1.68	53.8 ± 0.82
Superoxide radical scavenging assay	80.04 ± 2.40	63.93 ± 1.82	76.58 ± 1.20	55.02 ± 0.63
Hydroxyl radical scavenging assay	65.2 ± 0.75	52.1 ± 0.61	73.2 ± 1.65	50.8 ± 0.95
Nitric oxide radical scavenging assay	539.3 ± 50.01	441.9 ± 19.56	400.1 ± 9.72	381.4 ± 0.95

peak (Frc-2) and third peak (Frc-2) was 72 and 132 mL, respectively. The estimated native molecular masses of the other major peaks (Frc-1, 2, and 3) were calculated as 105, 65, and 9 kDa.

Figure 3 shows the bands of five protein samples. Marker lane shows bands of protein markers. Lane 2 shows crude proteins (CP) and more than 8 proteins were seen after staining of SDS-PAGE gel. Frc-1, Frc-2, and Frc-3 lanes show the purified protein. The molecular weight of these proteins were 105, 65, and 9 kDa, respectively.

The results obtained by DPPH assay are shown in Figure 4. *Chrysaora nematyocyst* proteins exhibited powerful DPPH radical scavenging activity. CP at a concentration from 20-120 µg/mL showed a scavenging effect on the DPPH radical from 18.0-70.7%. The results were found to be statistically significant ($P < 0.05$). IC_{50} values of all experiments are shown in Table 1. IC_{50} of CP, Frc-1, Frc-2, and Frc-3 was found to be 78.4, 59.7, 82.0, and 53.8 µg/mL, respectively.

The superoxide radical-scavenging activities of proteins are shown in Figure 5. The protein samples scavenge superoxide anion radical in a dose dependent manner. Frc-3 at a concentration from 20-120 µg/mL showed a scavenging effect on the superoxide anion radicals from 43.9 to 92.6%. The results were found to be statistically significant ($P < 0.05$).

Figure 6 shows the % scavenging effects on hydroxyl radical of CP, Frc-1, Frc-2, and Frc-3. IC_{50} of Frc-1 and Frc-3 was 52.1 and 50.8 µg/mL, respectively. Frc-1 at a concentration from 20-120 µg/mL, the % scavenging effect was from 50.7-88.0% and for Frc-3 at a concentration from 20-120 µg/mL, the % scavenging

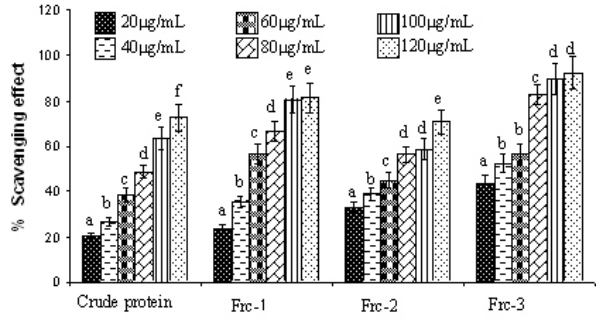

Figure 5. Scavenging effect of protein samples on superoxide radical. The superscripts a-f in the figure represents that the difference between the samples is less than 0.05 ($P < 0.05$) which is statistically significant.

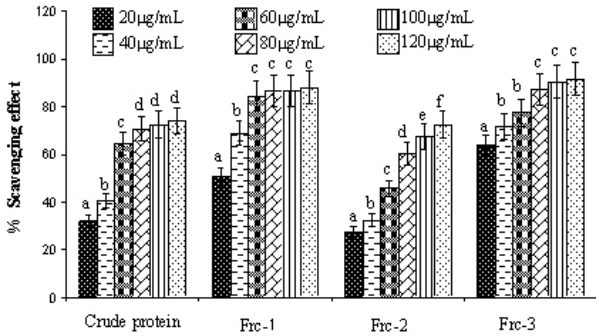

Figure 6. Scavenging effect of protein samples on hydroxyl radical. The superscripts a-f in the figure represents that the difference between the samples is less than 0.05 ($P < 0.05$) which is statistically significant.

effect was from 63.6-91.7%. Figure 7 shows the nitric oxide scavenging effects of CP, Frc-1, Frc-2, and Frc-3 at a concentration from 50-300 µg/mL. The results were found to be statistically significant ($P < 0.05$).

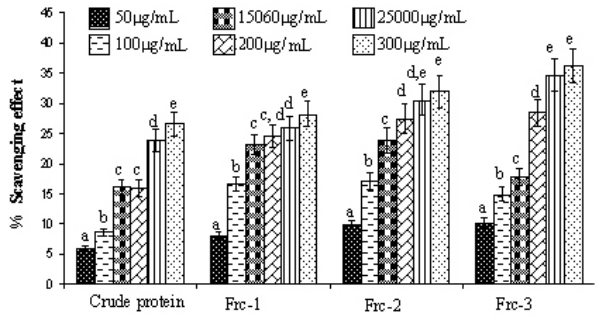

Figure 7. Scavenging effect of protein samples on nitric oxide radical. The superscripts a-e in the figure represents that the difference between the samples is less than 0.05 ($P < 0.05$) which is statistically significant.

4. Discussion

Separation of native *Chrysaora quinquecirrha* venom proteins was achieved by size-exclusion chromatography across the molecular mass separation range, 10-200 kDa. Reducing SDS-PAGE analysis of the crude venom and purified fractions revealed that all the three major fractions are significantly purified and the apparent molecular weight is same as calculated by size exclusion chromatography.

It is well known that the radical system used for antioxidant evaluation may influence the experimental results, and two or more radical systems are required to investigate the radical-scavenging capacities of a selected antioxidant (*32*). The DPPH radical has been widely used to test the ability of compounds as free-radical scavengers or hydrogen donors and to evaluate the antioxidative activity (*33,34*). DPPH is one kind of the compound that has a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (*35,36*). All the protein samples had strong DPPH scavenging activities. Frc-3 showed maximum scavenging effect on DPPH radical.

Superoxide anion is also an initial free radical formed from mitochondrial electron transport systems. Mitochondria generate energy using four electron chain reactions, reducing oxygen to water. Some of the electrons escaping from the chain reaction of mitochondria directly react with oxygen and form superoxide anion. It plays an important role in the formation of other reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (*37*). Therefore, studying the scavenging effects of sea nettle proteins on superoxide radicals is one of the most important ways of clarifying the mechanism of antioxidant activity.

The superoxide anion radicals scavenging activity of protein samples followed the order Frc-2 > CP > Frc-1 > Frc-3. IC_{50} of Frc-3 (53.8 µg/mL) is low when compared with other protein samples. Generally, the quenching of free radical has been attributed to the donation of hydrogen. Further, some amino acids such as His, Leu, Tyr, Met enhance the scavenging activities of peptides (*38*). These results indicated that *Chrysaora quinquecirrha* nematocyst proteins has a notable effect on scavenging of superoxide radicals.

Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron. Hydroxyl radicals react readily with lipid, polypeptides, proteins, and DNA, especially thiamine and guanosine. When a hydroxyl radical reacts with aromatic compounds, it can add across a double bond, resulting in hydroxycyclo-hexadienyl radical. The resulting radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical, or decompose to phenoxyl-type radicals by water elimination (*39*). Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of a living body against various diseases. The scavenging effect against hydroxyl radicals was investigated by using the 2-deoxyribose oxidation method. Of the four samples, Frc-1 and Frc-3 showed maximum scavenging effects. With this finding it can be concluded that *Chrysaora* proteins can be used as a good hydroxyl radical scavenger.

The NO• scavenging activity of a compound is of potential health interest as it has been proposed that NO• plays an important role in the progression of many diseases and pathological conditions such as septic shock, atherosclerosis, ischemia reperfusion, neurodegenerative disorders like Alzheimer's and Parkinson's diseases, cancer and diabetes (*40-43*). No significant nitric oxide scavenging effect was found at a concentration 20-40 µg/mL. The scavenging effect of protein samples followed the sequence CP > Frc-1 > Frc-2 > Frc-3.

The different protein fractions obtained by gel filtration exhibited different antioxidant and free radicals scavenging activities. Results revealed that Fra-3 has the highest antioxidant and free radicals scavenging activities. Frc-3 had the strongest radical scavenging activity for not only the small radicals (hydroxyl and superoxide) but also the relatively large species (DPPH), suggesting a nondiscriminating nature of the peptide antioxidants. Based on these findings, we believe that smaller peptides have a higher level of radical scavenging activity than larger proteins, a finding consistent with that of Moosman and Behl (*44*), where they have reported the antioxidant nature of smaller peptides. These findings are in agreement with observations from other studies and support the fact that functional properties of antioxidative peptides are highly influenced by properties such as molecular mass (*45,46*). The smaller fractions possibly contained some groups, which were electron donors and could react with free radicals to convert them to more stable

products and terminate the radical chain reaction. Neither the structure-activity relationship nor the antioxidant mechanism of peptides is fully understood (*47*).

The results of the present study showed that the purified proteins from *Chrysaora quinquecirrha* can be used as an easily accessible source of natural antioxidants. Furthermore, the observed radical scavenging activity can at least partially justify the therapeutic use of *Chrysaora quinquecirrha* proteins. Nevertheless, its potential toxicity should be addressed before any possible application on a practical scale.

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Original Article

Beneficial effects of combined administration of sodium molybdate with atorvastatin in hyperlipidemic hamsters

Amany A. E. Ahmed*

Department of pharmacology, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

ABSTRACT: This study aims to evaluate the benefit of combined administration of sodium molybdate with atorvastatin in management of hyperlipidemia. Hyperlipidemic male Serian golden hamsters were administered either atorvastatin (40 or 80 mg/kg, p.o.) sodium molybdate (100 mg/kg, p.o.) or combination of atorvastatin (40 mg/kg, p.o.) with sodium molybdate (100 mg/kg, p.o.) for 30 consecutive days. Blood lipids (total cholesterol, triglycerides, HDL-cholesterol, Non-HDL-cholesterol and anti-atherogenic index) in addition to the activities of liver transaminases (AST, ALT), as well as antioxidant status (lipid peroxidation, catalase, glutathione peroxidase) were estimated before and after 15 and 30 days of treatment. The results indicate that atorvastatin is effective in lowering the blood lipids with maximum effect achieved by the high dose (80 mg/kg, p.o.). However, this dose elevates the liver enzymes significantly after 15 and 30 days of treatment. Sodium molybdate lowers the blood lipids after 30 days from treatment without alteration in liver enzymes. Moreover, in this group, lipid peroxides were significantly reduced and activities of catalase as well as glutathione peroxidase were significantly elevated compared with the hyperlipidemic control group (saline). Combination of atorvastatin (40 mg/kg, p.o.) with sodium molybdate significantly reduced the elevated blood lipids in a similar degree as the high dose of atorvastatin. Meanwhile, in this group, the liver enzymes were not significantly elevated while, the antioxidants profile were significantly improved compared with that of control hyperlipidemic and atorvastatin groups. In conclusion, combination of sodium molybdate with atorvastatin is beneficial in management of hyperlipidemia as it allows maximum reduction in blood lipids, improves the antioxidant status with minimal disturbances in liver enzymes.

**Address correspondence to:*
Dr. Amany A. E. Ahmed, Department of Pharmacology, Faculty of Pharmacy, King Saud University, Riyadh, 11495, P.O. Box 22452, Saudi Arabia.
e-mail: amresearch2009@yahoo.com

Keywords: Hyperlipidemic Syrian golden hamsters, atorvastatin, sodium molybdate, blood lipids, liver transaminases, antioxidant status

1. Introduction

The elevation of plasma levels of low density lipoprotein-cholesterol, triglycerides, and reduction of high-density lipoprotein is often associated with the high incidence of atherosclerosis and coronary heart disease (*1*). Hyperlipidemia and accordingly atherosclerosis are correlated to the elevation of oxidative stress which is characterized by lipid and protein oxidation (*2*).

Statins or the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-Co-A) reductase inhibitors represent the main class of lipid lowering drugs (*3*). Statins are proved to be effective in reducing serum levels of total as well as low density lipoprotein-cholesterol, slowing the progression of atherosclerosis, reducing the incidence of cardiovascular events, and decreasing the mortality rate (*4*). The currently used statins are generally well tolerated and present a good safety profile (*5*). Nevertheless, many adverse effects of statins have been reported. The main reported adverse effects of statins may include hepatotoxicity, characterized by an increased level of transaminases (*6*), various forms of myotoxicity, myalgias and rhabdomyolysis (*3*). Other minor adverse effects such as generalized gastrointestinal discomfort and neuropathy are also known. These adverse effects may increase upon aging, physical exercise or when statins are given at high doses or when administrated with another medications that interfere with its metabolism (*7*). The most widely used statins are also suffered from limitations due to intolerance, partial effectiveness in lowering cholesterol levels, and the cost (*8*).

Atorvastatin is a long half-life, lipophilic statin metabolized by cytochrome P450 (CYP3A4) enzyme. Atorvastatin at doses of 10 to 80 mg is the leading prescribed statin in the world, providing an LDL reduction of 38% to 55%. However, the high doses

of atorvastatin have an incidence of liver enzyme elevation, approximately 2.5%, which is the highest of all the statins (*9*).

Syrian golden hamster has been used as a model for studying lipids as well as lipoprotein metabolism because of its similarities to humans with regards to cholesterol and bile metabolism (*10*), Non-HDL-cholesterol and triglycerides response to atherogenic diet (*11*), development of early atherosclerotic lesions (*12*), and exclusive hepatic production of apolipoprotein (apo) B-100 (*13,14*).

Molybdenum represents an important trace element involved in the structure of certain enzymes that catalyzing redox reactions. Although it can form complexes with numerous physiologically important compounds, it was supposed that this trace element is absorbed, transported and excreted in a simple molybdate form (*15*). Many simple as well as complex molybdenum compounds were found to significantly reduce the levels of blood glucose and free fatty acids (*16,17*). Moreover, sodium molybdate was proved to prevent oxidation of lipids and protects antioxidant systems in different experimental models (*17,18*).

Accordingly, the aim of the current study is to evaluate the effect of atorvastatin treatment either alone or in combination with sodium molybdate on blood lipids, liver enzymes, and antioxidant status in hyperlipidemic Syrian golden hamsters. The study also targets to improve the hypolipidemic effects of atorvastatin and to minimize its adverse actions in an experimental trial to suggest or recommend a safe and effective combined therapy to hyperlipidemia.

2. Materials and Methods

2.1. *Drugs and chemicals*

All chemicals used were of high analytical grade and were obtained from Sigma-Aldrich (St. Louis, Mo, USA). Atorvastatin was supplied from Parke-Davis, Germany. The selected drug doses were matched with that in the literature and were chosen after a preliminary experimental study in our laboratory. The dose of sodium molybdate was in accordance with that previously reported (*18*), and there were no toxic effects observed with this dose.

2.2. *Animals and experimental protocol*

Male Syrian golden hamsters weighing between 100-120 g, obtained from the animal facilities of King Saud University were used. The animals were randomly divided into groups and acclimatized for one week in colony cages (five hamsters per cage), at 22 ± 2ºC and under a 12-h light: dark cycle with free access to water and rodent chow. After acclimatization period, all hamsters were fed ad libitum with the hyperlipidemic diet consists of the standard diet containing 10% coconut oil and 0.2% cholesterol. The diet was prepared in the form of paste, and it was found to be effective for induction of hyperlipidemia in hamsters in our laboratory. After two weeks from hyperlipidemic diet blood samples were withdrawn from 16 h fasted animals, *via* the retro-orbital sinus into heparinized glass tubes, under light ether anesthesia. The separated plasma was used for estimation of total cholesterol (TC). The hamsters were then divided into five groups (n = 10) of matched blood cholesterol levels and body weights.

All groups were fed with the hyperlipidemic diet till the end of the experiment. Group 1 was administered saline for 30 days (control), and groups 2 and 3 were administered atorvastatin (40 or 80 mg/kg, p.o.), respectively. Group 4 was administered sodium molybdate (100 mg/kg, p.o.), group 5 was administered atorvastatin (40 mg/kg, p.o.) followed by sodium molybdate (100 mg/kg, p.o.) one hour latter. All treatments were continued daily for 30 consecutive days. Blood samples were withdrawn, into heparinized tubes, at 1, 15, and 30 days of treatment from hamsters deprived of food for 16 h. The separated plasma was used for determination of the biochemical parameters. All hamsters in each group survived the entire length of the study. All experiments were conducted according to the guidelines of the Animal Care and Use Committee Acts of King Saud University, and in accordance with the international guidelines of handling of laboratory animals.

2.3. *Measurement of biochemical parameters*

2.3.1. *Blood lipids determinations*

Total cholesterol (TC) and triglycerides (TG) were measured by CHOD-PAP and GPO-PAP methods, respectively, using commercial kits (Spinreact S.A., Sant Esteve de Bas, Spain). Measurement of high density lipoprotein (HDL) cholesterol in plasma was carried out in the supernatant after precipitation of low density, intermediate density and very low density containing lipoproteins (LDL, IDL and VLDL), with phosphotungstate reagent followed by centrifugation, using commercial kits (Spinreact S.A., Spain). Results were expressed as Non-HDL (VLDL + IDL+ LDL) cholesterol instead of LDL-cholesterol, because the Friedewald equation is not applicable to hamsters. The concentration of lipoprotein (Non-HDL) cholesterol was calculated by subtracting HDL-cholesterol concentrations from total plasma cholesterol. Anti-atherogenic index was calculated as ratio of high density lipoprotein cholesterol/total cholesterol of each sample.

2.3.2. *Estimation of plasma transaminases activities*

Plasma aspartate aminotransferase (AST) and alanine

aminotransferase (ALT) activities were determined using the commercial enzymatic kits (Bio Merieux, France) and following the instruction manual.

2.3.3. *Estimation of plasma lipid peroxides, glutathione peroxidase, and catalase activities*

Plasma lipid peroxides was assayed according to the method described by Ohakawa *et al.* (*19*), using thiobarbituric acid reaction. The results were expressed as malondialdehyde (MDA) in nmol/L. Glutathione peroxidase (GPx) activity was measured spectrophotometrically at 340 nm according to the method described by Paglia and Valentine (*20*) using a Cayman microplate assay kit (Cayman, Ann Arbor, MI, USA). GPx activity was expressed as nmol/min/mL. Catalase (CAT) activity was estimated in plasma following the method of Aebi (*21*). Catalase activities were measured at 240 nm and were expressed as µmol/L.

2.4. *Statistical analysis*

The data was analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Results were presented as means ± SEM, n = 10 hamsters. The statistical significance of differences between data means were determined by using one way analysis of variance (ANOVA), followed by post hoc Tukey's test. Values of $P < 0.05$ were considered as statistically significant.

3. Results

As shown in Table 1, administration of hypercholesterolemic diet to hamsters for 30 days resulted in a significant elevation in plasma TC, Non-HDL-C, and TG levels compared with the initial value (day 1 of experiment after two weeks hyperlipidemic diet). This effect was accompanied by a significant reduction in the anti-atherogenic index. Meanwhile, the HDL-C level was not affected by the hypercholesterolemic diet.

Treatment of the hyperlipidemic hamsters with atorvastatin (40 or 100 mg/kg) for 30 days, resulted in a gradual, dose related and significant decrease in plasma TC levels (to 52% and 61% compared with the control hyperlipidemic hamsters at 30 days, respectively). A similar response was also observed for plasma Non-HDL cholesterol levels (to 61% and 74%, respectively). Additionally, atorvastatin treatment produced a significant and dose related decrease in triglycerides levels (to 40% and 53%, compared with the control hyperlipidemic hamsters at 30 days, respectively). HDL-C was significantly elevated after 30 days treatment with atorvastain (to 21% and 38%, compared with the control hyperlipidemic hamsters at 30 days, respectively). The anti-atherogenic index was significantly improved upon treatment by both doses of atorvastatin (to 2.5 and 3.7 folds compared with control hyperlipiemic hamsters at 30 days, respectively) (Table 1).

Table 1. Effect of treatment on plasma lipid parameters in hyperlipidemic hamsters

Item	Day 1				
	HL	At 40	At 100	Mol 100	At 40 + Mol 100
TC (mg/dL)	276.7 ± 12.33	313.0 ± 16.75	312.1 ± 21.82	318.7 ± 20.13	319.0 ± 19.35
HDL-C (mg/dL)	37.6 ± 2.40	38.9 ± 2.53	41.5 ± 2.04	38.7 ± 2.99	42.8 ± 2.39
Non HDL-C (mg/dL)	239.1 ± 10.22	273.9 ± 14.34	270.6 ± 19.86	279.9 ± 17.27	276.3 ± 17.05
TG (mg/dL)	194.2 ± 7.88	237.3 ± 12.05	238.5 ± 12.09	241.0 ± 10.45	233.8 ± 14.74
Anti-ath Ind	0.14 ± 0.005	0.13 ± 0.011	0.14 ± 0.003	0.13 ± 0.014	0.13 ± 0.003
Item	Day 15				
	HL	At 40	At 100	Mol 100	At 40 + Mol 100
TC (mg/dL)	332.7 ± 9.57$^{\#}$	251.6 ± 13.70$^{*\#}$	215.1 ± 16.62$^{*\#}$	256.6 ± 16.08$^{*\#}$	187.6 ± 7.16$^{*\#ab}$
HDL-C (mg/dL)	41.9 ± 2.32	45.5 ± 2.14	49.2 ± 2.61	48.9 ± 2.75	58.3 ± 1.48$^{*\#ab}$
Non HDL-C (mg/dL)	290.8 ± 7.75$^{\#}$	206.1 ± 12.13$^{*\#}$	165.9 ± 15.29$^{*\#}$	207.7 ± 14.68$^{*\#}$	128.9 ± 6.23$^{*\#ab}$
TG (mg/dL)	231.3 ± 10.40$^{\#}$	207.2 ± 11.15	179.6 ± 8.37$^{*\#}$	205.6 ± 10.69$^{\#}$	138.3 ± 6.31$^{*\#ab}$
Anti-ath Ind	0.13 ± 0.005	0.19 ± 0.015$^{*\#}$	0.24 ± 0.016$^{*\#}$	0.19 ± 0.016$^{*\#}$	0.31 ± 0.011$^{*\#ab}$
Item	Day 30				
	HL	At 40	At 100	Mol 100	At 40 + Mol 100
TC (mg/dL)	398.5 ± 7.75$^{\#}$	190.8 ± 4.09$^{*\#}$	153.7 ± 10.10$^{*\#b}$	198.9 ± 9.15$^{*\#}$	131.0 ± 3.01$^{*\#ab}$
HDL-C (mg/dL)	44.8 ± 2.13	54.0 ± 1.99$^{\#}$	61.9 ± 1.93$^{*\#}$	65.1 ± 3.13$^{*\#}$	76.4 ± 2.56$^{*\#ab}$
Non HDL-C (mg/dL)	353.8 ± 6.29$^{\#}$	136.8 ± 4.60$^{*\#}$	91.8 ± 9.24$^{*\#b}$	133.9 ± 7.34$^{*\#}$	54.7 ± 2.76$^{*\#ab}$
TG (mg/dL)	256.5 ± 10.28$^{\#}$	151.0 ± 6.20$^{*\#}$	121.7 ± 4.94$^{*\#b}$	164.3 ± 5.24$^{*\#}$	98.6 ± 4.86$^{*\#ab}$
Anti-ath Ind	0.11 ± 0.004$^{\#}$	0.28 ± 0.012$^{*\#}$	0.41 ± 0.022$^{*\#b}$	0.33 ± 0.023$^{*\#}$	0.58 ± 0.017$^{*\#ab}$

Abbreviations: HL, hyperlipidemic control group; At 40, atorvastatine (40 mg/kg, p.o.); At 100, atorvastatine (100 mg/kg, p.o.); Mol 100, sodium molybdate (100 mg/kg, p.o.); At 40 + Mol 100, atorvastatine (40 mg/kg, p.o.) + sodium molybdate (100 mg/kg, p.o.); TC, total cholesterol; HDL-C, high density lipoprotein; Non HDL-C, non-high density lipoprotein; TG, triglycerides; Anti-ath Ind, anti-atherogenic index.
Values are expressed as mean ± SEM of 16 h fasted hamsters (n = 10). All treatments were continued for 30 consecutive days.
* Significantly different from HL group at corresponding time intervals at $P < 0.05$.
$^{\#}$ Significantly different from the corresponding group value at day 1 at $P < 0.05$.
^{a} Significantly different from Mol 100 group value at corresponding time intervals at $P < 0.05$.
^{b} Significantly different from At 40 group value at corresponding time intervals at $P < 0.05$.

Treatment of the hyperlipidemic hamsters for 30 days with sodium molybdate (100 mg/kg) resulted in a significant reduction in TC (50%), Non-HDL-C (62%), TG (36%) and a significant elevation in HDL-C (45%) compared with hyperlipidemic hamsters at 30 days. Moreover, sodium molybdate produced a 3 fold improvement in the anti-atherogenic index (Table 1).

Combined administration of sodium molybdate (100 mg/kg) with atorvastatin (40 mg/kg) for 30 days resulted in a better protection against diet-induced hyperlipidemia in hamsters which was indicated by the significant reduction in TC (67%), Non HDL-C (85%), TG (62%), and the significant elevation in HDL-C (71%) compared with hyperlipidemic hamsters at 30 days. Moreover, the anti-atherogenic index was significantly improved by 5 folds. Interestingly, all these values were found to be significantly different from the corresponding values of either atorvastatin (40 mg/kg) or sodium molybdate alone groups at 30 days treatment (Table 1).

As shown in Table 2, Hyperlipidemic diet did not significantly affect the liver transaminases (AST and ALT) activities all over the experiment. Treatment of the hyperlipidemic hamsters with atorvastatin (100 mg/kg) for 30 days resulted in a significant elevation in plasma activities of both AST (34%) and ALT (36%) compared with the hyperlipidemic group at 30 days. On the contrary, sodium molybdate produced no effect on the activities of transaminases during treatment. Similarly combined administration of sodium molybdate (100 mg/kg) with atorvastatin (40 mg/kg) for 30 days did not affect the activities of transaminases (Table 2).

The results of Table 3 showed that administration of hyperlipidemic diet for 30 days produced a significant elevation in plasma lipid peroxides measured as MDA. On the contrary, Administration of atorvastatin (40 or 100 mg/kg), sodium molybdate (100 mg/kg), and combination of sodium molybdate (100 mg/kg) with atorvastatin (40 mg/kg) for 30 days resulted in a significant reduction in plasma lipid peroxides levels (to 55%, 60%, 56%, and 66%, compared with the control hyperlipidemic hamsters at 30 days, respectively).

As presented in Table 3, hyperlipidemic diet did not significantly affect the plasma glutathione peroxidase activity all over the experiment. Meanwhile, administration of atorvastatin (40 or 100 mg/kg), sodium molybdate (100 mg/kg), and combination of sodium molybdate (100 mg/kg) with atorvastatin (40 mg/kg) for 30 days produced a significant elevation in plasma glutathione peroxidase (GPx) activity (to 2.5, 2.8, 3.5, and 3.8 folds compared with the control hyperlipidemic hamsters at 30 days, respectively).

The present results showed that hyperlipidemic diet did not significantly affect the catalase activity during the experiment. Meanwhile, 30 days treatment of hyperlipidemic hamsters with atorvastatin (100 mg/kg) significantly increased the catalase activities (to 96%, compared with the hyperlipidemic group at 30 days). Similarly, treatment with sodium molybdate significantly elevated the catalase activity (to 111%, compared with the hyperlipidemic group at 30 days). Additionally, combined administration of sodium molybdate (100 mg/kg) with atorvastatin (40 mg/kg) for 30 days produced a significant elevation in plasma catalase activity (to 141% compared with the hyperlipidemic group at 30 days) (Table 3).

4. Discussion

The significant and progressive increase in hamsters blood lipids (TC, Non-HDL-C, and TG) induced by hypercholesterolemic diet which was observed in the current study is in agreement with a previous report

Table 2. Effect of treatment on plasma AST and ALT activities in hyperlipidemic hamsters

Item	Day 1				
	HL	At 40	At 100	Mol 100	At 40 + Mol 100
AST (U/L)	82.4 ± 3.32	84.9 ± 4.37	81.0 ± 3.49	79.8 ± 5.17	86.7 ± 4.63
ALT (U/L)	38.6 ± 4.03	39.9 ± 3.00	42.1 ± 3.68	40.8 ± 3.13	41.0 ± 3.31
Item	Day 15				
	HL	At 40	At 100	Mol 100	At 40 + Mol 100
AST (U/L)	84.2 ± 3.52	94.5 ± 4.66	104.1 ± 5.17*#	88.3 ± 4.97	95.7 ± 4.89
ALT (U/L)	45.1 ± 3.21	48.5 ± 3.01	58.9 ± 3.85*#	46.1 ± 2.89	49.1 ± 3.39
Item	Day 30				
	HL	At 40	At 100	Mol 100	At 40 + Mol 100
AST (U/L)	90.9 ± 3.97	103.1 ± 3.83#	121.4 ± 6.01*#	94.7 ± 5.28	102.5 ± 4.49
ALT (U/L)	49.2 ± 2.91	55.2 ± 2.74#	66.7 ± 3.85*#	51.0 ± 3.11	52.7 ± 3.89

Abbreviations: HL, hyperlipidemic control group; At 40, atorvastatine (40 mg/kg, p.o.); At 100, atorvastatine (100 mg/kg, p.o.); Mol 100, sodium molybdate (100 mg/kg, p.o.); At 40 + Mol 100, atorvastatine (40 mg/kg, p.o.) + sodium molybdate (100 mg/kg, p.o.); AST, Aspartate aminotransferase; ALT, Alanine aminotransferase.

Values are expressed as mean ± SEM of 16 h fasted hamsters (n = 10). All treatments were continued for 30 consecutive days.

* Significantly different from HL group at corresponding time intervals at $P < 0.05$.

Significantly different from the corresponding group value at day 1 at $P < 0.05$.

Table 3. Effect of treatment on plasma MDA, glutathione peroxidase and catalase activities in hyperlipidemic hamsters

Item	Day 1				
	HL	At 40	At 100	Mol 100	At 40 + Mol 100
MDA (nmol/L)	444.2 ± 39.13	460.9 ± 52.13	432.6 ± 50.17	418.9 ± 41.67	442.4 ± 48.27
GPx (nmol/min/mL)	51.0 ± 5.59	63.7 ± 7.47	50.7 ± 5.67	65.6 ± 7.54	59.1 ± 6.22
Catalase (µmol/L)	13.9 ± 1.32	12.9 ± 1.64	14.1 ± 1.29	15.0 ± 1.76	13.2 ± 1.64
Item	Day 15				
	HL	At 40	At 100	Mol 100	At 40 + Mol 100
MDA (nmol/L)	606.2 ± 48.30[#]	417.7 ± 50.17[*]	346.2 ± 34.95[*]	329.8 ± 32.69[*]	296.6 ± 30.10[*#]
GPx (nmol/min/mL)	49.8 ± 4.43	78.3 ± 8.35	99.2 ± 9.54[*#]	121.1 ± 14.78[*#]	128.5 ± 15.94[*#b]
Catalase (µmol/L)	12.2 ± 1.27	13.7 ± 1.62	15.7 ± 1.48	16.9 ± 1.95	15.5 ± 1.66
Item	Day 30				
	HL	At 40	At 100	Mol 100	At 40 + Mol 100
MDA (nmol/L)	659.9 ± 49.46[#]	294.9 ± 38.25[*#]	266.7 ± 28.07[*#]	289.0 ± 31.30[*#]	221.9 ± 28.58[*#]
GPx (nmol/min/mL)	47.7 ± 4.30	119.5 ± 10.72[*#]	135.1 ± 14.71[*#]	171.6 ± 14.37[*#]	183.4 ± 18.29[*#b]
Catalase (µmol/L)	10.5 ± 1.01	18.2 ± 1.85	20.5 ± 1.92[*#]	22.0 ± 2.32[*]	25.2 ± 2.45[*#]

Abbreviations: HL, hyperlipidemic control group; At 40, atorvastatine (40 mg/kg, p.o.); At 100, atorvastatine (100 mg/kg, p.o.); Mol 100, sodium molybdate (100 mg/kg, p.o.); At 40 + Mol 100, atorvastatine (40 mg/kg, p.o.) + sodium molybdate (100 mg/kg, p.o.); MDA, malonedialdehyde; GPx, glutathione peroxidase.
Values are expressed as mean ± SEM of 16 h fasted hamsters (n = 10). All treatments were continued for 30 consecutive days.
[*] Significantly different from HL group at corresponding time intervals at $P < 0.05$.
[#] Significantly different from the corresponding group value at day 1 at $P < 0.05$.
[b] Significantly different from At 40 group value at the corresponding time intervals at $P < 0.05$.

by Moghadasian (*22*). Similarly, the increase of lipid peroxidation in animals fed a hyperlipidemic diet has been previously reported (*23-25*). It is known that, the hypercholesterolemic diet can change the *in vivo* antioxidant status of blood by increasing the generation of oxygen free radicals these exert their cytotoxic effect by causing lipid peroxidation (*23*) which promotes the cellular consumption of glutathione and inactivates glutathione peroxidase (*26*). Meanwhile, the free radicals generated during lipid peroxidation could inactivate catalase, and reduce the effectiveness of the cells to protect themselves from damage (*27*) which is concordant with the present results.

In the current study, transaminases activity was estimated to evaluate the possible damage of the liver which may occur during feeding the hyperlipidemic diet or upon treatment with atorvastatin or sodium molybdate. AST and ALT levels remain the most useful tests for detection of hepatic cell damage. Both enzymes are present in high concentration in hepatocytes and leak into the circulation when hepatocytes or their cell membranes are damaged (*28*).

It was reported that treatment with a high concentration of cholesterol can cause liver damage (*29*). However, the results of the current study revealed that AST and ALT activities did not significantly elevated by administration of hyperlipidemic diet. Furthermore, hamsters treated with sodium molybdate did not present any alteration in plasma AST and ALT levels. On the contrary, treatment with atorvastatin (100 mg/kg) significantly elevated the levels of both enzymes at 15 and 30 days of treatment compared with hyperlipidemic group at the same corresponding time. Meanwhile, treatment of the hamsters with the combination of sodium molybdate with atorvastatin did not significantly affect the liver enzymes all over the experiment. The results indicate a possibility of liver injury by the high dose of atorvastatin.

It was reported that, some patients have to discontinue statins therapy due to liver transaminases levels exceeding three times the upper reference limit especially at high doses (*30,31*). Similarly, hepatotoxicity related to atorvastatin was also reported previously (*32-34*). The risk of significant rise in serum transaminases, while using atorvastatin, is thought to be dose dependent (*35,36*). Atorvastatin is significantly longer acting compared with other statins this could explain the increased risk of hepatotoxicity in comparison to other statins (*35*). The pathogenesis of atorvastatin-associated liver dysfunction is unclear. However, statins-induced transaminases elevation was explained by the direct inhibition of mevalonate synthesis (*37*). Another explanation is through interference as well as disturbance of cholesterol-bile acid pathways. In this context, statins are known to reduce levels of oxidized cholesterol and cholesterol substrate for the hydroxylase, the enzyme involved in bile acid synthesis (*31*). Additionally, Dujovne (*38*) postulated that the pronounced lowering in serum low-density lipoprotein induced by atorvastatin could influence the structure of hepatocellular membrane leading to greater leakage of cellular enzymes including transaminases. Furthermore, some authors suggested that the induction of the CYP450 system may be central to these adverse events (*34,39*). Furthermore, an immunoallergic basis for such hepatotoxicity was previously suggested (*40*).

The lack of effect of sodium molybdate on liver

enzymes observed in the current study is in agreement with the results of Van Reen (*41*) after excessive feeding of sodium molybdate to rats.

Results of the present study indicated that, treatment of the hyperlipidemic hamsters with atorvastatin resulted in a significant decrease in plasma total cholesterol and Non-HDL cholesterol levels. Similar response was observed for plasma triglycerides levels. These effects were accompanied by a significant increase in HDL-C level (specially, at the high dose) and a significant improvement in the anti-atherogenic index.

Atorvastatin usually reduces LDL-C, in human, by 30-70% depending on the dose used (*31*,*42*). Similarly, four weeks atorvastatin therapy was reported to cause a significant decrease in oxidative stress, and LDL-C with an increase in HDL-C levels in hypercholesterolemic rabbits (*43*). Similarly, atorvastatin was reported to produce a significant lowering in TC, TG, and LDL-C accompanied by a reduction in the oxidative stress in STZ-diabetic rats (*44*).

Statins are proved to inhibit HMG-CoA reductase, the rate-limiting enzyme for cholesterol biosynthesis, and subsequently reduce cholesterol synthesis in the liver. This inhibition induces hepatocytes to increase their surface expression of LDL-receptors, so as to increase uptake of LDL and reduce plasma cholesterol as well as LDL-cholesterol (*45*). The elimination half life of atorvastatin is considered to be long, compared with other statins, this may explain its greater efficacy for lowering the LDL-cholesterol. Additionally, atorvastatin is unique in that its metabolites are active as inhibitors of HMG-CoA reductase as well as being potentially antioxidants (*46*).

The observed atorvastatin-induced reduction in hamster plasma level of triglycerides is consistent with the observation of the previous reports by Mangalogu, *et al.* (*47*) and Funatsu, *et al.* (*48*), using fructose-fed hamsters and sucrose fed rats, respectively. The mechanism of triglycerides lowering effect of atorvastatin may involve the marked inhibition of cholesterol synthesis which suppresses the assembly and secretion of hepatic VLDL (*49*,*50*). Another plausible mechanism is the increase in hepatic LDL receptors induced by atorvastatin leading to increase clearance not only of plasma LDL but also of VLDL remnant particles, and resulting in the reduction of both cholesterol and triglycerides levels (*51*). Furthermore, reduction of hepatic fatty acids levels, induced by long term treatment with atorvastatin, is responsible for reduction of hepatic triglycerides synthesis and secretion (*52*).

The Mechanism behind the HDL raising effects of statins is likely to involve gene transcription and phosphorylation of peroxisomal proliferating activator receptor-α (PPAR-α) (*31*).

In the current study, atorvastatin significantly reduced lipid peroxidation and increased the activities of glutathione peroxidase and catalase in hyperlipidemic hamsters. These effects could be correlated to the protective antioxidant effect, and/or the significant lipid lowering effect of atorvastatin. Furthermore, the reduction in lipid peroxides could be a direct consequence of the significant decrease in LDL-C which is more prone to oxidation (*43*). Statins have been shown in animal models to act as antioxidants by decreasing LDL oxidation (*53*) and to modulate oxidation of lipoproteins (*54)*, superoxide generation (*55*), and scavenger receptor expression (*56*). Moreover, the metabolites of atorvastatin were found to be potent antioxidant (*46*). Additionally, atorvastatin may provide protection from oxidative damage, induced by hyperlipidemia, indirectly *via* upregulating expression of the free radical scavenging enzyme catalase (*9*) which is concordant with the present results.

In the present study, treatment of hyperlipidemic hamsters with sodium molybdate resulted in a significant decrease in TC, Non HDL-C, and TG levels with a substantial increase in the serum HDL-C levels. On the other hand, sodium molybdate produced a significant decrease in lipid peroxidation and a marked increase in activities of glutathione peroxidase and catalase in plasma. The obtained results are concordant with those by Panneerselvam and Govindasamy (*18*) who found that, oral administration of molybdate for thirty days significantly reduced the levels of lipids like cholesterol, triglycerides, phospholipids and lipid peroxidation and simultaneously increased the activities of antioxidants like superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione (GSH) in diabetic rats. Similarly, normalization of lipid levels in STZ-diabetic rats by orally administered sodium molybdate was corroborated (*16*,*17*).

The alterations in lipids and antioxidant status of hyperlipidemic hamsters can be attributed to the increase in peroxidative damage of lipids induced by the free radicals. Accordingly, the suppression of lipid peroxidation may result from the anti-free radical activities of sodium molybdate. Sodium molybdate was previously reported to have the ability to increase the activities of antioxidant enzymes, and decrease the availability of lipid substrates by acting as a free radical scavenger (*57*). This effect occur through trapping of the free radicals by the cationic molybdate compound leading to reduction in lipid peroxidation and a lipid lowering effect (*18*). Furthermore, the lipid lowering action of sodium molybdate can be also explained through its insulin like action (*18*,*58)*. In this context, sodium molybdate was shown to inactivate glycogen synthase and increase glycolytic flux in rat hepatocytes (*59*) and to display synergistic stimulation of glucose uptake in rat adipocytes in the presence of H_2O_2 (*60*,*61*).

Catalase and glutathione peroxidase are enzymes which destroy the peroxides and play a significant role in providing antioxidant defense to an organism. Both are involved in the elimination of H_2O_2. The functions

of these enzymes are interconnected and a lowering of their activities results in the accumulation of lipid peroxides and increases oxidative stress in tissues. The observed increase in activities of these enzymes after administration of sodium molybdate can be attributed to the potent antioxidant property of molybdate (*57*). Molybdenum is present in the active site of various molybdoenzymes, including a variety of oxidases (*62*). Additionally, molybdates were reported to increase the activities of antioxidant enzymes like superoxide dismutase (*63*) as well as the activities of enzymes like glutathione peroxidase and catalase (*64*).

The current study showed that combined administration of sodium molybdate with the low dose of atorvastatin (40 mg/kg) produced a maximum lowering in blood lipids, compared with atorvastatin (100 mg/kg) dose, provided a better antioxidant picture, compared with each drug alone, and kept the integrity of the liver enzymes in the same time. The results indicated that the combined therapy is able to modify the hyperlipidemia apparently by improving the efficiency of the antioxidant defense system in hamsters.

5. Conclusion

Atorvastatin is a potent hypolipidemic drug, especially at high dose, it effectively reduces the elevated blood lipids (TC, Non HDL-C, TG), improves HDL-C, anti-atherogenic index and strengthen the antioxidant status of hyperlipidemic hamsters. However, these effects are accompanied by deterioration in liver function and elevation in transaminases.

Sodium molybdate prevents oxidation of lipids, significantly reduces hyperlipidemia, increases HDL-C, anti-atherogenic index, and protects antioxidant systems in experimental hyperlipidemia. Moreover, it did not affect the liver transaminases.

Combination of sodium molybdate with atorvastatin lead to a maximum reduction in plasma total cholesterol, triglycerides, Non-HDL cholesterol and a pronounced increase in HDL cholesterol levels apparently by improving the activity of the antioxidant defense system. Meanwhile, this combined therapy produces no deleterious effect on the liver enzymes. Accordingly, sodium molybdate may be a useful candidate for combined therapy with atorvastatin to maximize the lipid lowering effect and affording a good antioxidant status with simultaneous protection of the liver. This is the first experimental study, after searching in the literature, to investigate the benefits of combination of sodium molybdate with atorvastatin in hyperlipidemic models. The obtained results greatly recommend combination of both drugs in management of hyperlipidemia.

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Original Article

Effects of oral curcumin on indomethacin-induced small intestinal damage in the rat

Alessandro Menozzi[1,*], Cristina Pozzoli[2], Enzo Poli[2], Mario Martelli[3], Laura Martelli[3], Chiara Zullian[1], Simone Bertini[1]

[1] *Department of Animal Health, University of Parma, Parma, Italy;*
[2] *Department of Human Anatomy, Pharmacology and Forensic Medicine, University of Parma, Parma, Italy;*
[3] *Department of Chemical Sciences, University of Padova, Padova, Italy.*

ABSTRACT: Nonsteroidal anti-inflammatory drug (NSAID)-induced injury on gastrointestinal tract is well documented, and jejunal inflammation caused by indomethacin in rats is a broadly used experimental model of enteritis. We evaluated the effect of oral curcumin, a compound known to possess anti-inflammatory and anti-oxidant properties, on indomethacin-induced enteritis in the rat. Curcumin (50, 100, and 300 mg/kg) was given to rats by oral gavage 48, 24, and 1 h before enteritis was induced by intragastric administration of 20 mg/kg indomethacin. After 24 h, intestinal macroscopic lesions, myeloperoxidase activity and lipid peroxidation levels were assessed. Curcumin at the dose of 50 mg/kg was uneffective, while at the dose of 100 and 300 mg/kg significantly reduced macroscopic damage caused by indomethacin. By contrast, curcumin at all tested doses was unable to modify indomethacin-induced increases of myeloperoxidase and lipid peroxidation. Curcumin (100 and 300 mg/kg) significantly increased lipid peroxidation level in normal intestinal tissues of rats. Present data show that oral curcumin protects against macroscopic injury induced by indomethacin, leaving unaffected neutrophil infiltration and oxidative cell damage, thus suggesting that this beneficial effect is due to mechanisms not involving anti-inflammatory or anti-oxidant activities.

Keywords: Curcumin, indomethacin, rat, small intestine

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most used classes of drugs, despite their well documented tendency to induce gastroduodenal injury. It is now well established that NSAID use is associated with small intestine and colon damage and worsening of chronic intestinal inflammatory diseases such as ulcerative colitis (UC) or Crohn's disease (CD) (*1*,*2*). While prostaglandin (PG) depletion following cyclooxygenase (COX) inhibition is acknowledged as the crucial pathogenetic event of NSAID-induced gastric damage, the etiology of intestinal injury seems to be more complex, and other mechanisms are likely to be involved, such as mitochondrial dysfunction leading to increased mucosal permeability, luminal bacteria invasion of gut wall, neutrophil-induced oxidative damage and microvascular injury (*3*,*4*). However, a general consensus is gathered around the damaging effects of reactive oxygen species (ROS) released by activated leukocytes in the intestinal mucosa (*5-7*) leading to several studies about the therapeutic potential of antioxidant agents against NSAID-induced enteropathy.

Curcumin (diferuloylmethane) is a bioactive constituent of turmeric (*Curcuma longa*), which has been shown to possess many pharmacological properties, ranging from anti-inflammatory activity to anticancerogenic and antibacterial effects (*8-10*). In particular, curcumin was proven to be effective in protecting against oxidative stress by means of a direct scavenging action of ROS and by the activation of endogenous antioxidant enzymes like catalase, superoxide dismutase and glutathione transferase (*11*,*12*).

The aim of the present study was to evaluate the effects of curcumin on the small intestinal damage induced by acute administration of indomethacin in the rat. Indomethacin-induced enteritis is an experimental model widely used to test new treatments against NSAID-induced enteropathy, and for screening of novel drugs against inflammatory bowel disease (IBD), because of the histo-pathological and functional similarities between human CD and indomethacin jejunal lesions in rats (*13*).

**Address correspondence to:*
Dr. Alessandro Menozzi, Department of Animal Health, University of Parma, Via Del Taglio 10, 43100 Parma, Italy.
e-mail: alessandro.menozzi@unipr.it

2. Materials and Methods

2.1. *Animals*

Male Wistar rats (220-240 g) were purchased from Harlan-Italy (Milan, Italy). They were housed in a restricted access room with controlled temperature (23°C) and a light/dark (12 h:12 h) cycle, and allocated in wire mesh cages with a maximum of 4 subjects per cage. Food and water were provided ad *libitum*. The study was approved by the local Animal Ethic Committee of the Faculty of Medicine, University of Parma, Italy.

2.2. *Induction of intestinal damage*

Enteritis was induced in four groups (n = 6 for each) of unfasted rats, by means of a single intragastric administration of 20 mg/kg indomethacin, suspended in 1% carboxymethylcellulose (CMC), in a total volume of 5 mL/kg b.w. Three groups of indomethacin-treated rats were also pre-treated with curcumin by oral gavage at the dose of 50, 100 or 300 mg/kg. Curcumin was suspended in 5 mL/kg of 1% CMC and administered 48, 24, and 1 h before indomethacin. Control group received an equal volume of 1% CMC p.o. following the same protocol. In order to assess the effect on healthy intestine, separate groups of rats were given curcumin alone, at the same doses and times described above. Rats were sacrificed by cervical dislocation under ether anesthesia 24 h after indomethacin administration and the intestinal lesions were evaluated.

2.3. *Macroscopic evaluation of intestinal damage*

The small intestine was removed from each animal and the first 20 cm of the proximal region, starting from the pylorus, were discarded. The remaining portion of intestine was divided into 8-10 segments of about 5 cm length. Intestinal segments were opened along the antimesenteric border, gently rinsed of fecal contents, fixed on a slide and photographed for macroscopic evaluation of damage. Damaged area of each segment was calculated by means of a digital image analysis software (ImageJ, NIH) and summed per small intestine. The amount of intestinal injury was expressed as a percentage of damaged area over the total examined intestinal mucosa. The examiners were unaware of animal treatment.

2.4. *Myeloperoxidase activity*

Intestinal myeloperoxidase (MPO) activity was assumed as a quantitative index of mucosal inflammation and was measured according to a previously described method with minor modifications (*14*). Briefly, a 5 cm-long segment of jejunum from each rat was homogenized in 1 mL hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% in 50 mM phosphate buffer, pH 6.0) for each 50 mg tissue and centrifuged at 12,000 g for 15 min at 4°C. An aliquot of the supernatant (7 µL) from each sample was then added to 200 µL of a reaction mixture containing 0.167 mg/mL *O*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide in 50 mM phosphate buffer at pH 6.0. Changes in absorbance at 450 nm were measured using a microplate absorbance reader (Tecan Sunrise, Tecan Inc., Mannedorf, Switzerland). One unit of MPO was assumed as that degrading 1 µM hydrogen peroxide per minute at 25°C. Data were expressed as units of MPO per mg of tissue.

2.5. *Lipid peroxidation*

Lipid peroxidation was evaluated as an index of oxidative damage and was assessed by measuring thiobarbituric acid reactive substances in intestinal tissues, according to a previously described technique (*15*), with minor modifications. Samples of jejunum from treated rats were collected, homogenized in 0.15 M KCl (1 mL for 100 mg wet tissue) and centrifuged at 400 g for 10 min. Aliquots (0.5 mL) of supernatants were then mixed to 1 mL 0.6% thiobarbituric acid, 3 mL of 1% phosphoric acid and 83 µL of a 0.2% solution of 2,6-ditert-butyl-4-methylphenol in 95% ethanol. After heating at 85°C for 60 min, samples were then ice-cooled and centrifuged at 2,600 g for 15 min, and the absorbance of the supernatant was measured using a multiplate spectrophotometer (Tecan Sunrise, Tecan Inc., Mannedorf, Switzerland) at a wavelength of 530 nm. Results were expressed as µmoles of malondialdehyde (MDA) per mg tissue.

2.6. *Statistical analyses*

Results were expressed as means ± SEM from 6 rats. Differences among groups were evaluated by one-way analysis of variance, followed by Dunnett's test. A p value less than 0.05 was considered statistically significant. Calculations were performed by a commercial software (GraphPad Prism, ver.3.03, GraphPad Software Inc., San Diego, CA, USA).

2.7. *Drugs*

Drugs and reagents (indomethacin, curcumin and all the analytical chemicals) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All drugs were prepared immediately before use, as suspensions in 1% CMC, and administered by intragastric route in a volume of 5 mL/kg b.w.

3. Results

3.1. *Macroscopic damage*

Intragastric treatment with 20 mg/kg indomethacin resulted in a severe intestinal damage, characterized by hyperemia, segmental mucosal ulcerations extending along the mesenteric border of the jejunum and bowel thickening (Figure 1). The area of macroscopically visible damage induced by indomethacin extended for 8.96 ± 0.97% of the total examined intestinal area (Figure 2). Treatment with curcumin at the lowest dose (50 mg/kg) did not modify indomethacin-induced injury (Figure 2). By contrast, intestinal damaged area induced by indomethacin was significantly reduced by curcumin at 100 mg/kg (4.97 ± 0.84%; $p < 0.01$). The highest dose of curcumin employed (300 mg/kg) was able to ameliorate jejunal lesions (5.52 ± 0.78%; $p < 0.05$) even though the effect was not significantly different than that of the lower dose of 100 mg/kg (Figures 1 and 2). Curcumin alone left intestinal mucosa completely unaffected (data not shown).

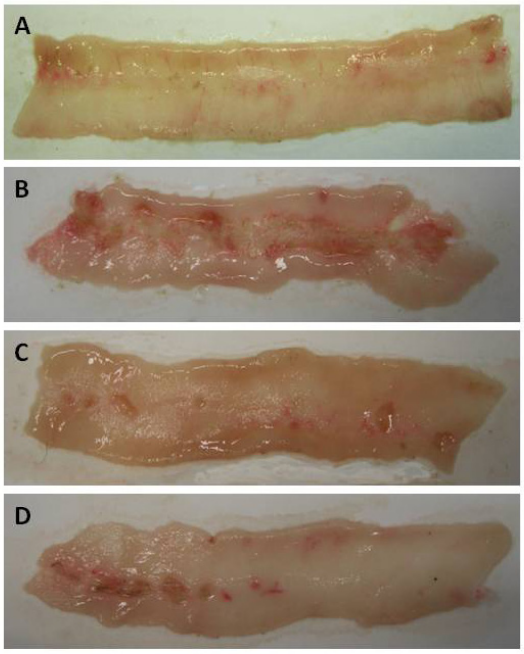

Figure 1. Macroscopic lesions of rat jejunum. A, normal intestine; **B**, indomethacin plus vehicle; **C**, indomethacin plus curcumin 100 mg/kg; **D**, indomethacin plus curcumin 300 mg/kg.

3.2. *Myeloperoxidase assay*

Indomethacin administration caused a more than three-fold increase in mucosal MPO activity (0.067 ± 0.01 U/mg; $p < 0.01$) compared to normal intestine (0.021 ± 0.002 U/mg) (Figure 3). Pre-treatment with curcumin at the dose of 50, 100 or 300 mg/kg did not affect MPO levels in a significant fashion with respect to indomethacin-treated rats (Figure 3). Curcumin at all tested doses did not modify MPO levels of normal intestinal tissues (data not shown).

3.3. *Lipid peroxidation*

Indomethacin administration enhanced lipid peroxidation in intestinal tissue, as demonstrated by the significant increase of MDA levels with respect to vehicle-treated rats (0.065 ± 0.02 *vs*. 0.029 ± 0.003 µmol/mg; $p < 0.01$). Curcumin administered at the dose of 100 mg/kg

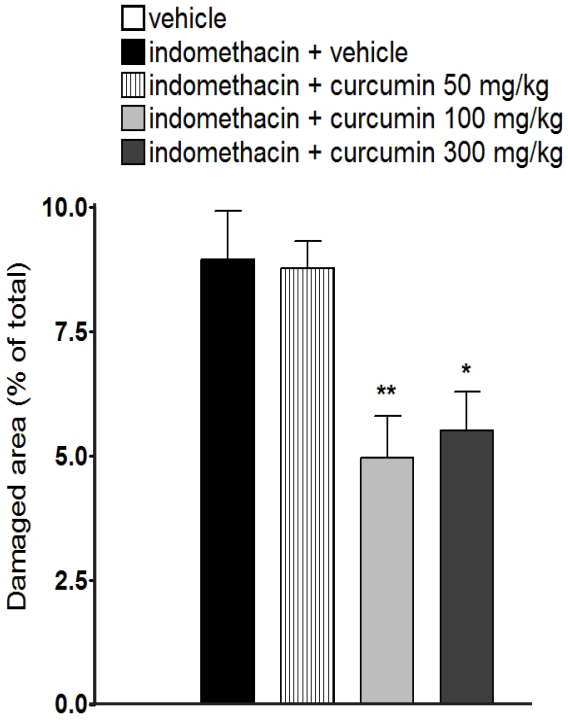

Figure 2. Effect of curcumin (50, 100, and 300 mg/kg) on macroscopic damage induced by indomethacin (20 mg/kg) in the small intestine of rats. Bars represent means ± SEM of 6 experiments. * $p < 0.05$ and ** $p < 0.01$ *vs*. indomethacin + vehicle.

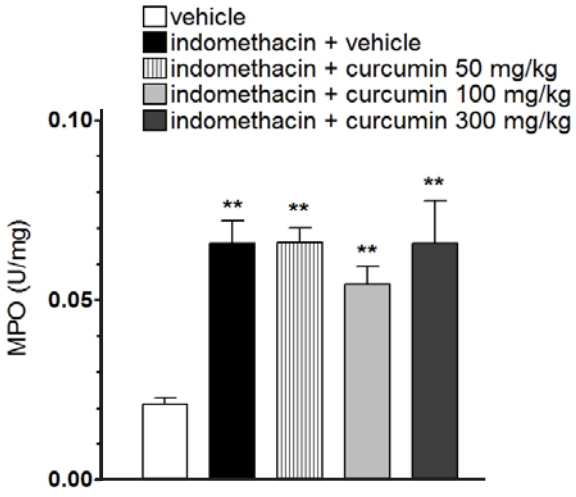

Figure 3. Effect of curcumin (50, 100, and 300 mg/kg) on myeloperoxidase (MPO) activity, as an index of neutrophil infiltration. Results are expressed as means ± SEM of 6 experiments. ** $p < 0.01$ *vs*. vehicle.

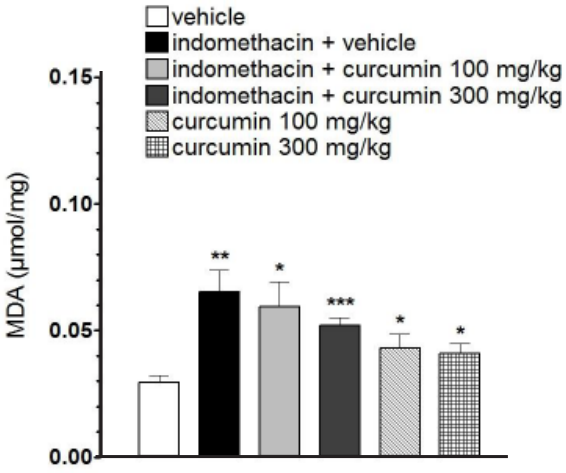

Figure 4. Effect of curcumin (100 and 300 mg/kg) on malondialdehyde (MDA) levels, as an assay of lipid peroxidation. Results are expressed as means ± SEM of 6 experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs*. vehicle.

and 300 mg/kg was devoid of significant effects on indomethacin-treated rats (Figure 4). Curcumin alone was able to significantly increase lipid peroxidation in healthy intestinal mucosa at both 100 and 300 mg/kg (0.043 ± 0.005; $p < 0.05$ and 0.041 ± 0.004; $p < 0.05$, respectively) (Figure 4).

4. Discussion

The purpose of this study was to evaluate the effects of oral curcumin on an experimental model of intestinal inflammation, the enteritis induced in rats by indomethacin administration. As in previous works (*3*,*5*), indomethacin induced a severe inflammation in the rat small intestine with hyperemia, erosions and linear ulcerations of the mucosa. Besides macroscopically visible damage, indomethacin caused a marked mucosal neutrophil infiltration and increased lipid peroxidation as evidenced by the enhanced MPO and MDA levels, respectively. Curcumin gavage at the dose of 50 mg/kg was unable to modify the area of damage, while at 100 or 300 mg/kg resulted effective in reducing the extension of macroscopic lesions, showing a dose-independent protecting effect. By contrast, all tested doses of curcumin were uneffective on indomethacin-induced increases of MPO activity or MDA levels. Interestingly, curcumin given alone, caused a significant increase of intestinal lipid peroxidation in normal rats both at 100 and 300 mg/kg, while leaving MPO activity unaffected.

Several authors have already demonstrated the efficacy of this compound against damage induced by various stimuli both in the stomach (*16*,*17*) and in the intestine (*18-20*) of experimental animals and there seems to be general consensus that the antioxidant properties of curcumin play an important role in its protective effects (*9-12*). Indomethacin-induced gastric damage was reduced by curcumin in rats and this beneficial effect was linked to the scavenging of ROS exerted by the drug and to a protective activity on endogenous antioxidant enzymes (*21*). Moreover, in previous studies, intestinal damage caused by indomethacin in rat small intestine was ameliorated by intraperitoneal administration of curcumin, which reduced MDA levels and restored catalase and glutathione peroxidase activity (*22*) or decreased oxidative stress in mithocondria (*23*). In our experiments, curcumin, administered by a different route, *i.e.* by oral gavage, was effective in reducing the extent of indomethacin-induced lesions, in accordance to data shown in the study by Sivalingam *et al*. (2007), even if the protective dose in our study was 5 times higher. This is not surprising, as it could be due to lower bioavailability by the intragastric route. Remarkably instead, oral curcumin failed to decrease neutrophil infiltration or lipid peroxidation in inflamed intestinal tissues of rats. Moreover, curcumin was able to increase MDA level in normal mucosa at both doses which were protective against indomethacin-induced macroscopic injury. Actually, curcumin has been shown to possess a dual behavior of anti-oxidant/pro-oxidant, being able to reduce the formation of ROS as well as to increase it, and to promote cell apoptosis by stimulating the production of oxidative radicals (*24-26*). The enhancement of lipid peroxidation in healthy jejunum of rats induced by curcumin could therefore explain the lack of efficacy of this drug on MDA levels in inflamed intestine, and suggest that ROS concentrations are not crucial in the protective effect exerted by curcumin on indomethacin-induced enteritis.

However, pathogenesis of NSAID-induced intestinal damage is unanimously considered multifactorial and several mechanisms other than peroxidation of membrane lipids by means of neutrophil-derived oxidative radicals are involved. Microvascular injury, due to the lack of vasodilation by PGs and to occlusions following the accumulation of inflammatory cells in the lumen of microvessels, which is thought to be fundamental for the developing of NSAID-induced lesions in the stomach (*27*,*28*), plays a role in the etiology of intestinal damage as well (*5*). The importance of vasodilation in the protection of gastrointestinal mucosa against the noxious effects of NSAIDs is demonstrated by the lack of lesivity of nitric oxide (NO)-donor NSAIDs (*29*) or by the protective effects of vasodilators on indomethacin-induced gastric and intestinal damage (*30*,*31*). We could therefore hypothesize that the beneficial effect of curcumin shown by our study was possibly due to a vasodilator activity and, by literature data, this compound was indeed shown to possess relaxant properties on isolated rat aorta and on porcine coronary arteria (*32*,*33*). Moreover, a previous study has shown that curcumin is effective in ameliorating dinitrobenzene sulfonic acid

(DNBS)-induced colitis in mice by acting as an agonist on transient potential vanilloid receptor 1 (TRPV1) (*34*) and could therefore induce the release of vasorelaxant mediators such as substance P, calcitonine gene – related peptide (CGRP) or NO (*35*) from sensory nerve terminals. There is also evidence that NSAIDs are able to block TRPV1 receptors (*36*) and this might contribute to the antiinflammatory and analgesic effects, as well as to the damaging activity, since it was demonstrated that TRPV1 mediates protection against experimental colitis in mice and rats (*37*,*38*). We cannot therefore exclude that these mechanisms may also be involved in the protective effect exerted by curcumin against indomethacin-induced enteritis. On the other hand, the TRPV1-mediated release of vasoactive substances could cause an increase of vascular permeability and subsequent neutrophil infiltration of intestinal mucosa, thus explaining the lack of efficacy of curcumin on MPO levels in our experimental conditions.

In conclusion, this study demonstrated that curcumin, given orally, is able to protect against acute macroscopic injury caused by indomethacin in rat small intestine and that this effect is not likely involving antioxidant properties. Further experiments, however, will be necessary to elucidate the mechanisms underlying the beneficial activity exerted by this drug on NSAID-induced enteritis.

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Original Article

Gaps in the information shared on consumer healthcare products

Makiko Hayashi[1], Shintaro Sengoku[2], Sakuo Hoshi[1,*], Hiromichi Kimura[1]

[1] *Pharmaco-Business Innovation Laboratory, Graduate School of Pharmaceutical Science, The University of Tokyo, Tokyo, Japan;*
[2] *Innovation Management Science Laboratory, The University of Kyoto, Kyoto, Japan.*

ABSTRACT: We conducted a questionnaire survey of visitors to the Japan Drugstore Show 2006 and an additional questionnaire survey of pharmacists in 2008 to ascertain the current information gaps between consumers and manufacturers of consumer healthcare products (CHPs). Three main gaps were apparent: first was a gap between information that consumers wanted to receive and information that was widely disclosed by manufacturers of CHPs, second was a gap between the advisors whom consumers regarded as appropriate and the advisors who consumers had actually consulted, and a gap between what consumers expect pharmacists to know and pharmacists' actual knowledge. Manufacturers' efforts alone will not be able to close these gaps because of the number of regulations. Thus, a new social system should be constructed to supply adequate information on CHPs and consumers should enjoy free access to this information.

Keywords: Consumer healthcare product, information sharing, gap

1. Introduction

Recently, more Japanese have become conscious of their health. The rapid aging of Japanese society unlike that in any other country may be a driving force behind these health concerns. A new health examination focusing on metabolic syndrome that started in April 2008 may also affect concerns about health. As a result, the market for health-related products has grown rapidly. The Ministry of Economy, Trade and Industry (METI) has estimated that the market for consumer healthcare products (CHPs) had reached 1.3 trillion yen in 2000 and will expand to about 3.2 trillion yen in 2010 (*1*). CHPs have now become a popular part of daily life. However, such a rapid growth in CHPs also causes trouble for consumers. Some CHPs have evidence indicating their efficacy at improving health, but others have dubious efficacy, safety, and quality. The confusion over foods for specific health uses, *i.e.* "health foods," may be one factor for this trouble. That is, foods for specific health uses are regulated by the METI, while "health foods" (Table 1) are simply "food," so permission, approval, and notification for their labeling claims are not regulated by the METI. Therefore, each consumer must obtain information on "health foods" and evaluate their efficacy, safety, and quality individually. That said, only a few consumers appear able to evaluate "health foods" correctly on the basis of adequate information. Most consumers seem to be influenced just by information deluging them without being able to evaluate those foods.

The Internet now allows free, instant access to news sources around the world with regard to CHPs. That said, some information seems to have dubious reliability and to contain exaggerated advertisements. Such information may cause consumer misunderstanding with regard to the efficacy, safety, and quality of CHPs. The following are examples of confusion-causing statements: "Some diseases can be treated by some CHPs," "The more we take CHPs, the more effective CHPs are," "CHPs cannot be harmful to humans because they are health food," and "All CHPs are officially guaranteed to be safe because they are marketed as health foods." Such statements are why some CHPs actually cause problems with consumer health.

Under these circumstances, the Ministry of Health, Labour and Welfare (MHLW) enhanced the regulatory and guidance systems for CHPs (*2*-*7*). In 2002, the MHLW issued a notice to promote the training of advisory personnel with special knowledge about CHPs (*8*). In the same year, the National Institute of Health and Nutrition, an "independent policy corporation," established a public qualification system for Nutrition Representatives. The MHLW has also reviewed systems for regulating CHPs since 2003 and established guidelines to manage the safety of CHPs in tablets or capsules in 2005 (*7*). These guidelines are expected to improve CHPs manufacturers' risk management and to

**Address correspondence to:*
Dr. Sakuo Hoshi, Pharmaco-Business Innovation Laboratory, Graduate School of Pharmaceutical Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
e-mail: shoshi@mol.f.u-tokyo.ac.jp

Table 1. Category of consumer healthcare products

Drugs	Foods			
	Foods with Health Claims			General Foods
Drug Quasi-drug	Functional Nutritional Foods	Foods for Specific Health Uses	"Health Foods"	Other
	Consumer Healthcare Product (CHP)			

A "consumer healthcare product (CHP)" is defined as shown in Table 1 (shown in gray). CHPs include foods with health claims, which include functional nutritional foods and foods for specific health uses, as well as "health foods." Foods for specific health uses are regulated " by the MHLW, and functional nutritional foods are officially defined. That said, the words "consumer healthcare product," "health food," and" health foods" are not officially defined.

perhaps also improve consumers' awareness of the risks of CHPs. That said, the rapid growth and expansion of the CHP market of and its rich variety of products, including foreign imports, mean that there should be limitations to regulate the wide variety of CHPs in accordance with the same guidelines.

Thus, the current study surveyed consumers to ascertain problems with CHPs. A questionnaire was distributed at the Japan Drugstore Show 2006, and several gaps in information sharing with regard to CHPs became apparent. An additional questionnaire was distributed among pharmacists in 2008. Another gap in the information sharing with regard to CHPs also became apparent. The results of the surveys are reported here, and a new way to eliminate gaps in information sharing with regard to CHPs is proposed.

2. Materials and Methods

The Japan Drugstore Show 2006 was held on February 11, 2006 at Makuhari in Chiba Prefecture. A questionnaire was distributed to the visitors of the Japan Drugstore Show. The completed survey was collected on the same day. Visitors were asked about (*i*) their individual attributes (gender, age, and occupation), (*ii*) their purchase histories with regard to CHPs, (*iii*) their motives for purchasing CHPs, (*iv*) the route by which they purchased CHPs, and (*v*) how they obtained information about CHPs. As a result of that survey, an additional questionnaire was distributed to new pharmacists at Ain Pharmacies, Ltd. on May 12, 2008. The completed survey was collected on the same day. This survey focused on the pharmacists' purchase histories and knowledge of CHPs. Table 1 shows the categories of CHPs.

3. Results

A total of 515 questionnaries was distributed to the visitors of the Japan Drugstore Show held on February 11, 2006 and a total of 501 responses was received, indicating a response rate of 97.3%. Of the 501 respondents, 134 were men (26.7%) and 367 were women (73.3%); 15 were teens (over 15 years old) (3.0%), 82 were in their twenties (16.4%), 156 were in their thirties (31.1%), 101 were in their forties (20.2%), 72 were in their fifties (14.4%), and 75 were over sixty (14.9%). Most respondents were housewives (36.9%). Age and occupation distributions are shown in Figure 1.

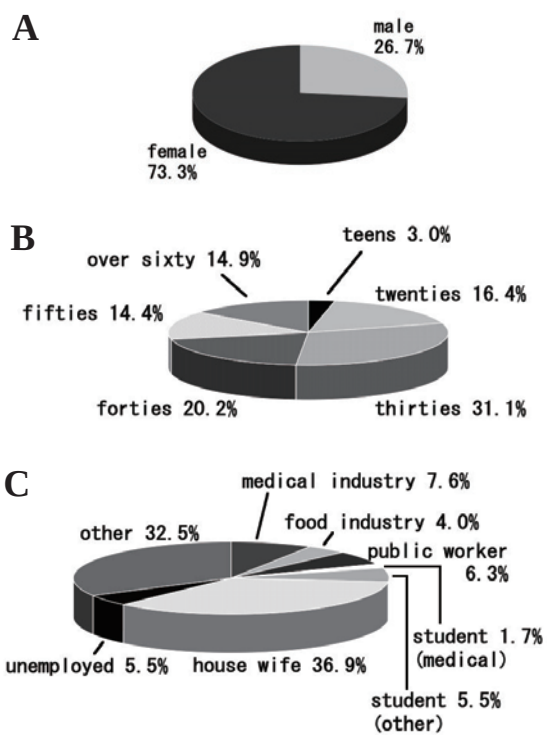

Figure 1. Attributes of respondents. (A) Gender of respondents, (B) Age of respondents, (C) Occupation of respondents. $n = 501$.

A total of 28.1% of respondents bought certain CHPs regularly, a total of 17.1% of respondents bought certain CHPs irregularly, a total of 21.5% of respondents bought different CHPs, and a total of 33.3% of respondents did not buy any CHPs (Figure 2). The age and occupation distributions were similar for each group (data not shown). Most people bought CHPs at drugstores and mainly received information about CHPs from drugstores, TV, and the Internet. Manufacturers or manufacturers' web sites were seldom used as sources of information on CHPs (Figure 3).

People mainly consulted with medical doctors, dentists, pharmacists, and other users. They wanted

to consult with advisory personnel though seldom did so (Figure 4). People obtained information on CHPs mainly in regard to the efficacy and safety of specific products or specific components or about the reputations of specific products or specific components (Figure 5). People who bought certain CHPs regularly or irregularly tended to obtain more information about efficacy than people who bought different CHPs or who did not buy CHPs (data not shown).

A total of 158 questionnaries was additionally distributed to new pharmacists at Ain Pharmacies, Ltd. on May 12, 2008. Of the 158 respondents, 77 were men (48.7%) and 81 were women (51.3%); 153 were in their twenties (96.8%) and 5 were in their thirties (3.2%). A total of 5.2% of respondents bought certain CHPs regularly, a total of 22.4% of respondents bought certain CHPs irregularly, a total of 39.7% of respondents bought different CHPs, and a total of 32.7% of respondents did not buy any CHPs (Figure 6). Though 96.2% of those pharmacists knew the names of CHPs, only 38.0% of the pharmacists could explain the categories of CHPs (data not shown), and only 10.9% indicated that they could explain the effects of CHPs (Figure 7). Those who were able to explain about CHPs obtained their knowledge mainly from lectures at university, followed by TV or magazines (data not shown).

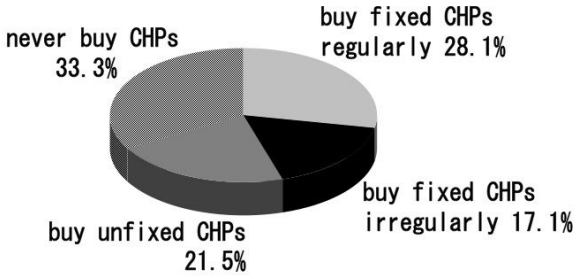

Figure 2. Purchase histories with regard to CHPs. *n* = 501.

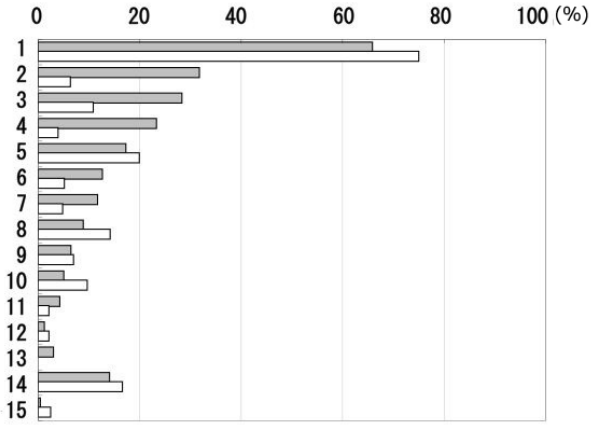

Figure 3. Means of buying CHPs and means of obtaining information on CHPs. ■, Buying CHPs (*n* = 324); □, Obtaining information on CHPs (*n* = 496). 1, drugstore; 2, television; 3, Internet; 4, magazine; 5, catalog; 6, hospital/clinic; 7, newspaper; 8, pharmacy; 9, convenience store; 10, speciality store; 11, web site; 12, door-to-door sales; 13, did not receive information; 14, other; 15, no answer.

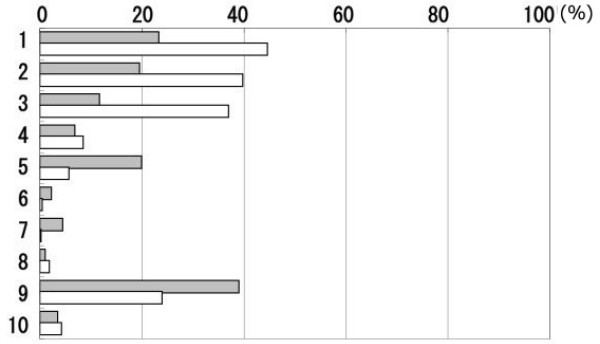

Figure 4. Advisors with regard to CHPs. ■, Advisors whom respondents actually consulted (*n* = 481); □, Advisors whom respondents regarded as appropriate (*n* = 477). 1, medical doctor or dentist; 2, pharmacist; 3, advisory personnel; 4, customer service; 5, other user; 6, door-to-door salesperson; 7, entertainer; 8, researcher; 9, other; 10, no answer.

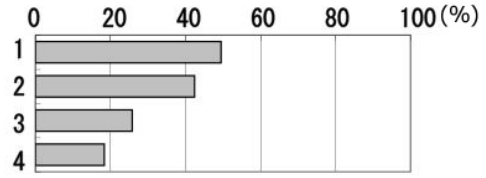

Figure 5. Information that respondents received about CHPs. *n* = 498. 1, efficacy and safety of specific products; 2, efficacy and safety of specific components; 3, reputations of specific products; 4, reputations of specific components.

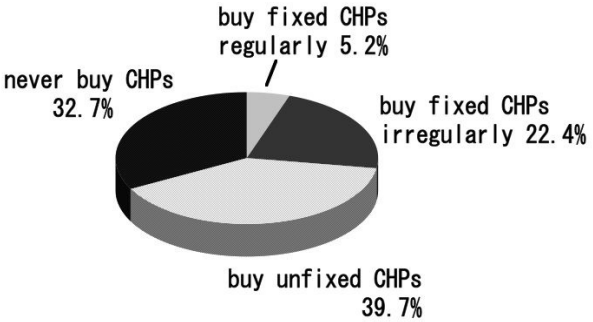

Figure 6. Purchase histories with regard to CHPs (An additional questionnaire survey of pharmacists). *n* = 158.

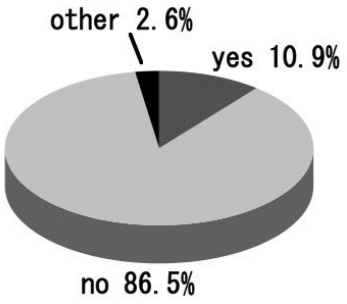

Figure 7. Knowledge of the health effects of CHPs (An additional questionnaire survey of pharmacists). *n* = 158.

4. Discussion

In Japan, an increase in health consciousness is thought to have resulted in a rapid expansion of the market for CHPs, though the market is reaching maturity. That said, the market will expand again as a result of a new mandatory health checkup system (health checkup/ health guidance) focusing on metabolic syndrome that was introduced in April 2008. Of course, manufacturers' effort have also contributed to the expansion of the market for CHPs. For example, Calpis Co., Ltd. has spent ten years evaluating the efficacy and safety of Ameal S®, a food for specific health uses (*9*). Otsuka Pharmaceutical Co., Ltd. chartered an airplane and conducted simulations on a long-distance international flight to evaluate the health effects of Pocari Sweat® in terms of preventing a traveler's thrombosis (economy class syndrome) (*9,10*). Such manufacturers' efforts should contribute to the prevalence of CHPs and their sound use. That said, there are also a number of reported cases where CHPs caused harm (*11*).

Many questionnaires have surveyed consumers about their knowledge and use of CHPs (*12-15*). To the extent known, however, no questionnaires have surveyed consumers about their awareness of CHPs to investigate gaps between consumers and manufacturers. The current study conducted questionnaire surveys regarding CHPs to specifically investigate information gaps between consumers and manufacturers. In this study, CHPs were used under the broad categories shown in Table 1. General foods, drugs, and quasi-drugs were excluded from consideration. This study did not separate CHPs according to the categories shown in Table 1 but considered them as a whole. This was because consumers were considered to be generally unaware of the categories of CHPs themselves. That view was supported by the results of an additional questionnaire for pharmacists. Less than 40% of the pharmacists knew the categories of CHPs (data not shown). Consumer unawareness of the categories of CHPs may also cause gaps in information sharing with regard to CHPs, so a survey separating CHPs by category is planned for the near future.

The current study showed that respondent attributes included different age groups and various occupations, but females, respondents in their thirties, and housewives were dominant (Figure 1). In the actual market, slightly more females buy supplements than males (*16,17*). Thus, the current study may somewhat highlight the opinions of females. The data showed that about 70% of consumers bought CHPs and about 30% of consumers bought certain CHPs regularly (Figure 2). Consumers bought CHPs mainly at drugstores and obtained information about CHPs mainly at drugstores or from TV and the Internet. Manufacturers' web sites, which were thought to be rich in information, were the least used source (Figure 3). In this study, consumers were more interested in the "efficacy and safety" of "each CHP" while they were less interested in the "reputations" of "each component of a CHP" (Figure 5). With "health foods", however, manufacturers are restricted in terms of labeling or advertising of health claims by the Pharmaceutical Affairs Law. Only on functional nutritional foods and specific health uses are manufacturers permitted to label or advertise nutrition claims. In short, manufacturers of CHPs are usually unable to provide consumers with information on the health effects of their products. This is why few consumers obtain information directly from manufacturers (Figure 3). Obviously, however, manufacturers of CHPs have the most information on those products. Thus, one can conclude that is a gap between information that consumers want and information that manufacturers can provide. Needless to say, regulations should exist, but manufacturers should be better utilized as resources.

Medical doctors or dentists, pharmacists, and advisory personnel were advisors for CHPs who consumers felt were appropriate for consultation. Of these individuals, advisory personnel should be most appropriate since they have received specific training in CHPs, but advisory personnel were actually consulted the least (Figure 4). Given the great variety of CHPs on the market and the huge amount of information on CHPs deluging consumers, consumers should take advantage of advisory personnel since they are well versed in CHPs. They know, for instance, what each component of a CHP does and its effectiveness and how to use the CHPs and they can provide consumers with correct and adequate information on CHPs. The MHLW is promoting the training of advisory personnel (*8*), and Japan now has more than ten training systems for advisory personnel, national training systems, public training systems, and private training systems. The results of the current study, however, indicate that consumers consult medical doctors or pharmacists rather than advisory personnel. That may be because advisory personnel are not qualified and not well known. Thus, there is a gap between the advisors that consumers actually consult and the advisors that consumers consider appropriate.

An additional survey showed that about 70% of pharmacists bought some CHPs, which was at almost the same rate as general consumers. Fewer pharmacists regularly bought certain CHPs, *i.e.* 5.2% of pharmacists bought certain CHPs regularly while 28.1% of general consumers did (Figures 2 and 6). Moreover, pharmacists were considered an appropriate source of information by consumers, but in reality only 38.0% of pharmacists knew the categories of CHPs (data not shown) and only 10.9% were able to explain the health effects of CHPs (Figures 4 and 7). Thus, there is a gap between what consumers expect pharmacists to know and pharmacists' actual knowledge.

In conclusion, three gaps were identified. First was the gap between information that consumers want and information that manufacturers can provide, second was the gap between the advisors that consumers actually consulted and the advisors that consumers considered appropriate, and third was the gap between what consumers expected pharmacists to know and pharmacists' actual knowledge. Several steps could be taken to close these gaps. Lectures on CHPs should be included in the curricula of medical and pharmaceutical courses more often and incentives to study CHPs should be given. Qualifications for advisory personnel should be established and standardized. If possible, advisory personnel should be obligatory at drug stores. Qualified salespersons will be obligatory at drug stores, so education of those salespersons in CHPs should be an effective solution.

Needless to say, CHP advertisement should be regulated under the current law. That said, manufacturers may be intentionally flooding the Internet with questionable information or exaggerated advertisements. Moreover, many CHPs are now being imported officially and individually in today's borderless world. Voluntary management by manufacturers should have limitations. Finally, current consumer centers in Japan should allow consumers free access and inform them of what is correct and what is wrong with CHPs. All permitted information should be provided, regardless of whether there is evidence or not, pursuant to regulations for specialists at consumer centers. If consumer centers cannot fulfill this role, a center to provide comprehensive information on CHPs should be established. In addition to establishing an information provision system, consumers should be correctly informed of the categories of CHPs since 38.0% of pharmacists knew the categories of CHPs (data not shown). The newly established consumer affairs office should take the initiative in providing appropriate information on CHPs.

In the current study, only a domestic survey was performed. Of course, similar gaps in the information sharing with regard to CHPs are likely to exist in the United States. In the United States, however, foods are simply categorized as conventional foods and dietary supplements, so there are no such categories of CHPs as exist in Japan. Furthermore, forms of dietary supplements are restricted to pills, tablets, capsules, or liquids. Additionally, labels on dietary supplements are legally required (*18*). Thus, gaps in information sharing with regard to CHPs are assumed to be smaller in the United States than in Japan.

In the current study, the survey focused only on CHPs. There are, however, numerous complementary and alternative medicines (CAM) (*19*) to improve health. With a CAM, consumers and providers usually contact each other directly. Thus, gaps in information sharing with regard to CAM are assumed to be smaller than those with regard to CHPs. The authors intend to focus on gaps in information sharing with regard to CAM in the near future.

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Drug Discoveries & Therapeutics

Guide for Authors

1. Scope of Articles

Drug Discoveries & Therapeutics mainly publishes articles related to basic and clinical pharmaceutical research such as pharmaceutical and therapeutical chemistry, pharmacology, pharmacy, pharmacokinetics, industrial pharmacy, pharmaceutical manufacturing, pharmaceutical technology, drug delivery, toxicology, and traditional herb medicine. Studies on drug-related fields such as biology, biochemistry, physiology, microbiology, and immunology are also within the scope of this journal.

2. Submission Types

Original Articles should be reports new, significant, innovative, and original findings. An Article should contain the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, References, Figure legends, and Tables. There are no specific length restrictions for the overall manuscript or individual sections. However, we expect authors to present and discuss their findings concisely.

Brief Reports should be short and clear reports on new original findings and not exceed 4000 words with no more than two display items. *Drug Discoveries & Therapeutics* encourages younger researchers and doctors to report their research findings. Case reports are included in this category. A Brief Report contains the same sections as an Original Article, but Results and Discussion sections must be combined.

Reviews should include educational overviews for general researchers and doctors, and review articles for more specialized readers.

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Title page. The title page must include 1) the title of the paper, 2) name(s) and affiliation(s) of the author(s), 3) a statement indicating to whom correspondence and proofs should be sent along with a complete mailing address, telephone/fax numbers, and e-mail address, and 4) up to five key words or phrases.

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Abbreviations. All nonstandard abbreviations must be listed in alphabetical order, giving each abbreviation followed by its spelled-out version. Spell out the term upon first mention and follow it with the abbreviated form in parentheses. Thereafter, use the abbreviated form.

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Editorial Office
TSUIN-IKIZAKA 410,
2-17-5 Hongo, Bunkyo-ku,
Tokyo 113-0033, Japan

Tel: 03-5840-9697
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