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Policy Forum

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Systematic evidence-based clinical practice guidelines are ushering in a new stage of standardized management of hepatocellular carcinoma in Japan

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Summary **Since the European Association for the Study of the Liver published their guidelines for hepatocellular carcinoma HCC (EASL Guideline) in 2001, there have been many explorations of "transferring best current evidence into clinical decision-making" around the worldwide. Comparative analysis on current 17 characteristic guidelines for HCC indicated that evidence-based clinical practice guidelines for HCC are urgently needed and appropriate constructing approach is the factor most significantly influencing their implementation. The construction of evidence-based clinical practice guidelines for HCC in Japan made a good example of this practice. In accordance with evidence-based medicine (EBM), the first version of the J-HCC Guidelines was published in 2005, then revised in 2009, and the third version has just been published on October 15, 2013 with the incorporation of new evidence, which marks the construction of evidence-based clinical practice guidelines for HCC step into a systematic process in Japan. In order to make a more clear description on how to construct evidence-based clinical practice guidelines for HCC in Japan, the three versions of the J-HCC Guidelines were comparatively analyzed in this paper. Focus on methodology used to develop the updated version, the decision tree of 2013 J-HCC Guideline and its features were also revealed. It is expected that J-HCC Guidelines could be useful not only for Japanese physicians and patients in decision making at every clinical step, but also to benefit users internationally with the accumulated evidence and its interpretation in the guidelines.**

Keywords: Hepatocellular carcinoma (HCC), clinical guideline, evidence-based medicine (EBM), evaluation

1. Introduction

Eagerly anticipated by Japanese hepatologists and liver surgeons, an updated version of the evidence-based Japanese Hepatocellular Carcinoma (HCC) Guidelines (the J-HCC Guidelines, 2013 version, in Japanese, chaired by Professor Norihiro Kokudo) was published in Japan on October 15, 2013 (*1*). This is the second update since the first evidence-based clinical practice guidelines for HCC were published in Japan (chaired by Professor Masatoshi Makuuchi) in 2005. This event marks the construction of evidence-based clinical practice guidelines for HCC step into a systematic process, and is believed to push standardized management of HCC in Japan into a new stage.

With the development of evidence-based medicine (EBM), the concept of "transferring best current evidence into clinical decision-making" has garnered substantial attention worldwide (*2,3*). About how to get "best current evidence" to influence clinical decision-making, there have been many explorations through the approach of constructing evidence-based clinical practice guidelines for HCC (*4*). Globally, since the European Association for the Study of the Liver published their guidelines for HCC (Conclusions of the Barcelona-2000 EASL conference) in 2001 (*5*), many such guidelines have been published in order to promote standardized management

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of HCC to reduce incidence and mortality as well as to improve healthcare quality of patients.

Based on the selection criteria of credibility, influence, and whether the guidelines were multi-faceted, the current 17 characteristic guidelines for HCC – including guidelines established by American Association for the Study of Liver Disease (AASLD), National Comprehensive Cancer Network (NCCN), American College of Surgeons (ACS), Asian Pacific Association for the Study of the Liver (APASL), and Japan Society of Hepatology (JSH) – were selected (Table 1). Comparative analysis indicated that evidence-based clinical practice guidelines for HCC are urgently needed and appropriate constructing approach is the factor most significantly influencing their implementation (*22,23*). Of the 17 guidelines, 5 were formulated based on a systematic analysis of the literature that resulted in recommendations for the management of HCC supported by data while the remaining 12 were formulated through a consensus of experts that yielded recommendations for the management of HCC based on experience. While most guidelines were drafted by hepatologists, only 2 guidelines were the result of experts consisting of radiologists, statisticians, and other experts besides hepatologists. In terms of content, all 17 guidelines dealt with diagnosis and treatment, only 10 guidelines mentioned epidemiology, 8 mentioned prevention, 11 mentioned surveillance, and 1 mentioned follow-ups. In terms of evaluation measures, 8 guidelines had evidence categories and recommendation grades, 3 had dissemination evaluation and 2 had resource-based recommendations.

2. Characteristics of J-HCC Guidelines

According to EBM, clinical practice guidelines should be updated every 3-4 years with the incorporation of new evidence, and some guidelines for HCC have also been

Table 1. Characteristics of current 17 guidelines for HCC around the world

Areas /Year	Guidelines/Approach	Content	Evaluation measures	Draft by
America				
2005	AASLD Guideline/LA (*13*)	D&T+S	evidence categories and recommendation grades; dissemination evaluation	American Association for the Study of Liver Disease
2007	ACS Guideline/EC (*14*)	D&T	—	American College of Surgeons
2009	NCCN Guideline/EC (*15*)	D&T+E+S	consensus categories	National Comprehensive Cancer Network
2010	WGO Guideline/EC (*16*)	D&T+E+P+S	resource-based recommendations	World Gastroenterology Organisation
2010	NCI Guideline/EC (*17*)	D&T+E	—	United States National Cancer Institute
Asia				
2004	Korean Guideline/LA (*18*)	D&T	evidence categories and recommendation grades	Korean Liver Cancer Study Group and National Cancer Center
2005	J-HCC Guideline/LA† (*19*)	D&T+P+S	evidence categories and recommendation grades; dissemination evaluation draft; evaluation prior to publication	Japanese Ministry of Health, Labor, and Welfare
2006	SGA Guideline/LA (*20*)	D&T+E+P	evidence categories and recommendation grades	Saudi Gastroenterology Association
2007	JSH Guideline/EC (*21*)	D&T+S	question and answer analyser system	Japan Society of Hepatology
2009	AOS Guideline/EC (*22*)	D&T+P+S	evidence categories and recommendation grades; resource-based recommendations	Asian Oncology Summit 2009
2009	Chinese Guideline/EC (*23*)	D&T	—	Chinese Society of Liver Cancer, Chinese Society of Clinical Oncology, Chinese Society of Hepatology Liver Cancer Study Group
2010	APASL Guideline/EC† (*24*)	D&T+E+P+S	evidence categories and consensus grade	Asian-Pacific Association for the Study of the Liver
Europe				
2001	EASL Guideline/EC (*11*)	D&T+E+P+S	—	European Association for the Study of the Liver
2003	BSG Guideline/LA (*25*)	D&T+E+S	evidence categories and recommendation grades	British Society of Gastroenterology
2004	BASL Guideline/EC (*26*)	D&T+E+P+S	—	Belgian Association for the Study of the Liver
2008	ESMO Guideline/EC (*27*)	D&T+E+P+S+F	dissemination evaluation	European Society for Medical Oncology
2009	GOIM Guideline/EC (*28*)	D&T+E	—	Italian Southern Oncological Group

LA, literature analysis; EC, expert consensus; † Experts consist of radiologists, statisticians, and other experts besides hepatologists; the others were drafted by hepatologists; E, epidemiology; P, prevention; S, surveillance; D&T, diagnosis and treatment; E, epidemiology; P, prevention; S, surveillance; F, follow-up.

updated, such as the revised version of NCCN Guideline (*24*) published on 2010, updated AASLD Guideline (*25*) and JSH Guideline (*26*) published on 2011. The J-HCC Guidelines are a good example of this practice. The first version of the J-HCC Guidelines was published in 2005, a second was published in 2009, and the third version has just been published in 2013. This marks the construction of evidence-based clinical practice guidelines for HCC step into a systematic process in Japan. The three versions of the J-HCC Guidelines were comparatively analyzed (Table 2). Doing so revealed the following characteristics of the J-HCC Guidelines.

2.1. *Involvement of a multi-disciplinary expert panel*

As mentioned earlier, only 2 of the 17 guidelines for HCC (the J-HCC Guidelines and guidelines drafted by Asian Pacific Association for the Study of the Liver) were drafted by experts consisting of radiologists, statisticians, and other experts besides hepatologists (*12,15*). With the support of the Japanese Ministry of Health, Labor, and Welfare, the 2005 version of the J-HCC Guidelines was compiled by an expert panel consisting of 5 surgeons, 4 internists, 3 radiologists, and 1 statistician who oversaw their own specialties. Most were executive board members of the Liver Cancer Study Group of Japan. A total of 26 experts in treating HCC also joined as members of a task force to help collect evidence and evaluate the guidelines (*27*).

The two updated versions of the J-HCC Guidelines better delineated tasks and included more experts: in addition to Executive Members consisting of surgeons,

Table 2. Comparative analysis of the formulation of three versions of the J-HCC Guidelines

Items	2005 version	2009 version	2013 version
Date published	February 28, 2005	November 24, 2009	October 15, 2013
Expert panel	Committees: 5 surgeons 4 internists 3 radiologists 1 statistician Co-members: 26 experts	Executive Members: 6 surgeons 4 internists 4 radiologists 1 statistician Advisory Members: 7 experts Co-members: 11 experts Paramedical Members: 1 nurse 1 clinical radiologist	Executive Members: 7 surgeons 5 internists 4 radiologists 1 statistician 1 expert in health care economics Advisory Members: 15 experts Co-members: 17 experts
Literature reviewed (dates)	7,192 articles (1966- Nov. 2002)	2,950 articles (Dec. 2002-June 2007)	6,750 articles (July 2007-Dec. 2011)
Second round of selection	334 articles	532 articles	596 articles
Evidence levels	General evidence categories (levels 1 to 6, high to low) Levels of evidence from articles on diagnostic examinations (levels 1 to 3, high to low) Sub-grading of evidence from articles on treatments (levels 1 to 7, high to low)		
Clinical questions (CQs)	58 CQs	51 CQs: 2 previous CQs 42 revised CQs 7 new CQs	57 CQ: 17 previous CQs 21 revised CQs 19 new CQs
Recommendation grades	Grade A: strongly recommended Grade B: recommended Grade C1: may be worth considering, but evidence is insufficient Grade C2: not recommended due to lack of evidence Grade D: recommended against		
General content	Prevention Diagnosis and Surveillance Surgery Chemotherapy TA(C)E Percutaneous local therapy	Prevention Diagnosis and surveillance Surgery Chemotherapy and radiotherapy TACE Local aspiration therapy	Prevention Diagnosis and surveillance Surgery Local aspiration therapy TACE Chemotherapy Radiotherapy Follow-up, prevention of recurrence, and treatment of recurrence
Systematic evaluation	Internal evaluation by 101 councilors of the Liver Cancer Study Group of Japan External evaluation by an external review board using the AGREE instrument, the Shaneyfelt instrument, and the COGS checklist a questionnaire survey was conducted to determine the level of awareness and impact of the Guidelines	Internal evaluation by Experts attending the 45th Conference of the Japan Society of Hepatology External evaluation online by members of the Japan Society of Hepatology and the public	Internal evaluation by Experts attending the 49th Conference of the Japan Society of Hepatology External evaluation online by members of the Japan Society of Hepatology and the public

internists, radiologists, and a statistician, the expert panel for the updated versions included Advisory Members (7 experts for the 2009 version, and 15 experts for the 2013 version) and Co-members (11 experts for the 2009 version, and 17 experts for the 2013 version) as executive partners.

Specially, for constructing 2009 version, 2 Paramedical Members (1 nurse and 1 clinical radiologist) newly joined the expert panel to review revisions overall and to offer their own perspectives; and in 2013 version, 1 health care economic expert newly joined the expert panel as an Executive Member to offer perspectives from that field.

2.2. *Evidence-based constructing approach*

In terms of their EBM methodology, the three versions of the J-HCC Guidelines were formulated based on a systematic review of literature mainly from MEDLINE and a second round of selection to evaluate sources. The approach used to search the literature has been disclosed and described in detail to ensure that evidence can be reproducibly collected.

For the 2005 version, 7,192 articles published from 1966 to November 2002 were initially selected; after the second round of selection, 334 articles were chosen (*28*). For the 2009 version, 2,950 articles published from December 2002 to June 2007 as well as articles chosen for the 2005 version were systematically reviewed; after the second round of selection, 532 articles were ultimately chosen (282 articles had previously been included in the 2005 version while 250 articles were new) (*29*). Similarly, revising of the J-HCC Guidelines (2009 version) began in September 2011. A total of 6,750 articles were initially selected, and 596 articles were ultimately chosen (245 articles had previously been included in the 2005 version and 2009 version while 351 articles were new). The second updated version was published in October 2013 (*1*).

2.3. *Revised grading criteria for evidence levels*

The general evidence categories for the systematic review of the literature were based on recommendations from the US Department of Health and Human Services. Evidence was divided into 6 levels (levels 1 to 6, high to low), with a level of 1 indicating a meta-analysis of randomized controlled studies and a level of 6 indicating personal opinions of specialists. These evidence levels were poorly suited to gauging a number of articles on diagnostic examinations, so the expert panel drafting the J-HCC Guidelines devised another set of evidence levels for articles on diagnostic examinations (levels 1 to 3, high to low). A level of 1 indicates a new diagnostic examination conducted concurrently with a gold-standard examination and evaluation of the characteristics of the examinations in a blinded fashion while a level of 3 indicates a new diagnostic examination by itself with no comparison.

Furthermore, evidence from articles on treatments was also sub-graded according to the number of patients, duration of the follow up, and the percentage of dropouts to help select articles for the second round of selection (levels 1 to 7, high to low). A level of 1 indicates at least 200 patients, a mean follow-up of at least 5 years, and a dropout rate below 10% while a level of 7 indicates a dropout rate of 10% or higher, regardless of the number of patients and duration of the follow-up.

Since the evidence levels as previously described were used to collect evidence for the first version of the J-HCC Guidelines, strict grading criteria have been included in the subsequent versions (2009 version and 2013 version) to ensure that articles are selected by each member of the expert panel in as uniform a manner as possible.

2.4. *Targeted clinical questions with recommendation grades*

In accordance with the revised grading criteria for evidence levels, the expert panel with its highly specialized knowledge was mobilized to pose targeted clinical questions (CQs) to cover a general overview for the management of HCC. As new evidence was incorporated, the targeted CQs were re-evaluated, deleted, combined, or created in the two updated versions of the J-HCC Guidelines. Accordingly, different grades of recommendation (grades A to D, from "strongly recommended" to "recommended against") were also assigned in accordance with the level of evidence.

For the 2005 version, the second round of selection yielded 334 articles. Based on these sources, 58 pairs of CQs and different grades of recommendation were devised (*30*). For the 2009 version, these 58 pairs of CQs and recommendations from 2005 version were re-evaluated and 532 articles were ultimately chosen. As a result, 51 pairs of CQs and recommendations were devised (*29*). Similarly, 596 articles were chosen for the 2013 version. The CQs and recommendations from the 2009 version were re-evaluated, and ultimately 57 pairs of CQs and different grades of recommendation were devised. These included 17 previous CQs from the 2009 version, 21 revised CQs, and 19 new CQs (*1*). With these CQs and recommendations, specialists can better understand the guidelines and make suitable clinical decisions for individual patients.

2.5. *Resource-based surveillance, diagnosis, and treatment algorithms*

In general, the J-HCC Guidelines cover 6 areas that include prevention, diagnosis and surveillance, surgery, chemotherapy, transcatheter arterial chemoembolization

(TACE), and local treatment. In addition, radiotherapy was added to the 2009 version and was described independently in the 2013 version. Content describing follow-up, prevention of recurrence, and treatment of recurrence was added to the 2013 version.

A main feature of the J-HCC Guidelines is the inclusion of algorithms for surveillance and diagnosis and for treatment of HCC for practical use (*27,29,31*). These algorithms were based on evidence from chosen articles and they were modified in accordance with the current status of medical practices in Japan: *i*) HCC is often detected in its early stages because high-risk patients are routinely followed by hepatologists; *ii*) liver resection to treat HCC is regarded as safe, with a mortality rate of less than 1%; *iii*) an indocyanine green (ICG) test is widely used as a precise liver function test; and *iv*) there is a dearth of cadaveric donors for liver transplantation.

Moreover, the resource-based algorithms for surveillance, diagnosis, and treatment of HCC also take the Japanese health insurance system into account: *i*) most of the costs of treating HCC as recommended by the J-HCC Guidelines are covered by universal health insurance in Japan, except for liver transplantation to treat HCC outside the Milan criteria; *ii*) the cost of tumor markers (monthly measurement of up to two different markers in high-risk patients is covered by all forms of Japanese health insurance, making the surveillance algorithm feasible); and *iii*) all methods of diagnostic imaging for HCC are also covered by Japanese health insurance.

However, the cost-effectiveness analysis for HCC screening and surveillance as well as the options of diagnostic tools and therapies have not yet been established. But for constructing 2013 version of J-HCC Guidelines, one expert specializing in health care economic newly joined the expert panel as Executive Member, and the concept of "cost-effectiveness analysis" has raised concerns, we expect the well cost-effectiveness analysis for standardized management of HCC will be created in the future version of J-HCC Guidelines.

2.6. *Systematic evaluation to promote implementation of the guidelines*

Internal evaluation The first draft of the J-HCC Guidelines (2005 version) was completed in June 2004. Prior to publication, the draft was sent to 101 councilors of the Liver Cancer Study Group of Japan to solicit their comments during a symposium on the guidelines held in June 2004. Similarly, the 2009 revision of the J-HCC Guidelines was evaluated by the 45th Conference of the Japan Society of Hepatology before publication, and the 2013 revision was evaluated by the 49th Conference of the Japan Society of Hepatology. Public comments from members of the Japan Society of Hepatology were obtained approximately one month after the conferences in 2009 and 2013.

External evaluation In November 2004, an external review board was formed to evaluate the validity of the J-HCC Guidelines (2005 version) and their potential for dissemination. The board consisted of two HCC specialists (a surgeon and an internist), two non-specialists familiar with other clinical guidelines, a medical statistician, and a patient who had undergone HCC surgery. After a thorough examination using the Appraisal of Guidelines for Research and Evaluation (AGREE) instrument, the Shaneyfelt instrument, and the Conference on Guidelines Standardization (COGS) checklist, the external review board gave the J-HCC Guidelines high marks (more than 80%) for clarity of subject, aims, structure, and recommendations (*27*). Differ from that, the 2009 and 2013 versions of the J-HCC Guidelines were evaluated by members of the Japan Society of Hepatology as well as by the public by posting of the guidelines online.

Implementation evaluation In March 2006, approximately a year after the publication of the J-HCC Guidelines (2005 version), a questionnaire survey of 2,279 members of the Liver Cancer Study Group of Japan as well as 689 primary care physicians in Osaka and Hyogo prefectures was conducted to determine the level of awareness and impact of the guidelines (*32*). Of the 1,175 respondents (39.6%), 71.9% of hepatologists, 75.6% of surgeons, and 61.0% of primary care physicians were aware of the J-HCC Guidelines, offering insight into the extent to which the J-HCC Guidelines had been implemented. However, the survey had a relatively low response rate (39.6%), so a survey of a larger sample with a higher response rate should be conducted in the future.

3. Features of the J-HCC Guidelines (2013 version)

In order to make a more clear description on how to construct evidence-based clinical practice guidelines for HCC in Japan, the decision tree of the 2013 J-HCC Guideline was revealed in Figure 1. Specially, focus on methodology used to develop that updated version and based on the comparative analysis of the formulation of three versions of the J-HCC Guidelines, the 2013 version of the J-HCC Guidelines features: *i*) involvement of a multi-disciplinary expert panel with better delineated tasks, inclusion of experts in health care economics to promote the concept of "cost-effectiveness analysis"; *ii*) a systematic review of the literature and search approach described in detail to ensure that evidence can be reproducibly collected; *iii*) consistent grading criteria for evidence levels to ensure that evidence is collected by each member of the expert panel in as uniform a manner as possible; *iv*) revising of targeted CQs with recommendation grades to help specialists better understand the guidelines and make appropriate clinical decisions for individual patients;

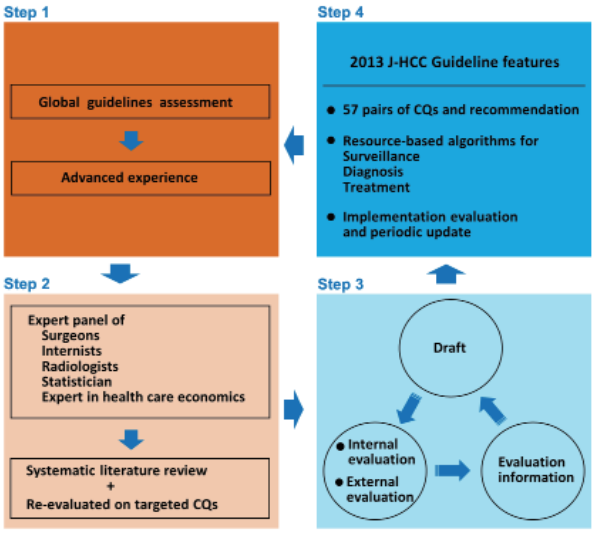

Figure 1. The decision tree for constructing 2013 version of J-HCC Guideline. CQs, clinical questions.

v) new content describing follow-up, prevention of recurrence, and treatment of recurrence to promote the systematic management of HCC; *vi*) resource-based algorithms for surveillance, diagnosis, and treatment of HCC that take the Japanese health insurance system into account; and *vii*) internal evaluation and external evaluation prior to publication to allow comments and modification of the draft guidelines.

The 2013 version of the J-HCC Guidelines is not without areas needing improvement. The 2013 version does not feature cost-effectiveness analysis of HCC screening and surveillance or options for diagnostic tools and therapies. Moreover, a survey of a larger sample with a higher response rate should be conducted in the future to determine the level of awareness and impact of the guidelines. Furthermore, the implementation evaluation should not only include the evaluation on awareness and influence, but also the evaluation on outcomes of adhering to J-HCC Guidelines for patients, as well as the effectiveness to promote health resources better allocation.

In conclusion, the construction of evidence-based clinical practice guidelines for HCC in Japan made a good example of translating "best current evidence" into clinical practice. Comparative analysis of the formulation of three versions of the J-HCC Guidelines indicated the construction of evidence-based clinical practice guidelines for HCC step into a systematic process, and is believed to push standardized management of HCC into a new stage in Japan. The systematic process of formulating evidence-based clinical practice guidelines for HCC has resulted in the present J-HCC Guidelines featuring the most precise treatment strategies for HCC that reflecting present practices in Japan. As such, the guidelines should be updated further and incorporate new evidence, especially that from cost-effectiveness analysis and evaluation of the guidelines' implementation. Furthermore, although the main users of the J-HCC Guidelines will most likely be Japanese physicians and patients, the accumulated evidence and interpretations of that evidence in the guidelines may also benefit users internationally.

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Review

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Daily hydroxyl radical scavenging capacity of mammals

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Summary **Both the formation and reactions of hydroxyl radical (•OH) are quantitative chemical reactions even in mammalians, and so we can reproduce such *in vivo* reactions in test tubes. Daily urinary excretions of some reaction products have been used to estimate the amount of •OH produced daily. Although urinary 8-hydroxydeoxyguanosine (8-OHdG) is a well-known marker of •OH, we have shown that creatol (CTL: 5-hydroxycreatinine), an •OH adduct of creatinine (Crn), and its metabolite, methylguanidine (MG), are better markers, because the amount of •OH scavenged by deoxyguanosine (dG) in the body is negligible. We measured CTL and MG together with Crn in 24-h urine, and calculated their molar sum, CTL + MG, providing a daily estimate of moles of •OH scavenged with Crn, and, from the molar ratio (CTL + MG)/Crn, we can calculate the percentage of Crn that was used to scavenge •OH. Healthy subjects and normal rats were indicated to use *circa* (ca.) 0.2 and 0.3% of Crn in order to scavenge •OH, respectively, because the corresponding ratios, scavenged •OH/Crn, were 2.2 and 3.0 mmole/mole (24-h urine) (Crn scavenged ca. 20-25 μmole and ca. 200 pmole of •OH in healthy subjects and normal rats, respectively). Since 8-OHdG/Crn has been reported to be 1.9 μmole/mole (24-h urine), the daily scavenging capacity with Crn is 10^3-fold more than dG. In patients with chronic renal failure (CRF) or chronic kidney disease (CKD) at stages 3-5: glomerular filtration rate (GFR) < 60 mL/min/1.73 m^2, •OH levels increased in proportion to the severity of CKD: up to ca. 3% of Crn was used daily in order to scavenge •OH. Although the accumulation of MG in organs has not been reported except for the brain and skin tissues in normal animals, •OH increases markedly and MG becomes detectable in all organs such as the kidney, liver, and heart in CRF rats.**

Keywords: Hydroxyl radical, creatinine, creatol, methylguanidine, 8-hydroxydeoxyguanosine

1. Introduction

Since creatinine (Crn) is one of main intrinsic hydroxyl radical (•OH) scavengers (*1*), we aimed to quantitatively show how much Crn scavenges •OH daily. Crn reacts with •OH to scavenge •OH and produce creatol (CTL: 5-hydroxycreatinine; •OH adduct to Crn, which partially decomposes to methylguanidine (MG) or demethylcreatinine (DMC)) (Figure 1A), and then they are excreted together with Crn into urine (*1-10*). Although we can detect *in vitro* creatones A and B as intermediates from CTL to MG (*11*), we do not introduce them herein, because they are not detectable *in vivo*. CTL and MG in serum and urine have been recognized as *in vivo* markers of •OH (*1-3,5-9*), although they were initially known as markers for chronic renal failure (CRF) (*12-14*). We can estimate their daily scavenging capacity for •OH using the urinary Crn-related metabolites of CTL, MG, and DMC. Since we know that urinary (CTL + MG) and DMC are roughly in a one to one ratio (*1,5*), we estimated the amount of DMC from the corresponding measured amount of CTL plus MG: the total sum might be nearly equal to 2 × (CTL + MG). Furthermore, we wanted to show in this mini-review that the well-known urinary level of 8-hydroxydeoxyguanosine (8-OHdG) (Figure 1B) (*15,16*) is not suitable as an *in*

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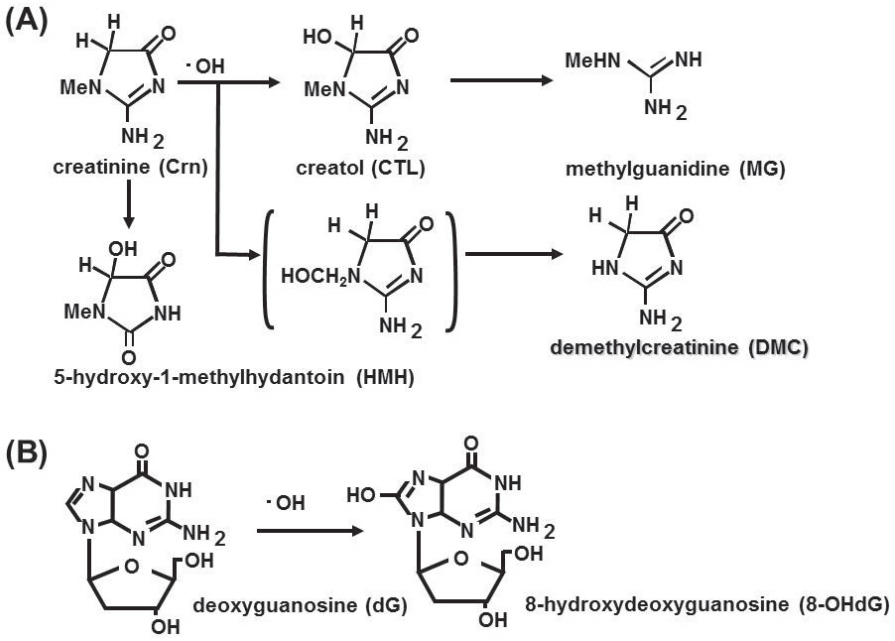

Figure 1. Metabolic pathways of Crn (A) and dG (B).

vivo marker of •OH in comparison with those of Crn-related markers, because their level is negligible (*17,18*). However, we wanted to cite papers reporting that 8-OHdG could be a useful marker of •OH inside the nucleus and mitochondria (*19*).

In order to show sites where •OH is detectable, we referred to reports on the distribution of MG in normal mammals (*1,20-22*). We distinguish, at the same time, organs where Crn is or is not distributed easily (*1,20,21*). We also illustrate that •OH accumulates in organs of mammals with CRF (*1,20,21*): chronic kidney disease (CKD) stages 3-5 (GFR: glomerular filtration rate: 60 mL/min/1.73 m^2) (*1,23*). We hope that this mini-review will clarify how much •OH might be produced daily, at the very least in mammals.

2. Detection of •OH

Because the •OH radical is so reactive, it is difficult to monitor directly. Therefore, an indirect monitoring method using a biomarker of •OH would be useful for patients with various diseases. In order to estimate the amount of •OH produced daily, the •OH-adducts and/or reactive products with •OH have to be measured as •OH biomarkers. Daily urinary excretions of such products have been used to estimate the amount of •OH produced daily. The molar ratio, (such metabolites)/Crn, in spot urine and 24-h urine has also been used in •OH-monitoring.

3. Daily amount of •OH scavenged by deoxyguanosine (dG) and Crn

We use •OH adducts of metabolites as markers of •OH.

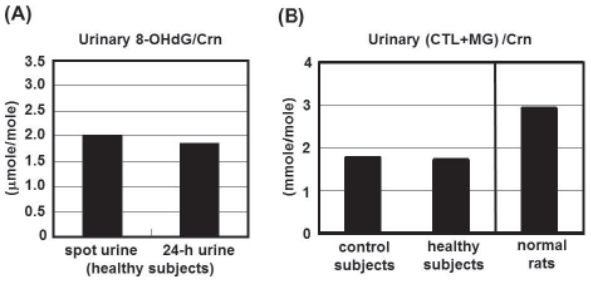

Figure 2. How much •OH is scavenged daily? Comparison of urinary levels of 8-OHdG/Crn (A) and (CTL + MG)/Crn (B).

One of the most frequently used biomarkers for •OH is 8-OHdG (*15-17*). However, their use should be limited, because only the amount of •OH formed inside nuclei and mitochondria can be shown (*1,17*). Furthermore, before the determination of 8-OHdG in urine, there are several reaction-steps from 8-OHdG-containing nucleotides. Therefore, we quantitatively compare the daily scavenged •OH in urine by dG with that by Crn. Since Crn is distributed throughout the whole body (*20*), its metabolites (especially CTL and MG) in urine can be monitored easily. In fact, the reported daily level of 8-OHdG excretion in urine of healthy subjects is fairly low; 8-OHdG/Crn: 2.03 ± 1.21 and 1.86 ± 1.09 μmole/mole (spot urine and 24-h urine, respectively: n = 67) (Figure 2A) (*17*); urinary 8-OHdG amount was also reported *circa* (ca.) 2.2 (1.7-2.8) and 6.05 (3.12-15.38) nmole/24 h, for control subjects (n = 85) and patients (n = 222), respectively (*18*). This means that dG scavenges 2-16 nmoles of •OH daily. Therefore, our estimation (see below) that the daily scavenged amount of •OH by Crn is ca. 50-500 μmoles in healthy subjects and severe CKD patients is very high. Daily

scavenging capacity with Crn might be at least 10^4-fold more than dG.

4. One of the best indices of *in vivo* •OH, the molar sum, CTL + MG, or its ratio to Crn, (CTL + MG)/Crn

Both the molar ratio of (CTL + MG)/Crn in 24-h urine and the urinary mole of (CTL + MG) against human subjects were reproducible to be 2.0 mmole/mole and ca. 20 μmole, respectively (Figure 2B) (*6,14*). Each one mole-detection of CTL or MG means that one mole of •OH, reacted with Crn, has been scavenged. Since both CTL and MG are directly detectable in the urine of mammals but not the serum of healthy individuals or normal mammals (*1,3,22*), we used urinary values.

Because Crn is distributed not only in nuclei and mitochondria but also in the cytosol and outside cells, the amounts of CTL and MG show how much •OH is scavenged by Crn therein. Theoretically, the molar sum, CTL + MG, and its ratio to Crn, (CTL + MG)/Crn, may be the best indices for the precise •OH level *in vivo*. We show that up to ca. 3% of Crn is used daily in order to scavenge •OH.

5. Increase in CTL and MG in patients with CKD

In patients with CKD at stages 3-5 (GFR < 60 mL/min/1.73 m^2), CTL and MG levels increase markedly (Figure 3) and •OH also increases in proportion to the severity of CKD. Both CTL and MG in serum and urine were initially indicated to be markers for CRF (*1,12-14*). However, both were later recognized to be markers of •OH (*1-3,5-9*).

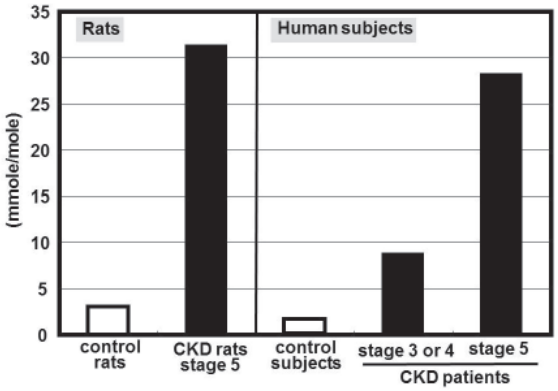

Figure 3. Amounts of •OH scavenged by Crn in mammals (rats and human subjects) in the presence of CRF.

6. Increase in •OH in patients with CKD

Healthy subjects and normal rats were indicated to use ca. 0.2 and 0.3% of Crn in order to scavenge •OH, respectively, because corresponding ratios, scavenged •OH/Crn, were 2.2 and 3.0 mmole/mole (24-h urine) (Crn scavenged ca. 25 μmole and ca. 200 pmole of •OH in healthy subjects and normal rats, respectively) (Figures 2 and 3). However, the production of •OH is increased in proportion to the severity of CKD, as shown in Figure 3. Because CTL/Crn and MG/Crn (mole/mole), for CKD patients in comparison with control subjects (eGFR > 60 mL/min/1.73 m^2), had been reported (*1*), we calculated their sum, (CTL + MG)/Crn (Figures 2 and 3), after CKD patients were classified into corresponding stages (Table 1).

Since MG levels in 48-h urine together with measured GFR values with time following adenine loading of rats had been reported (*22*), we assigned a stage of CKD for each sample and then mole of MG and the molar ratio of MG/Crn, and illustrated these in the previous review (*1*). From the molar ratio X (mmole/mmole), (CTL + MG)/Crn (Figure 3), the percentage of Crn used to scavenge •OH could be calculated as 10X %.

7. Prediction of sites where Crn scavenges •OH in mammals

Based on the reported Crn levels in rat organs (Figure 4) (*7*), and the reported MG levels in rat organs induced by Crn injection (*21,24*) and autoradiogram ^{14}C-Crn (*20*), sites and organs where Crn scavenges •OH in mammals was presumed (Figure 5). One-way flow of Crn from muscles and the brain, where Crn is synthesized and its concentration remains at a high level, into blood vessels was observed. In contrast, although Crn could be detectable in other organs (*21*) such as the kidney, liver, and heart, where it could not be synthesized, both in- and out-flows of Crn were observed. Sites where •OH is reacted with Crn to be scavenged, are ones

Table 1. Stages of CKD in mammals

Stage	Description*	Clinical GFR*, ** (mL/min/1.73 m^2)	Relative GFR (GFR/GFR_0)	Rat GFR******* (mL/min/kg)
1	Kidney damage with normal or GFR↑	> 90		15-17
2	Kidney damage with mild GFR↓	60-89		18-21
3	Moderate GFR↓	30-59	0.30-0.59	0.17-0.33
4	Severe GFR↓	15-29	0.15-0.29	0.08-0.16
5	Kidney failure	< 15 (or dialysis)	< 0.15	< 0.08

* The National Kidney Foundation K/DQI Clinical practice guidelines on CKD (2002).
** Clinical GFR_0, GFR of normal subjects, has been reported to be about 100 mL/min/1.73 m^2.
*** Rat GFR_0, GFR of normal rats, has been reported to be about ca. 0.55 mL/min/kg.
**** We classified CKD stages of rats based on rat GFR (Ienaga & Yokozawa, 2010).

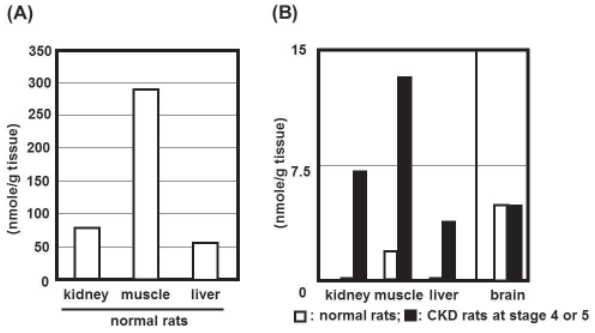

Figure 4. Crn and its •OH-product, MG, in organs of normal rats (A) and rats with CKD at stage 4 or 5 (B).

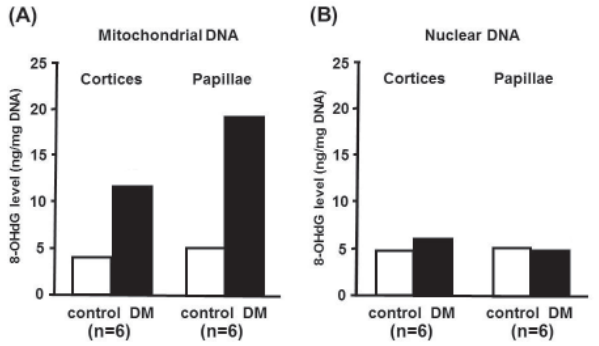

Figure 6. Changes in •OH inside mitochondria (A) and/or the nucleus (B) can be shown as changes in 8-OHdG levels.

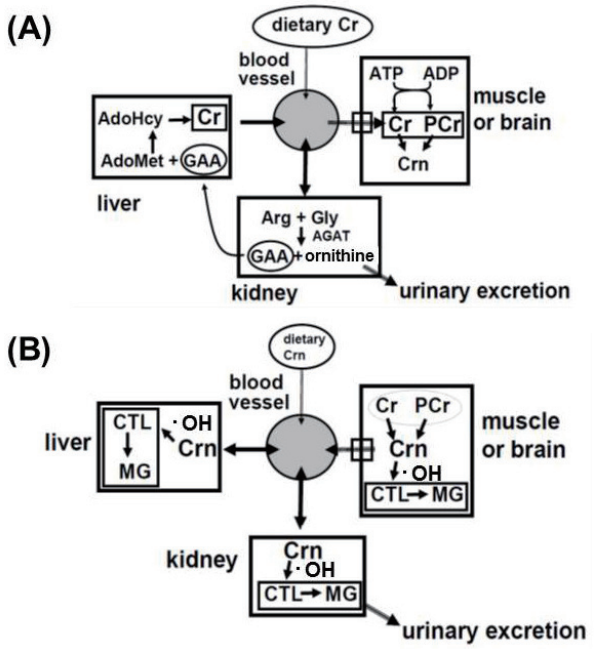

Figure 5. Flow of creatine (Cr) (A) and Crn (B) in mammals and organs where •OH is detectable.

where CTL, MG *etc*. are detected. In normal rats, only muscles and the brain had detectable levels of CTL and MG. However, in all organs in rats with CKD at stage 4 or 5, CTL and MG could be detected (Figure 4) (*21*).

8. Merits of measurement of 8-OHdG compared with Crn-related markers such as CTL + MG/Crn in mammals

If we want to know the total amount of •OH in mammals, Crn-related markers are likely to be more reliable than 8-OHdG for urinalysis. Absolute amounts of the former are ~10^4-fold higher than in the latter, and the former markers are determined directly without any further degradation process, whereas the latter are indirect, requiring not only degradation from the nucleotide chain but also excretion from the nucleus or mitochondria from cells into the urine *via* the cytoplasm and blood. However, for the estimation of DNA damage by •OH inside the nucleus or mitochondria, 8-OHdG is likely to be the better marker. Using a specific antiserum against 8-OHdG, we could show the difference in DNA damage between the nucleus and mitochondria. For example, at 8 weeks after the onset of diabetes, levels of 8-OHdG were significantly increased in DNA of mitochondria from the kidney of diabetic rats but not in nuclear DNA, suggesting the predominant damage of mitochondrial DNA (Figure 6) (*19*). If we want to further clarify the •OH levels scavenged by Crn in the nucleus and mitochondria, as well as in organs, we need a specific antibody against CTL and/or MG.

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Brief Report

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Design, synthesis and biological evaluation of 4-chromanone derivatives as I_{Kr} inhibitors

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Summary **Cardiac arrhythmia is a major cause of death in the world. Among many delayed rectifier potassium currents, the rapid delayed rectifier K current (I_{Kr}) plays an important role in the repolarization of cardiac tissue. The inhibition of I_{Kr} can delay repolarization and lead to an increase in the QT interval of the electrocardiogram, which is the treatment mechanism of Class III antiarrhythmic agents. Therefore, I_{Kr} can be considered as the drug target for the treatment of cardiac arrhythmia. In the current study, a series of 4-chromanone compounds (WR1-WR12) were well designed and synthesized as I_{Kr} inhibitors. The results disclosed that two compounds displayed potent inhibitory activities against I_{Kr}. Moreover, our structure-activity relationship results might provide necessary information for the rational design of inhibitors for I_{Kr}.**

Keywords: I_{Kr}, inhibitors, 4-chromanone, arrhythmia

1. Introduction

Potassium ion (K^+) channels consist of a ubiquitous family of membrane proteins that play critical roles in a wide variety of physiological processes, such as the regulation of neuronal excitability, cell proliferation, muscle contraction, and insulin secretion (*1*). K^+ channels have long been attractive targets for the rational drug design, due to their pivotal functions in biological systems (*2*). So far, various small-molecule compounds and toxins have been discovered as K^+ channel modulators (*3*).

Multiple delayed rectifier potassium currents play an important role in the repolarization and termination of the cardiac action potential. Inhibition of these potassium currents prolongs action potential duration (*4*), delays repolarization, and produces an antiarrhythmic effect (*5*). As the rapid component of cardiac delayed rectifier potassium current, the I_{Kr} potassium channel is mainly encoded by hERG (human ether-a-go-go-related gene) (*6,7*), and its electrophysiological properties can be regulated by its auxiliary subunit KCNE1 and KCNE2 (*8*). Besides in heart, it was reported that the expressing level of hERG in cancer cells was greatly increased (*9*). As a result, I_{Kr} encoded by hERG channel may be a potential cancer therapeutic target (*10*).

I_{Kr} is highly sensitive to blockade by many structurally diverse compounds (*11*), such as astemizole, imipramine, and dofetilide (*12*). However, there is still an urgent demand on I_{Kr} inhibitors for examining the mechanism of inhibition of I_{Kr}. In the current research, we designed and synthesized a series of 4-chromanone compounds, and evaluated their inhibitory activity against I_{Kr} using a radio-ligand based assay. The experimental results revealed that several compounds exhibited respectable activity against I_{Kr}. Moreover, analysis of the structure–activity relationship on these compounds could contribute to designing new I_{Kr} blocker for preventing arrhythmia and/or cancer therapy.

2. Materials and Methods

2.1. *Chemicals*

In summary, a series of 4-chromanone derivatives were well designed and synthesized. The synthetic route is outlined in Scheme 1. In this case, 4-substituted phenol

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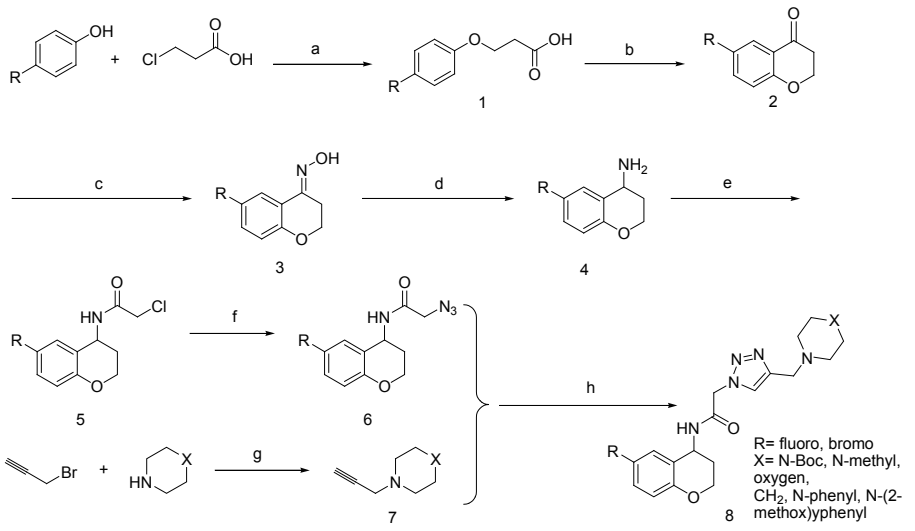


Scheme 1. Synthetic route of WR1-WR12. Reagents and conditions: **(a)** KOH, H_2O, reflux 4 h; **(b)** PPA, 60°C, 4 h; **(c)** Hydroxylamine hydrochloride, Na_2CO_3, EtOH, H_2O, 0°C→ r.t., 12 h; **(d)** CH_3COOH, Zn, 0°C→ r.t., 24 h; **(e)** Chloroacetyl chloride, K_2CO_3, CH_2Cl_2, 0°C→ r.t., 4 h; **(f)** NaN_3, KI, CH_3CN, reflux 6 h; **(g)** K_2CO_3, CH_2Cl_2, 0°C→ r.t., 4 h; **(h)** Sodium ascorbate, $CuSO_4$, MeOH, 0 °C→ r.t., 24 h.

and 3-chloropropionic acid gave compound 1, which was cyclized to form compound 2 by polyphosphoric acid (PPA). Compound 2 is then conveniently converted to compound 3 under oximation reaction. Hydrogenation of compound 3 with CH_3COOH/Zn gave compound 4, subsequently provided the key intermediate compound 6 *via* acylation with chloroacetyl chloride and substitution with sodium azide. Finally, reaction of compound 6 with the corresponding alkyne presented compound WR1-WR12 *via* click reaction, which was catalyzed by $CuSO_4$ and sodium ascorbate.

2.2. I_{Kr} *inhibition assay*

The inhibitory activities of these 12 compounds against I_{Kr} were evaluated by testing their affinities with hERG in the presence of 9 nM [^{3}H] dofetilide. Astermizole (Cat. No. #A2861-10MG; Sigma-Aldrich, St. Louis, MO, USA) and atropin was selected as positive and negative controls, respectively. The affinity with *h*ERG potassium channel was accessed in the presence of 9 nM [^{3}H]-dofetilide. Their binding abilities with the *h*ERG were exhibited and compared with the positive and negative controls.

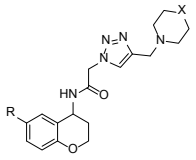
In brief, each compound was dissolved in DMSO as a stock solution (1 mM), which was diluted with binding buffers (10 folds, 6 points) when applied to the binding assays. Cell membranes were prepared as the instruction (GenScript USA Inc.). First, each well of Uni-filter 96 GF/C microplate was incubated with 100 µL 0.5% PEI (Polyethyleneimine，Sigma-Aldrich, dissolved in milli-Q water) at 4°C for 30-60 min. PEI was then discarded, and plates were washed with 2 mL/well wash buffer (50 mM Tris-HCl, pH 7.4; filtered and stored at 4°C). The reaction mixtures, including membrane (10 µg/well), each compound and [^{3}H]-dofetilide ligand (9 nM), were prepared in 24-well plates in a final volume of 100 µL (binding buffer: 10 mM Hepes, 130 mM NaCl, 60 mM KCl, 0.8 mM $MgCl_2$, 1 mM NaEGTA, 10 mM glucose, 0.1% BSA, pH7.4; filtered and stored at 4°C) and incubated at 25°C for 2 h with a shaking speed of 530 RPM. The reaction system was transferred into the filter plates and filtered with a Millipore vacuum manifold. The wells were washed with 2 mL/well cold wash buffer and dried at room temperature for 120 min. The bottom of the plates was sealed with Bottom seal™ (opaque) (Perkin Elmer) and 50 µL MicroScint 20™ (Perkin Elmer) was added to each well. Finally, the plates were sealed with Topseal A (Perkin Elmer) and counted on TopCount NXT for 1 min/well. IC_{50} values were calculated by GraphPad Prism 4 using the Cheng-Prusoff equation.

The binding data were converted to % displacement according to the below equation: % displacement = 100 × (1 – (sample CPM/Total binding CPM)) (in which total binding CPM values were obtained by testing binding of [^{3}H]-dofetilide to the target channel without competitors).

3. Results and Discussion

In order to study the influence of different substituents in the phenyl ring and piperazine ring in 4-chromanone-based compounds on the inhibitory activity, we design and synthesize twelve compounds (WR1-WR12) with different R and X group to examine the importance of phenyl ring and piperazine ring, respectively. All inhibition results are presented in Table 1. No compound exhibits a comparable replacement percentage (56% at 10 µM) with astemizole; however, several compounds could still prevent the binding

Table 1. Structures and inhibitory activities against I_{Kr} of compounds.



Numbers	R	X	IC_{50}/(μM)	% Displacement at 10 μM
Astemizole			0.0084	56.61 ± 4.29
Atropine				7.41 ± 3.53
WR1	F	N-phenyl	NA	29.08 ± 0.27
WR2	F	N-(2-methoxyl)phenyl	NA	24.69 ± 0.27
WR3	F	O	NA	2.26 ± 0.89
WR4	F	C	NA	31.66 ± 1.29
WR5	F	N-methyl	NA	3.22 ± 1.27
WR6	F	N-Boc	NA	6.20 ± 2.36
WR7	Br	N-phenyl	NA	34.98 ± 2.33
WR8	Br	N-(2-methoxyl)phenyl	9.16	25.79 ± 2.81
WR9	Br	O	NA	23.49 ± 0.06
WR10	Br	C	NA	17.95 ± 5.86
WR11	Br	N-methyl	NA	11.08 ± 9.16
WR12	Br	N-Boc	1.07	25.85 ± 2.88

NA: No significant dose response curve fitted.

of the ligand to the channel with > 20% replacement percentage when their concentrations are 10 μM (Table 1). We then calculated the inhibitory IC_{50} values for providing the exact value of inhibitory activity against I_{Kr}. As a result, two compounds with bromine rather than fluorine displayed potent inhibitory activities against I_{Kr}, which proposes that R group should be low electronegativity. While compare the X group we can find that phenyl and 2-methoxyphenyl are superior to other substituents to a piperazine ring, such as O, N-methyl, *etc*., which suggests that phenylpiperazine is highly fitted with the binding pocket of hERG.

On the basis of the result of I_{Kr} inhibition assay, we harvested several compounds with I_{Kr} inhibitory activities. In our opinions, these compounds are a new series of I_{Kr} inhibitors with novel chemical structures, so that can serve as lead compounds for the development of new I_{Kr} inhibitors.

4. Conclusion

In conclusion, a series of 4-chromanone derivatives were well designed and synthesized in the current study. After biological evaluation, two compounds exhibited moderate inhibition against I_{Kr}. It should be noted that these compounds are new I_{Kr} inhibitors with novel structure, so that can serve as lead compounds for further development. SAR analysis revealed that both the R group and the X group might play an important role in anti-I_{Kr} activity, and substituents of a phenyl ring should have a high influence on the activity. The proof-of-concept in this study may provide essential information for the future design of inhibitors for I_{Kr}. In our follow-up study, we will continue to manipulate the chemical structures of these compounds for improving the activity against I_{Kr}, as well as to take an attempt to employ other methods to test the inhibition of compound against I_{Kr} and to explore their possibility of preventing cardiac arrhythmia.

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Appendix

Chemistry: general procedures

All materials were purchased from commercial suppliers and used without further purification. Twice-distilled water was used throughout all experiments. Solvents were distilled prior to use, and all the reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60GF-254) and visualized with UV light or ninhydrin. Mass spectra were performed by the analytical and the mass spectrometry facilities in Drug Analysis Center at Shandong University on Agilent Technologies 1100 infinity HPLC, Applied Biosystems API4000. ^{1}H-NMR spectra were recorded on the Bruker 300 MHz NMR and 600 MHz NMR spectrometers. Chemical shifts are reported in delta (δ) units, parts per million (ppm) downfield from trimethylsilane. Melting points were determined on an electrothermal melting point apparatus (uncorrected).

1.1. *Phenyl-4-propynylpiperazine (7a)*

Propargyl bromide (1.19 g, 10 mmol) was added slowly to a mixture of 1-phenylpiperazine (1.95 g, 12 mmol) and K_2CO_3 (2.76 g, 20 mmol) in acetone. The mixture was stirred for 16 h at room temperature, filtrated, and the precipitation was washed with acetone. The combined filtrate was evaporated under vacuum. Water was added to the residue, and the mixture was extracted with CH_2Cl_2 (40 mL). The combined organic layer was washed with 5% $NaHCO_3$ (3 × 50 mL), saturated brine (2 × 50 mL), and then dried over $MgSO_4$. The solution was filtrated and evaporated. The crude product was then purified by column chromatography to obtain a white solid, yield 80%, m.p: 46-48°C. ESI-MS calcd for $C_{13}H_{16}N_2$ ($M + H^+$): 201.1; found: 201.4. ^{1}H-NMR (600 MHz, DMSO-d6): δ = 7.19 (dd, J_1 = 9.0, 7.8 Hz, 2H), 6.92 (d, J = 7.8 Hz, 2H), 6.76 (t, J = 7.2 Hz, 1H), 3.36 (s, 2H), 3.22 (s, 1H), 3.15 (s, 4H), 2.62 (s, 4H).

1.2. *1-(2-Methoxyphenyl)-4-propynylpiperazine (7b)*

Compound 7b was synthesized following the procedure described in 1.1. White solid, yield 76%, m.p: 75-77°C. ESI-MS calcd for $C_{14}H_{18}N_2O$ ($M + H^+$): 231.1; found: 231.2. ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 7.01 (m, 1H), 6.96 (d, J = 1.2 Hz, 1H), 6.92 (t, J = 7.2 Hz, 1H), 6.86 (d, J = 7.8 Hz, 1H), 3.87 (s, 3H), 3.36 (d, J = 1.8 Hz, 4H), 2.79 (s, 4H), 2.27 (s, 1H).

1.3. *N-propargylmorpholine (7c)*

Compound 7c was synthesized following the procedure described in 1.1. White solid, yield 51%, m.p: 161-163°C. ESI-MS calcd for $C_7H_{11}NO$ ($M + H^+$): 126.1; found: 126.0. ^{1}H-NMR (600 MHz, D_2O) δ: 3.95-3.90 (m,

4H), 3.77 (s, 2H), 3.43 (s, 2H), 3.13 (s, 2H), 2.95 (t, J = 2.4 Hz, 1H).

1.4. *1-(Prop-2-ynyl)piperidine (7d)*

Compound 7d was synthesized following the procedure described in 1.1. White solid, yield 46%, m.p: 178-180°C. ESI-MS calcd for $C_8H_{13}N$ (M + H^+): 124.1; found: 124.3. ^{1}H-NMR (600 MHz, D_2O) δ: 3.79 (s, 2H), 3.45-3.43 (m, 2H), 2.90-2.83 (m, 3H), 1.80-1.78 (m, 2H), 1.65-1.63 (m, 1H), 1.57-1.50 (m, 2H), 1.29-1.26 (m, 1H).

1.5. *1-Methyl-4-propargylpiperazine (7e)*

Compound 7e was synthesized following the procedure described in 1.1. White solid, yield 72%, m.p: 210°C. ESI-MS calcd for $C_8H_{14}N_2$ (M + H^+): 139.1; found: 139.1. ^{1}H-NMR (600 MHz, D_2O) δ: 3.96 (s, 2H), 3.69 (s, 4H), 3.35 (s, 4H), 2.98 (d, J = 2.4 Hz, 1H), 2.85 (s, 3H).

1.6. *Tert-butyl 4-propargylpiperazine-1-carb-oxylate (7f)* (*13*)

A solution of di-*tert*-butyl dicarbonate (4.34 g, 20 mmol) in CH_2Cl_2 (25 mL) was slowly added to a stirring solution of piperazine (3.46 g, 40 mmol) in CH_2Cl_2 (50 mL) at 0°C. The mixture was then stirred for 24 h at room temperature, and the solvent removed in vacuum. The crude solid was redissolved in diethyl ether (100 mL) with warming, and the white precipitate was filtered to. The product was extracted from the mother liquor with 1M citric acid solution (3 × 50 mL), and the aqueous layer was washed with CH_2Cl_2 (3 × 50 mL), basified with Na_2CO_3 (pH 11), and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layer was dried over $MgSO_4$ and evaporated in vacuum to give *tert*-butyl 1-piperazinecarboxylate as a waxy white solid (2.82 g, yield 75.6%). Propargyl bromide (2.38 g, 20 mmol) was added slowly to a mixture of *tert*-butyl 1-piperazinecarboxylate (4.4 mg, 24 mmol) and K_2CO_3 (3.32 g, 24 mmol) in acetone. The mixture was stirred for 12 h at room temperature. CH_2Cl_2 (40 mL) was then added, and the solution obtained was washed with 5% $NaHCO_3$ (3 × 50 mL), saturated brine (2 × 50 mL), and then dried over $MgSO_4$. The solution was filtrated and evaporated to dryness. The residue was crystallized from ethanol and gave *tert*-butyl 4-propargylpiperazine-1-carboxylate *7f* (4.2 g, yield 78%), m.p: 98-100°C. ESI-MS calcd for $C_{12}H_{20}N_2O_2$ (M + H^+): 224.1; found: 225.1. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 3.41(s, 4H), 3.26 (s, 2H), 2.46 (s, 4H), 2.22 (s, 1H), 1.42 (s, 9H).

2.1. *3-(4-Fluorophenoxy)propanoic acid (1a)* (*14*)

A mixture of potassium hydroxide (12.34 g, 220 mmol), 4-fluorophenol (11.21 g, 100 mmol), 3-chloropropionic acid (10.85 g, 100 mmol) and ethanol (2 mL) in water (40 mL) were refluxed 6 h. After cooling, the solution was acidified with concentrated hydrochloric acid to pH = 2 and extracted with ethyl acetate. The organic layer was washed with saturated aqueous solution of sodium bicarbonate. The aqueous phase was then acidified with concentrated hydrochloric acid and extracted with ethyl acetate. The final organic layer was dried over magnesium sulfate, filtrated, and evaporated under vacuum. The title product was recrystallized in ethanol. The resulting precipitate was collected by filtration, washed with hexane, and dried (3.87g, yield 21%), m.p: 84-86°C (Lit (*15*) m.p: 84-86°C). ESI-MS calcd for $C_9H_9FO_3$ (M + H^+): 185.1; found: 185.3.

2.2. *3-(4-Bromophenoxy)propanoic acid (1b)*

Compound 1b was synthesized following the procedure described in 2.1. White solid, yield 24%, and m.p: 148-150°C (Lit (*16*) m.p: 145-147°C). ESI-MS calcd for $C_9H_9BrO_3$ (M + H^+): 243.9; found: 243.8.

3.1. *6-Fluorochroman-4-one (2a)*

A mixture of 3-(4-fluorophenoxy)propanoic acid (3.68 g, 20 mmol) and polyphosphoric acid (PPA) (100 g) was placed into a three-neck round-bottom flask. The mixture was heated and stirred with a blade stirrer at 60°C for 4 h. One hundred mL ice water was added to the mixture after the temperature of the mixture was equal to room temperature, and then the aqueous layer was extracted with ethyl acetate for three times. The combined organic layer was dried overnight with anhydrous magnesium sulphate, filtrated, and evaporated under vacuum. The title product was recrystallized in ethanol. The resulting precipitate was collected by filtration, washed with hexane, and dried (2.76 g, yield 83%), and m.p: 118-120°C (Lit (*15*) m.p: 113-116°C). ESI-MS calcd for $C_9H_7FO_2$ (M + H^+): 167.0; found: 167.0.

3.2. *6-Bromochroman-4-one (2b)*

Compound 2b was synthesized following the procedure described in 3.1. White solid, yield 78%, and m.p: 77-78°C (Lit (*17*) m.p: 77°C). ESI-MS calcd for $C_9H_7BrO_2$ (M + H^+): 227.0; found: 227.1.

4.1. *(E)-6-fluorochroman-4-one oxime (3a)* (*18*)

To a suspension of hydroxylamine hydrochloride (1.39g, 20 mmol) in ethanol (50 mL), 6-fluorochroman-4-one (1.66g, 10 mmol) was added. Then, a solution of Na_2CO_3 (1.05 g, 20 mmol) in water (10 mL) was added dropwise. The resulting solution was stirred overnight. Most of the ethanol was evaporated, and then 60 mL water was added. The aqueous phase was extracted

with ethyl acetate (3 × 50 mL), and the combined organic layer was dried over Na_2SO_4. The solvents were removed under vacuum, and the residue was recrystallized. The resulting precipitate was collected by filtration, washed with hexane, and dried (1.54 g, yield 85%), m.p: 108-110°C. ESI-MS calcd for $C_9H_8FNO_2$ (M + H^+): 182.1; found: 182.0. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.25 (s, 1H), 7.51 (dd, *J* = 9.3 Hz, *J* = 3 Hz, 1H), 7.01 (ddd, *J* = 10.8 Hz, *J* = 7.8 Hz, *J* = 3.0 Hz, 1H), 6.89 (dd, *J* = 9 Hz, *J* = 4.5 Hz, 1H), 4.25 (t, *J* = 6.3 Hz, 2H), 3.00 (t, *J* = 6.0 Hz, 2H).

4.2. *(E)-6-bromochroman-4-one oxime (3b)*

Compound 3b was synthesized following the procedure described in 4.1. White solid, yield 91%, m.p: 139-141°C. ESI-MS calcd for $C_9H_8BrNO_2$ (M + H^+): 241.9; found: 241.7. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.46 (s, 1H), 7.95 (d, *J* = 2.4 Hz, 1H), 7.36 (dd, *J* = 9.0 Hz, *J* = 2.4 Hz, 1H), 6.81 (d, *J* = 8.7 Hz, 1H), 4.26 (t, *J* = 6.3 Hz, 2H), 3.00 (t, *J* = 6.3 Hz, 2H).

5.1. *2-Chloro-N-(6-fluorochroman-4-yl)acetamide (5a)* (*19,20*)

To a suspension of (E)-6-fluorochroman-4-one oxime (1.81 g, 10 mmol) in CH_3COOH (20 mL) was added the Zn (dust, 3.27 g, 50 mmol). The resulting solution was stirred 24 h. The Zn dust was filter off and washed by ethanol (20 mL) three times. The filtrate was collected and evaporated under vacuum. Then the product without purification was transferred to a mixture of K_2CO_3 (2.76 g，20 mmol) and CH_2Cl_2 (30 mL), and chloroacetyl chloride (2.26 g，20 mmol) was added in the mixture at room temperature. The reaction mixture was stirred for 4 h, subsequently slowly poured into 100 mL of ice water. The aqueous solution was extracted with CH_2Cl_2 (40 mL × 3), the organic phase was dried over Na_2SO_4, filtered and the solvent was evaporated to furnish a solid residue which was purified by crystallization from ethanol. The resulting precipitate was collected by filtration, washed with hexane, and dried (1.27 g, yield 52%), m.p: 129-131°C. ESI-MS calcd for $C_{11}H_{11}ClFNO_2$ (M + H^+): 244.1; found: 244.2. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.95-6.89 (m, 2H), 6.83-6.80 (m, 2H), 5.19 (dt, *J* = 13.8 Hz, *J* = 6.3 Hz, 1H), 4.29-4.22 (m, 1H), 4.19-4.15 (m, 1H), 4.12 (d, *J* = 2.4 Hz, 2H), 2.29-2.19 (m, 1H), 2. 90-2.00 (m, 1H).

5.2. *2-Chloro-N-(6-bromochroman-4-yl)acetamide (5b)*

Compound 5b was synthesized following the procedure described in 5.1. White solid, yield 50%, m.p: 176-178°C. ESI-MS calcd for $C_{11}H_{11}BrClNO_2$ (M + H^+): 304.0; found: 304.2. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.31-7.26 (m, 2H), 6.76 (d, *J* = 6.0 Hz, 2H), 5.18 (dt, *J* = 13.5 Hz, *J* = 6.3 Hz, 1H), 4.31-4.24 (m, 1H), 4.20-4.15(m, 1H), 4.13 (d, *J* = 4.2 Hz, 2H), 2.29-2.18 (m, 1H), 2.10-2.00 (m, 1H).

6.1. *N-(7-fluorochroman-4-yl)-2-(4-((4-phenylpiperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamide (WR1)*

Compound 5a (0.24 g, 1.0 mmol), KI (0.17 g, 1 mmol), sodium azide (2.2 mmol), 15 mL acetonitrile and 10 mL H_2O were added to a round bottom flask. After stirring overnight at 80°C, the reaction mixture was cooled to room temperature and most of the acetonitrile was evaporated and then 60 mL water was added. The product (6a) was extracted by CH_2Cl_2 and removed the solvent under reduced pressure. Without purification, 6a was dissolved into 20 mL methanol and 2 mL water, and compound 7a (0.2 g, 1.0 mmol), an aqueous sodium ascorbate solution (0.1 g, 0.5 mmol, dissolved in 2 mL water) and an aqueous solution of copper(II) sulfate pentahydrate (0.1 mL, 0.05 mmol) was added to this stirred solution. After another 24 h of stirring, the solvent was removed under vacuum, and the crude product was purified by flash chromatography (CH_2Cl_2/MeOH, 10:1) to afford the product WR1 (0.2 g, yield 52%), and m.p: 97-99°C. ESI-HRMS calcd for $C_{24}H_{27}FN_6O_2$ (M + H^+): 451.2258; found: 451.2277. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.75 (s, 1H), 7.03-6.98 (m, 2H), 6.93 (d, *J* = 4.2 Hz, 2H), 6.89-6.83 (m, 2H), 6.80-6.72 (m, 2H), 6.70 (d, *J* = 7.8 Hz, 1H), 5.17-5.11(m, 3H), 4.23-4.16 (m, 1H), 4.11-4.04 (m, 1H), 3.78 (s, 2H), 3.09 (br.s, 4H), 2.75 (br.s, 4H), 2.24-2.14 (m, 1H), 2.03-1.93 (m, 1H).

6.2. *N-(6-fluorochroman-4-yl)-2-(4-((4-(2-methoxyphenyl) piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamide (WR2)*

Compound WR2 was synthesized as white solid following the procedure described in 6.1, yield 41%, m.p: 152-154°C. ESI-HRMS calcd for $C_{25}H_{29}FN_6O_3$ (M + H^+): 481.2363; found: 481.2389. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.80 (s, 1H), 7.04-6.67 (m, 1H), 6.93 (d, *J* = 4.2 Hz, 2H), 6.90-6.82 (m, 2H), 6.80 (dt, *J* = 8.1 Hz, *J* = 3 Hz, 2H), 6.59 (d, *J* = 8.1 Hz, 1H) 5.18-5.12 (m, 3H), 4.23-4.16 (m, 1H), 4.11-4.04 (m, 1H), 3.86 (s, 3H), 3.82 (s, 2H), 3.12 (br.s, 4H), 2.78 (br.s, 4H), 2.25-2.14 (m, 1H), 2.04-1.93 (m, 1H).

6.3. *N-(6-fluorochroman-4-yl)-2-(4-(morpholinomethyl)-1H-1,2,3-triazol-1-yl)acetamide (WR3)*

Compound WR3 was synthesized following the procedure described in 6.1. White solid, yield 38%, m.p: 152-154°C. ESI-HRMS calcd for $C_{18}H_{22}FN_5O_3$ (M + H^+): 376.1785; found: 376.1787. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 9.02 (d, *J* = 8.1 Hz, 1H), 8.37 (s, 1H), 7.07-7.00 (m, 2H), 6.85 (dd, *J* = 8.7 Hz, *J* = 4.8 Hz, 1H), 5.36 (dt, *J* = 25.2 Hz, *J* = 6.3 Hz, 2H), 5.04 (dt, *J* = 13.2 Hz,

J = 6.3 Hz, 1H), 4.49 (s, 2H), 4.26-4.15 (m, 2H), 3.98-3.95 (m, 2H), 3.77-3.70 (m, 2H), 3.38-3.30 (m, 2H), 3.11-3.02 (m, 2H), 2.11-2.03 (m, 1H), 1.96-1.86 (m, 1H).

6.4. *N-(6-fluorochroman-4-yl)-2-(4-(piperidin-1-ylmethyl)-1H-1,2,3-triazol-1-yl)acetamide (WR4)*

Compound WR4 was synthesized following the procedure described in 6.1. White solid, yield 46%, m.p: 138-140°C. ESI-HRMS calcd for $C_{19}H_{24}FN_5O_2$ (M + H^+): 374.1992; found: 374.1994. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.90 (d, *J* = 7.8 Hz, 1H), 7.96 (s, 1H), 7.07-7.00 (m, 2H), 6.85 (dd, *J* = 8.7 Hz, *J* = 5.1 Hz, 1H), 5.20 (dt, *J* = 26.7 Hz, *J* = 16.2 Hz, 2H), 5.04 (dt, *J* = 13.2 Hz, *J* = 6.3 Hz, 1H), 4.27-4.14 (m, 2H), 3.55 (s, 2H), 2.50 (br.s, 4H), 2.12-2.02 (m, 1H), 1.95-1.85 (m, 1H), 1.52-1.45 (m, 4H), 1.38-1.36 (m, 2H).

6.5. *N-(6-fluorochroman-4-yl)-2-(4-((4-methylpiperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamide (WR5)*

Compound WR5 was synthesized following the procedure described in 6.1. White solid, yield 39%, and m.p: 223-225°C. ESI-HRMS calcd for $C_{19}H_{25}FN_6O_2$ (M + H^+): 389.2101; found: 389.2113. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 9.06 (d, *J* = 8.1 Hz, 1H), 8.36 (s, 1H), 7.07-7.00 (m, 2H), 6.85(dd, *J* = 8.7 Hz, *J* = 4.8 Hz, 1H), 5.33 (dt, *J* = 24.6 Hz, *J* = 15.0 Hz, 2H), 5.04 (dt, *J* = 13.5 Hz, *J* = 6.3 Hz, 1H), 4.48 (s, 2H), 4.28-4.17 (m, 2H), 3.80-3.77 (m, 4H), 3.62-3.58 (m, 4H), 2.81 (s, 3H), 2.13-2.03 (m, 1H), 1.97-1.87 (m, 1H).

6.6. *Tert-butyl4-((1-(2-((6-fluorochroman-4-yl)amino)-2-oxoethyl)-1H-1,2,3-triazol-4-yl)methyl)piperazine-1-carboxylate (WR6)*

Compound WR6 was synthesized following the procedure described in 6.1. White solid, yield 44%, m.p: 224-226°C. ESI-HRMS calcd for $C_{23}H_{31}FN_6O_4$ (M + H^+): 475.2469; found: 475.2473. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 9.06 (d, *J* = 7.8 Hz, 1H), 8.38 (d, *J* = 4.2 Hz, 1H), 7.07-7.00 (m, 2H), 6.85(dd, *J* = 8.7 Hz, *J* = 4.8Hz, 1H), 5.34 (dt, *J* = 26.4 Hz, *J* = 15.9 Hz, 2H), 5.04 (dt, *J* = 13.2 Hz, *J* = 6.3 Hz, 1H), 4.49 (s, 2H), 4.26-4.17 (m, 2H), 3.51-3.01 (m, 8H), 2.13-2.03 (m, 1H), 1.95-1.88 (m, 1H), 1.47(s, 9H).

6.7. *N-(6-bromochroman-4-yl)-2-(4-((4-phenylpiperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamide (WR7)*

Compound WR7 was synthesized following the procedure described in 6.1. White solid, yield 40%, and m.p: 148-150°C. ESI-HRMS calcd for $C_{24}H_{27}BrN_6O_2$ (M + H^+): 511.1457, 513.1437; found, 511.1448, 513.1430. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.89 (d, *J* = 8.1 Hz, 1H), 8.01 (s, 1H), 7.36-7.32 (m, 2H), 7.27-7.17 (m, 2H), 6.94 (d, *J* = 8.1 Hz, 2H), 6.80-6.74 (m, 2H), 5.23 (dt, *J* = 31.2 Hz, *J* = 15.9 Hz, 2H), 5.04 (dt, *J* = 12.9 Hz, *J* = 5.7 Hz, 1H), 4.30-4.15 (m, 2H), 3.64 (s, 2H),3.14-3.11 (m, 4H), 2.58-2.55 (m, 4H), 2.13-2.03 (m, 1H), 1.98-1.88 (m, 1H).

6.8. *N-(6-bromochroman-4-yl)-2-(4-((4-(2-methoxyphenyl) piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamide (WR8)*

Compound WR8 was synthesized following the procedure described in 6.1. White solid, yield 42%, m.p: 106-108°C. ESI-HRMS calcd for $C_{25}H_{29}BrN_6O_3$ (M + H^+): 541.1485, 543.1464; found, 541.1540, 543.1527. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.89 (d, *J* = 8.1 Hz, 1H), 8.03 (s, 1H), 7.35-7.32 (m, 2H), 6.94-6.85 (m, 4H), 6.82-6.77 (m, 1H), 5.23 (dt, *J* = 31.2 Hz, *J* = 16.2 Hz, 2H), 5.04 (dt, *J* = 13.5 Hz, *J* = 6.3 Hz, 1H), 4.26-4.16 (m, 2H), 3.76 (s, 3H), 3.64 (s, 2H), 2.95 (br.s, 4H), 2.56 (br.s, 4H), 2.10-2.03 (m, 1H), 1.95-1.88 (m, 1H).

6.9. *N-(6-bromochroman-4-yl)-2-(4-(morpholinomethyl)-1H-1,2,3-triazol-1-yl)acetamide (WR9)*

Compound WR9 was synthesized following the procedure described in 6.1. White solid, yield 39%, m.p: 166-168°C. ESI-HRMS calcd for $C_{18}H_{22}BrN_5O_3$ (M + H^+): 436.0984, 438.0964; found, 436.0999, 438.0980. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 9.01 (d, *J* = 7.8 Hz, 1H), 8.36 (s, 1H), 7.36-7.32 (m, 2H), 6.81-6.77 (m, 1H), 5.34 (dt, *J* = 31.5 Hz, *J* = 16.2 Hz, 2H), 5.04 (dt, *J* = 13.5 Hz, *J* = 6.3 Hz, 1H), 4.49 (s, 2H), 4.30-4.17 (m, 2H), 4.07-3.95 (m, 2H), 3.76-3.69 (m, 2H), 3.37-3.30 (m, 2H), 3.12 (s, 2H), 2.13-2.03 (m, 1H), 1.97-1.87 (m, 1H).

6.10. *N-(6-bromochroman-4-yl)-2-(4-(piperidin-1-ylmethyl)-1H-1,2,3-triazol-1-yl)acetamide (WR10)*

Compound WR10 was synthesized following the procedure described in 6.1. White solid, yield 43%, m.p: 215-216°C. ESI-HRMS calcd for $C_{19}H_{24}BrN_5O_2$ (M + H^+): 434.1192, 436.1171; found, 434.1203, 436.1185. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.71 (s, 1H), 7.26-7.22 (m, 1H), 7.18 (d, *J* = 2.1 Hz, 1H), 6.72-6.67 (m, 2H), 5.15-5.10 (m, 3H), 4.24-4.17 (m, 1H), 4.11-4.03 (m, 1H), 3.64 (s, 2H), 2.44 (br.s, 4H), 2.22-2.12 (m, 1H), 2.06-1.93 (m, 1H), 1.61-1.54 (m, 4H), 1. 46-1.42 (m, 2H).

6.11. *N-(6-bromochroman-4-yl)-2-(4-((4-methylpiperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)acetamide (WR11)*

Compound WR11 was synthesized following the procedure described in 6.1. White solid, yield 34%, m.p: 202-204°C. ESI-HRMS calcd for $C_{19}H_{25}BrN_6O_2$ (M + H^+): 449.1301, 451.1280; found, 449.1293, 451.1270.

^{1}H-NMR (300 MHz, $CDCl_3$) δ: 9.03 (d, *J* = 7.8 Hz, 1H), 8.32 (s, 1H), 7.36-7.32 (m, 2H), 6.81-6.78 (m, 1H), 5.34 (dt, *J* = 30.9 Hz, *J* = 16.2Hz, 2H), 5.04 (dt, *J* = 13.5 Hz, J = 6.3 Hz, 1H), 4.65 (s, 2H), 4.24-4.17 (m, 2H), 3.60-3.40 (m, 4H), 3.30-3. 04 (m, 4H), 2.81 (s, 3H), 2.16-1.99 (m, 1H), 1.97-1.87 (m, 1H).

6.12. *Tert-butyl4-((1-(2-((6-bromochroman-4-yl)amino)-2-oxoethyl)-1H-1,2,3-triazol-4-yl)methyl)piperazine-1-carboxylate (WR12)*

Compound WR12 was synthesized following the procedure described in 6.1. White solid, yield 42%, m.p: 206-207°C. ESI-HRMS calcd for $C_{23}H_{31}BrN_6O_4$ (M + H^+): 535.1668, 537.1648; found, 535.1654, 537.1635. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 9.58 (s, 1H), 9.03 (d, *J* = 8.4 Hz, 1H), 7.36-7.32 (m, 2H), 6.81-6.77 (m, 1H), 5.34 (dt, *J* = 31.2 Hz, *J* = 16.2Hz, 2H), 5.04 (dt, *J* = 13.2 Hz, *J* = 6.0 Hz, 1H), 4.49 (s, 2H), 4.30-4.13 (m, 2H), 4.06-4.02 (m, 2H), 3.47-3. 00 (m, 6H), 2.13-2.00 (m, 1H), 1.97-1.87 (m, 1H), 1.41(s, 9H).

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Metabolites from *Aspergillus versicolor*, an endolichenic fungus from the lichen *Lobaria retigera*

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Summary **Three new anthraquinone derivatives (1-3) and one new artifact (4) were isolated, along with six known anthraquinone derivatives (5-10) and three xanthones (11-13), from a culture of an endolichenic fungus, *Aspergillus versicolor*, that was isolated from the lichen *Lobaria retigera*. The structures of these substances were determined on the basis of 1D and 2D (COSY, HMQC, and HMBC) NMR and MS analyses. The substances 1-4 were also tested for their cytotoxic activity.**

Keywords: Endolichenic fungus, *Aspergillus versicolor*, *Lobaria retigera*, anthraquinone

1. Introduction

Lichens are composite organisms of a fungus (the mycobiont) and an algal partner (the photobiont or phycobiont) growing together in a symbiotic relationship. Endolichenic fungi, an emerging group of endosymbiotic microorganisms, consist of fungal strains that live within asymptomatic lichen thalli, much in the same way as endophytic fungi live within healthy plant tissues (*1*). In contrast to endophytic fungi that have been studied chemically a number of times (*2-4*), only a limited number of endolichenic fungi have thus far been investigated for their secondary metabolites (*5,6*), but studies have shown that endolichenic fungi are also rich sources of structurally diverse and biologically active small-molecule metabolites. In the course of the current authors' ongoing efforts to discover bioactive compounds from endophytic and endolichenic microorganisms, four new anthraquinone derivatives (**1-4**) (Figure 1) were obtained, along with six known anthraquinones (**5-10**) and three xanthones (**11-13**) (Supplemental data, *http://www.ddtjournal.com/docindex.php?year=2014&kanno=2*), from a culture of *Aspergillus versicolor*, an endolichenic strain of the lichen *Lobaria retigera*. The structures of these substances were elucidated using spectroscopic methods, including 1D and 2D nuclear magnetic resonance (NMR) and high-resolution electrospray ionization mass spectrometry (HRESIMS). Substances **1**-**4** were tested for their cytotoxic activity, with **1** and **2** displaying weak inhibitory activity towards the PC-3 and H460 human cancer cell lines. Reported here are the isolation of these compounds, elucidation of their structures, and determination of their bioactivity.

2. Materials and Methods

2.1. *General experimental procedures*

Optical rotations were measured on a GYROMAT-HP

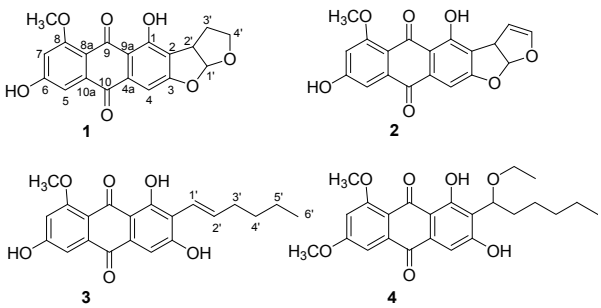

Figure 1. Structures of compounds 1-4

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polarimeter (Shimadzu Corporation, Kyoto, Japan). Infrared spectra (IR) were recorded on a Nicolet iN 10 Micro FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) in transmission mode. Ultraviolet (UV) spectra were obtained with a Shimadzu UV-2550 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). NMR spectra were recorded on a Bruker Avance DRX-600 spectrometer (Bruker BioSpin Group, Billerica, MA, USA) operating at 600 (^{1}H) and 150 (^{13}C) MHz with tetramethylsilane (TMS) as an internal standard. HRESIMS was performed on an LTQ Orbitrap XL (Thermo Fisher Scientific Inc., Waltham, MA, USA). All solvents used were of analytical grade (Laiyang Chemical Reagent Co., Ltd., Shandong, China). High-performance liquid chromatography (HPLC) was performed on an Agilent 1100 G1310A isopump equipped with an Agilent 1100 G1322A degasser, an Agilent 1100 G1314A VWD detector (254 nm), and an Agilent ZORBAX SB-C_{18} column (9.4 mm × 250 mm, 5 μm) (Agilent Technologies, Inc., Santa Clara, CA, USA). Silica gel (200-300 mesh; Qingdao Haiyang Chemical Co., Ltd., Qingdao, Shandong, China), C_{18} reversed-phase silica gel (YMC ODS-A gel, YMC Co., Ltd., Kyoto, Japan), and Sephadex LH-20 (GE Health, Uppsala, Sweden) were used for column chromatography (CC). Thin-layer chromatography (TLC) was performed with high-performance TLC plates precoated with silica gel GF254 (Qingdao Haiyang Chemical Co., Ltd.). Spots of TLC were visualized within iodine vapor or by spraying with H_2SO_4-EtOH (1:9) followed by heating.

2.2. *Microorganism*

The strain of fungus was isolated from the lichen *Lobaria retigera* collected from Mount Laojun, Yunnan Province, China. Nuclear ITS rDNA sequencing revealed that it was *Aspergillus versicolor*. The fungus (accession no. 2011-WX-35b) was deposited in the Key Laboratory of Chemical Biology (Ministry of Education).

2.3. *Fermentation, extraction, and isolation*

The fungal strain was grown on slants of potato dextrose agar (PDA) at 25°C for 15 days. The fungus was then placed in five Erlenmeyer flasks (300 mL) that each contained 120 mL of potato dextrose broth (PDB). These flasks were incubated at 25°C on a rotary shaker (120 rpm) for seven days to obtain the seed culture. The seed broth was added to 40 flasks (500 mL) that each contained an autoclaved culture medium of rice (80 g) and water (120 mL). Afterwards, the flasks were left to stand at room temperature for 40 days until the solid medium had almost disappeared. At harvest, the culture medium containing the mycelium was cut into small pieces, extracted with EtOAc for two days at room temperature, and then filtered. The solvent was evaporated under reduced pressure at 38°C to yield a crude extract (36.0 g) that was separated using CC with silica gel eluted with a gradient of petroleum ether (PE) and acetone from 100:0 to 0:100 (v/v) to yield eleven fractions (Fr. A-Fr. K). Fr. D (771.7 mg) was separated using CC with silica gel (PE/acetone, 80:1 to 2:1) to yield six subfractions (Fr. D1-Fr. D6). Compound **11** (101.5 mg) was obtained from Fr. D2 after recrystallization in CH_2Cl_2. Fr. D4 (230.0 mg) was loaded onto a Sephadex LH-20 column (CH_2Cl_2/MeOH, 1:1) to yield **4** (10.0 mg) and **5** (28.3 mg). Fraction E (80.0 mg) was separated using CC with a Sephadex LH-20 column (CH_2Cl_2/MeOH, 1:1) to yield the primary portion (16.2 mg), which was further purified using HPLC (MeOH-H_2O, 90:10, 1.5 mL/min) to yield **9** (3.0 mg, t_R = 10.9 min). Fraction G (2.0 g) was separated using CC with silica gel eluted with a gradient of CH_2Cl_2-MeOH (60:1 to pure MeOH) to yield seven subfractions (Fr. G1-Fr. G7). After recrystallization in CH_2Cl_2, compounds **6** (8.2 mg) and **7** (10.3 mg) were obtained from Fr. G3 (26.0 mg) and Fr. G2 (29.4 mg), respectively. Fr. G6 (638.7 mg) was subjected to CC with C18 reversed-phase silica gel eluted with a gradient of MeOH-H_2O (50:50 to 100% MeOH) to yield the primary portion, which was further separated using HPLC (MeOH-H_2O, 67:33, 1.5 mL/min) to yield **13** (2.1 mg, t_R = 34.2 min) and **12** (4.7 mg, t_R = 23.4 min). Fraction H (3.6 g) was subjected to CC with silica gel eluted with a gradient of CH_2Cl_2-MeOH (80:1 to 5:1) to yield eight subfractions (Fr. H1-Fr. H8). Fr. H4 (430.9 mg) was first separated on silica gel eluted with a gradient of PE-EtOAc (40:1 to 10:1) to yield the primary portion and then separated on C18 reversed-phase silica gel eluted with a gradient of MeOH-H_2O (50:50 to pure MeOH) to yield six subfractions (Fr. H4-1-Fr. H4-6). Fr. H4-4 (53.1 mg) was purified using HPLC (CH_3CN-H_2O, 50:50, 1.5 mL/min) to yield **1** (3.2 mg, t_R = 19.6 min) and **2** (2.0 mg, t_R = 21.4 min). Fr. H4-5 (38.4 mg) yielded **10** (5.1 mg, t_R = 34.2 min) after HPLC (MeOH-H_2O, 75:25, 1.5 mL/min) purification. Fr. H5 (637.4 mg) was separated on a silica gel column eluted with a gradient of PE-EtOAc (20:1 to 1:1) to yield five subfractions (Fr. H5-1-Fr. H5-5). Fr. H5-2 (44.9 mg) was purified using HPLC (CH_3CN-H_2O, 70:30, 1.5 mL/min) to yield **8** (20.0 mg, t_R = 28.2 min), while Fr. H5-4 (25.0 mg) yielded **3** (4.8 mg, t_R = 24.2 min) after HPLC (CH_3CN-H_2O, 64:36, 1.5 mL/min) purification.

8-*O*-methylversicolorin B (**1**): Orange solid. $[\alpha]_D^{20}$ = –75.76 (*c* 0.132, MeOH). UV (MeOH) λ_{max} (logε) nm: 197 (1.65), 223 (2.08), 287 (1.53), 310 (0.96). IR (KBr) ν_{max} cm^{-1}: 3,494, 2,946, 2,851, 1,620, 1,598, 1,242, 949. HRESIMS (negative mode) *m/z* 353.0635 [*M*-H]$^-$ (calcd. for $C_{19}H_{13}O_7$, 353.0661). For ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

8-*O*-methylversicolorin A (**2**): Orange solid. $[\alpha]_D^{20}$ =

Table 1. ^{1}H-NMR data for compounds 1-4 (at 600 MHz, δ in ppm, J in Hz)

Position	1[a]	2[a]	3[a]	4[b]
4	6.99 (1H, *s*)	7.14 (1H, *s*)	7.26 (1H, *s*)	7.08 (1H, *s*)
5	7.26 (1H, *s*)	7.33 (1H, *s*)	7.31 (1H, *s*)	7.45 (1H, *s*)
7	6.70 (1H, *s*)	6.92 (1H, *s*)	6.91 (1H, *s*)	6.78 (1H, *s*)
1-OH	14.44 (1H, *brs*)	13.89 (1H, *s*)	14.58 (1H, *s*)	13.76 (1H, *s*)
3-OH				9.72 (1H, *s*)
6-OCH_3				4.00 (3H, *s*)
8-OCH_3	3.89 (3H, *s*)	3.97 (3H, *s*)	3.97 (3H, *s*)	4.02 (3H, *s*)
1'	6.45 (1H, *d*, *J* = 4.2)	6.9 (1H, *d*, *J* = 4.2)	6.74 (1H, *d*, *J* = 16.2)	5.03 (1H, *m*)
2'	4.15 (1H, *t*, *J* = 6.6)	4.80 (1H, *m*)	7.02 (1H, *dt*, *J* = 16.2, 7.2)	1.75 (2H, *m*)
3'	2.23 (1H, *m*) (β-H); 2.28 (1H, *m*) (α-H)	5.45 (1H, *m*)	2.28 (1H, *q*, *J* = 7.2)	1.70 (2H, *m*)
4'	3.58 (1H, *m*) (β-H); 4.09 (1H, *t*, *J* = 7.8) (α-H)	6.65 (1H, *s*)	1.49 (2H , *m*)	1.30 (2H, *m*)
5'			1.40 (2H, *m*)	1.25 (2H, *m*)
6'			0.94 (3H, *t*, *J* = 7.2)	0.87 (3H, *m*)
1''				3.60 (2H, *m*)
2''				1.26 (3H, *m*)

[a] Measured in acetone-d_6; [b] Measured in $CDCl_3$

Table 2. ^{13}C-NMR data for compounds 1-4 (at 150 MHz, δ in ppm)

Position	1[a]	2[a]	3[a]	4[b]
1	165.2 (C)	n.o.[c]	161.1 (C)	162.0 (C)
2	120.8 (C)	120.9 (C)	119.2 (C)	119.7 (C)
3	160.7 (C)	164.1 (C)	164.0 (C)	162.4 (C)
4	99.97 (CH)	100.2 (CH)	107.2 (CH)	108.6 (CH)
4a	136.0 (C)	134.7 (C)	132.0 (C)	132.9 (C)
5	110.5 (CH)	107.0 (CH)	107.6 (CH)	103.8 (CH)
6	169.7 (C)	164.8 (C)	163.5 (C)	164.9 (C)
7	106.2 (CH)	104.9 (CH)	105.7 (CH)	104.9 (CH)
8	165.3 (C)	163.2 (C)	164.8 (C)	162.8 (C)
8a	112.0 (C)	n.o.[c]	114.6 (C)	137.6 (C)
9	186.8 (C)	186.6 (C)	187.6 (C)	186.8 (C)
9a	113.7 (C)	n.o.[c]	111.3 (C)	110.2 (C)
10	183.8 (C)	181.9 (C)	182.8 (C)	182.6 (C)
10a	138.0 (C)	137.3 (C)	132.0 (C)	115.4 (C)
6-OCH_3				56.0 (CH_3)
8-OCH_3	56.4 (CH_3)	55.9 (CH_3)	56.8 (CH_3)	56.7 (CH_3)
1'	114.0 (CH)	113.2 (CH)	120.4 (CH)	77.6 (CH)
2'	45.2 (CH)	48.9 (CH)	139.1 (CH)	34.8 (CH_2)
3'	31.5 (CH_2)	101.7 (CH)	35.4 (CH_2)	31.6 (CH_2)
4'	68.1 (CH_2)	145.5 (CH)	32.6 (CH_2)	25.1 (CH_2)
5'			23.1 (CH_2)	22.6 (CH_2)
6'			14.0 (CH_3)	14.1 (CH_3)
1''				66.3 (CH_2)
2''				15.0 (CH_3)

[a] Recorded in acetone-d_6; [b] Measured in $CDCl_3$; [c] These quaternary carbons were not observed.

– 95.24 (*c* 0.063, MeOH). UV (MeOH) λ_{max} (logε) nm: 197 (1.16), 223 (1.06), 285 (0.81). IR (KBr) ν_{max} cm^{-1}: 3,366, 2,921, 2,851, 1,738, 1,630, 1,598, 1,347, 1,294, 1,223, 973. HRESIMS (negative mode) *m/z* 351.0503 $[M\text{-H}]^-$ (calcd. for $C_{19}H_{11}O_7$, 351.0505). For ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

8-*O*-methylaverythin (**3**): Orange solid. UV (MeOH) λ_{max} (logε) nm: 195 (2.30), 225 (2.45), 295 (2.90). IR (KBr) ν_{max} cm^{-1}: 3,529, 3,377, 3,089, 3,027, 2,956, 2,927, 2,856, 1,618, 1,583, 1,336, 1,300, 1,258, 1,061, 978. HRESIMS (negative mode) *m/z* 367.1177 $[M\text{-H}]^-$ (calcd. for $C_{21}H_{19}O_6$, 367.1182). For ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

1'-*O*-ethyl-6,8-di-*O*-methylaverantin (**4**): Yellow solid. $[\alpha]_D^{20}$ = – 11.63 (*c* 0.086, MeOH). UV (MeOH) λ_{max} (logε) nm: 197 (3.02), 224 (3.79), 286 (3.14). IR (KBr) ν_{max} cm^{-1}: 3,219, 2,926, 2,854, 1,736, 1,670, 1,622, 1,595, 1,333, 1,265, 1,162, 1,063. HRESIMS (positive mode) *m/z* 429.1908 $[M\text{+H}]^+$ (calcd. for $C_{24}H_{29}O_7$, 429.1913). For ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

2.4. *Cytotoxicity assay*

A tetrazolium-based colorimetric assay (MTT assay) was used to determine cell viability. PC-3 cells (ATCC CRL-1435 human prostate adenocarcinoma) and NCI-H460 cells (ATCC HTB 177 human lung carcinoma) were used. The cell culture and cytotoxic activity assay followed the same procedures as previously described (*7*). Doxorubicin was used as a positive control, for which the IC_{50} values were 0.2 and 0.3 μM against PC-3 and NCI-H460, respectively.

3. Results and Discussion

Compound **1** was obtained as an orange powder. HRESIMS revealed a pseudo molecular ion $[M\text{-H}]^-$ peak at *m/z* 353.0635 (calcd. 353.0661) compatible with the molecular formula $C_{19}H_{14}O_7$, indicating 13 degrees of unsaturation. Evident in the ^{1}H-NMR spectrum for this compound (Table 1) was the resonance of one hydro-bonded phenolic hydroxyl group at δ_H 14.44 (s), three aromatic protons at δ_H 7.26 (s), 6.99 (s), and 6.70 (s), an oxygenated methine at δ_H 6.45 (d, *J* = 4.2 Hz), a methine at δ_H 4.15 (t, *J* = 6.6 Hz), a pair of oxygen-bearing methylene protons at δ_H 4.09 (t, *J* = 7.8 Hz) and δ_H 3.58 (m), another methylene at δ_H 2.28 (m) and δ_H 2.23 (m), and one oxygenated methyl group at δ_H 3.89 (3H, s). Evident in the ^{13}C-NMR spectrum (Table 2) was resonance of two carbonyls (δ_C 186.8 and 183.8), three aromatic methines, and nine aromatic quarternary

carbons (including four oxygenated) with chemical shifts attributable to a highly substituted anthraquinone scaffold (*8*). In addition, two methylenes (including one oxygenated), two methines, and one oxygenated methyl groups were observed. These data indicated that **1** was similar to versicolorin B (*9*), except that a methoxy group was present at C-6 in **1** instead of the 6-OH group in versicolorin B. The relative configuration of **1** was determined by analysis of NOESY correlations. Therefore, compound **1** was determined to be 8-*O*-methylversicolorin B.

Compound **2** was also an orange amorphous solid. HRESIMS revealed an $[M-H]^-$ ion peak at *m/z* 351.0503 (calcd. 351.0505) corresponding to the molecular formula $C_{19}H_{12}O_7$. The ^{1}H- and ^{13}C-NMR data (Tables 1 and 2) for **2** closely resembled those for **1**, except for the presence of an additional double bond between C-3' (δ_C 101.7) and C-4' (δ_C 145.5) in **2**. This was verified by the HMBC correlations of H-3' (δ_H 5.45)/C-1' and H-1'/C-2', C-3', and C-4' along with the ^{1}H-^{1}H COSY correlations of H-2'/H-3' and H-3'/H-4'. Therefore, compound **2** was identified as 8-*O*-methylversicolorin A.

Compound **3** was also an orange solid. A molecular formula of $C_{21}H_{20}O_6$ was assigned to **3** based on the quasimolecular ion $[M-H]^-$ peak at *m/z* 367.1177 (calcd. 367.1182) according to HRESIMS. The ^{1}H- and ^{13}C-NMR spectra for **3** (Tables 1 and 2) were similar to those previously reported for averythin (**8**) (*8*). The only difference was replacement of 8-OH by a methoxy group (δ_H 3.97, 3H, s). This was confirmed by HMBC correlations from 8-OMe to C-7 (δ_C 105.7) and C-8 (δ_C 164.8). Therefore, compound **3** was determined to be 8-*O*-methylaverythin.

Compound **4** was obtained as a yellow solid. It was assigned a molecular formula of $C_{24}H_{28}O_7$ based on the $[M+H]^+$ ion peak at *m/z* 429.1908 (calcd. 429.1913) according to HRESIMS. The NMR data for **4** (Table 1) were similar to those for **5** (*10*), except that the 1'-methoxy group in **5** was replaced by an ethoxy moiety in **4**. This was verified by the HMBC correlations of H-2" (δ_H 1.26)/C-1" (δ_C 66.3) and H-1" (δ_H 3.60)/C-1'. Hence, compound **4** was determined to be 1'-*O*-ethyl-6,8-di-*O*-methylaverantin. Compound **4** may be an artifact since EtOH was used in the separation process.

The known compounds 6,8,1'-tri-*O*-methylaverantin (**5**) (*10*), aversin (**6**) (*11-13*), 6,8-di-*O*-methylversicolorin A (**7**) (*11,14*), averythin (**8**) (*8,9,15,16*), 6,8-di-*O*-methylaverufin (**9**) (*10*), 6,8-di-*O*-methylnidurufin (**10**) (*10*) sterigmatocystin (**11**) (*13,17*), 5-methoxysterigmatocystin (**12**) (*13*), and dihydrosterigmatocystin (**13**) (*17*) were identified by comparing their NMR data (see Supplemental Data) to those reported in the literature.

The *in vitro* inhibition of cell proliferation of two cancer cell lines, PC-3 (human prostate cancer cells) and H460 (human lung cancer cells), was evaluated using the new compounds **1**–**4**. Compounds **1** and **2** had weak cytotoxic activity, with IC_{50} values of 19.5 and 12.6 μM against PC-3 cells and 27.2 and 17.3 μM against H460 cells. The other compounds had no activity against PC-3 and H460 cells (IC_{50} > 50 μM).

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Original Article

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Exploring the influence of renal dysfunction on the pharmacokinetics of ribavirin after oral and intravenous dosing

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Summary **Although ribavirin is minimally cleared by renal elimination, its pharmacokinetics are substantially altered in patients with chronic renal impairment. This open-label study assessed the pharmacokinetics of single 400-mg oral and intravenous (IV) doses of ribavirin in two healthy volunteers and 12 patients with varying degrees of chronic renal impairment. Blood and urine samples were collected pre-dose and up to 168 h post-dose for pharmacokinetic analyses. Ribavirin area under the plasma concentration-time curve from time zero to time of final quantifiable sample and maximum plasma concentration values were increased, and total plasma clearance (CL), renal clearance (CL_r), non-renal clearance (CL_{nr}), volume of distribution at steady state (Vd_{ss}), and amount excreted values were reduced in patients with renal dysfunction compared with those who had normal renal function. Following IV administration, mean CL_r was 54%, 23%, and 10% in patients with mild, moderate, and severe renal dysfunction, respectively, relative to control subjects, and was 56%, 28%, and 9% of control values after oral dosing. After IV dosing, mean CL_{nr} was 94%, 76%, and 75% of control values in patients with mild, moderate, and severe renal dysfunction, respectively, and was 54%, 48%, and 27% of control values after oral dosing. Mean oral bioavailability of ribavirin was 35%, 60%, 57%, and 71% in control subjects and patients with mild, moderate, and severe renal dysfunction, respectively. These data indicate that there are multiple mechanisms (increased oral bioavailability, reduced CL_r and CL_{nr}, reduced Vd) contributing to altered ribavirin pharmacokinetics in chronic renal impairment.**

Keywords: Intravenous, oral, pharmacokinetics, ribavirin, renal dysfunction, creatinine clearance, bioavailability, excretion

1. Introduction

Ribavirin is a broad-spectrum antiviral agent that is active against a number of viruses, including hepatitis C virus (HCV) (*1-6*). In combination with pegylated interferon, ribavirin is an established treatment for chronic HCV infection (*7,8*), and more recently has also become a component of protease inhibitor–based triple therapy regimens with boceprevir or telaprevir (*9-12*).

Ribavirin is contraindicated in patients with creatinine clearance < 50 mL/min (*13*). Pharmacokinetic studies in healthy volunteers and patients with HCV infection indicate that although both renal and hepatic pathways contribute to ribavirin elimination, the relative contribution of renal pathways is comparatively low, accounting for only 5-15% of the total elimination (*14-19*). It is therefore somewhat surprising that ribavirin has been shown to accumulate in patients with renal failure (*20,21*). A recent pharmacokinetic study of single-dose oral ribavirin in patients with renal dysfunction highlighted substantial alterations in ribavirin pharmacokinetics associated with declining renal function (*22*). The authors proposed that changes in ribavirin metabolism associated with renal impairment might underlie the altered pharmacokinetics seen in this patient group. The objective of the present study was to compare ribavirin pharmacokinetics after single oral and intravenous (IV) doses in patients with renal dysfunction, to further evaluate mechanisms contributing to the altered pharmacokinetics.

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2. Materials and Methods

This was an open-label, parallel-group, single-dose, and 2-stage study. The first stage was designed to assess the pharmacokinetic properties of a single oral 400-mg dose of ribavirin, whereas the second stage assessed the pharmacokinetics of a single 400-mg IV dose, in subjects with varying degrees of stable chronic renal insufficiency. Subjects who had received oral ribavirin during the first stage of the study and who continued to meet the enrollment criteria were permitted to enroll in the second phase of the study. The two stages of the study were separated by a washout period of several months. The study was conducted in accordance with the Principles of Good Clinical Practice and the Declaration of Helsinki. All subjects provided written informed consent to participate in this study, and the protocol was approved by the Research Consultants Review Committee, Austin, Texas.

2.1. *Study population*

Male and female subjects, 18 to 65 years of age, who had normal renal function or varying degrees of stable chronic renal insufficiency were enrolled. Subjects with normal renal function (creatinine clearance [CL_{cr}] > 90 mL/min) were excluded if they had a history of cardiovascular, neurologic, hematologic, gastrointestinal, cerebrovascular, respiratory, hepatic, or renal disease, or any other disorder requiring physician care. Subjects with evidence of HIV or hepatitis B coinfection, or urinary traces of drugs of abuse, were also excluded. Subjects with compromised renal function (CL_{cr} < 90 mL/min) were excluded if they had significant medical disorders unrelated to their renal disorder that would substantially interfere with their ability to participate in the study.

2.2. *Study design*

During stage 1, participants fasted overnight and then received a single oral 400-mg dose of ribavirin (2 × 200-mg capsules) with 200 mL water. For analysis, participants were divided into 4 groups according to CL_{cr} (based on a 24-h urinary collection): group I, CL_{cr} > 90 mL/min/1.73 m^2 (normal); group II, CL_{cr} > 61 and ≤ 90 mL/min/1.73 m^2 (mild renal dysfunction); group III, CL_{cr} ≥ 31 and ≤ 60 mL/min/1.73 m^2 (moderate renal dysfunction); and group IV, CL_{cr} 10-30 mL/min/1.73 m^2 (severe renal dysfunction). During stage 2, participants again fasted overnight and then received a single IV 400-mg dose of ribavirin solution (13.3 mL of 30 mg/mL solution), infused over 15 min (0.89 mL/min) using a syringe pump. For analysis, participants were again divided into groups I to IV as described for stage 1. Participants continued fasting until 4 hours after the dose and were discharged from the study center after 48 h. Subsequent samples were collected on an outpatient basis.

2.3. *Sample collection and assay for pharmacokinetic assessments*

After oral dosing, blood samples for determination of plasma ribavirin concentrations were obtained immediately prior to drug administration and then at specified time intervals until 168 h after the dose. After IV infusion dosing, additional blood samples were collected at 0.08, 0.16, 0.25, and 0.5 h after the dose and then again at the same time intervals as for the oral dose until 168 h after the dose. After collection, the plasma was separated from blood samples and frozen at – 80°C until analysis.

Block urine samples were collected immediately prior to drug administration and at 12- to 24-h intervals until 168 h following the dose. Plasma and urine concentrations of ribavirin were determined using high-performance liquid chromatography/tandem mass spectrometry, as previously described (*22*).

2.4. *Pharmacokinetic analysis*

Plasma and urine ribavirin concentrations above the limit of quantitation (plasma, 50 ng/mL; urine, 250 ng/mL) were used to calculate pharmacokinetic parameters using model-independent methods (*23*). The maximum plasma concentration (C_{max}) and time to maximum plasma concentration (T_{max}) were the observed values. The area under the plasma concentration-time curve from time zero to the time of the final quantifiable sample (AUC_{tf}) was calculated using the linear trapezoidal method. Individual terminal rate constants could not be determined with precision; therefore, the elimination half-life ($t_{½}$) and area under the plasma concentration-time curve from time zero to infinity (AUC_I) were not reported. Pharmacokinetic analysis was consequently limited to estimation of individual AUC_{tf} value instead of AUC_I. Total plasma clearance (CL) was calculated by dividing the dose by AUC_{tf} after IV dosing. Renal clearance (CL_r) was calculated by dividing the amount excreted (A_e) in the urine from time 0 to 168 h by the AUC_{tf}. Nonrenal clearance (CL_{nr}) was the difference between CL and CL_r.

Absolute bioavailability (F) was calculated (as percentage) for each subject as [AUC^{PO} (tf, po) × $dose^{IV}$]/[AUC^{IV} (tf, po) × $dose^{PO}$], where *tf, po* was the *tf* for that subject following oral administration, and AUC^{PO} (tf, po) and AUC^{IV} (tf, po) were AUC values from time zero to *tf*, for *po* dose following administration of oral and IV doses to the same subjects, respectively. Absolute bioavailability (F) was expressed as a percentage and was not calculated from urine data, because most subjects had renal impairment and could not provide adequate urine samples.

The volume of distribution at steady state (Vd_{ss}) was calculated using the following equation: (CL) × MRT_{tf} – CL × 1/2 (infusion duration), where MRT_{tf} was the mean residence time obtained from the ratio of area under the first moment curve from time zero to tf ($AUMC_{tf}$) and AUC_{tf}. CL_r was calculated by dividing A_e in urine over 168 h after IV dosing by AUC_{tf}.

2.5. *Safety*

Safety was assessed based on the results of vital signs, which were measured at screening, immediately prior to dosing (0 h), and then at regular intervals until 168 h following the dose. Electrocardiograms and laboratory assessments were obtained at screening and 168 h after dosing. The intensity (severity) of adverse events was assessed according to the Common Toxicity Criteria (CTC) grading system where applicable, or graded as mild, moderate, severe, or life threatening as defined in the protocol.

2.6 *Statistical analyses*

Summary statistics were determined for the pharmacokinetic parameters of each group. Linear regression analyses were used to determine the relationship between CL_{cr} and CL_r, Vd_{ss}, and F.

3. Results

3.1. *Patient characteristics*

All 14 subjects enrolled received single oral and IV doses of ribavirin (Table 1). Data from all subjects were included in the pharmacokinetic analyses and in evaluations of safety and tolerability.

3.2. *Pharmacokinetic assessment*

After a single oral dose of ribavirin, mean AUC_{tf} increased with the severity of renal dysfunction; however, there was no notable change in C_{max} associated with declining renal function (Table 2). After a single IV dose of ribavirin, mean C_{max} values (at end of ribavirin infusion) were similar in groups I, II, and IV, and slightly higher in group III. In contrast, renal dysfunction was associated with a corresponding increase in mean AUC_{tf} of 1.3-, 2.0-, and 2.0-fold in

Table 1. Demographic and baseline characteristics of study participants

	Group I (*n* = 2)	Group II (*n* = 5)	Group III (*n* = 3)	Group IV (*n* = 4)
Mean age, y (range)	43 (34-52)	52 (30-64)	48 (27-64)	40 (30-50)
Women/men (*n*)	2/0	1/4	2/1	2/2
Race (*n*)				
White	2	3	2	2
Black	0	1	0	1
Hispanic	0	1	1	1
Mean weight, kg (range)	83.5 (74-93)	81.6 (71-92)	68.7 (55-93)	77.3 (47-103)
Mean height, cm (range)	164.5 (160-169)	173.6 (168-180)	169.3 (158-187)	166.8 (157-173)
CLcr (mL/min [%CV])	114 (–)	74 (13)	44 (23)	19 (22)

CLcr, creatinine clearance; %CV, % coefficient of variation.

Table 2. Mean (%CV) pharmacokinetic parameters of ribavirin after single oral and IV 400-mg doses in patients with varying degrees of renal insufficiency

	Group I (*n* = 2) CL_{cr} > 90 mL/min		Group II (*n* = 5) CL_{cr} 61-90 mL/min		Group III (*n* = 3) CL_{cr} 31-60 mL/min		Group IV (*n* = 4) CL_{cr} 10-30 mL/min	
	Oral	IV	Oral	IV	Oral	IV	Oral	IV
CL_{cr} (mL/min)	114[a]		74 (13)		44 (23)		19 (22)	
C_{max} (ng/mL)	339[a]	12,044[a]	829 (53)	13,043 (17)	994 (57)	16,305 (45)	1,274 (24)	1,0791 (37)
AUC_{tf} (ng•h/mL)	5,555[a]	22,924[a]	15,610 (44)	29,341 (20)	25,857 (52)	46,882 (42)	32,574 (21)	47,442 (31)
CL (mL/min)	–	296[a]	–	236 (23)	–	168 (55)	–	151 (30)
CL_r (mL/min)	147.6[a]	107[a]	76.8 (55)	58.1 (36)	26.5 (33)	24.6 (41)	11.8 (30)	10.2 (42)
CL_{nr} (mL/min)	–	189[a]	–	178 (25)	–	143 (59)	–	141 (30)
Vd_{ss} (L)	–	639[a]	–	634 (15)	–	506 (42)	–	469 (25)
A_e (mg)	49.1[a]	145[a]	58.2 (20)	98.6 (31)	36.5 (18)	62.7 (39)	21.9 (21)	26.5 (24)
% Dose	12[a]	36[a]	15 (20)	25 (31)	4 (13)	15.7 (39)	5 (8)	6.6 (24)
%F	35[a]	–	60 (35)	–	57 (30	–	71 (20)	–

AUC_{tf}, area under the serum concentration-time curve from time zero to time of final quantifiable sample; A_e, amount excreted in urine; CL, total plasma clearance; CL_r, renal clearance; CL_{cr}, creatinine clearance; CL_{nr}, non-renal clearance; C_{max}, maximum plasma concentration; IV, intravenous; Vd_{ss}, volume of distribution at steady state; F, absolute bioavailability. [a] %CV not determined when $n < 3$.

groups II, III, and IV, respectively, compared with the mean AUC_{tf} in subjects with normal renal function.

In patients receiving IV ribavirin, mean CL decreased as CL_{cr} declined (mean CL relative to controls was 80%, 57%, and 51% in groups II, III, and IV, respectively). Regression of CL against CL_{cr} was highly statistically significant ($CL = 102 + 1.81 \times CL_{cr}$; $r^2 = 0.64$, $p < 0.001$).

In subjects with normal renal function receiving IV ribavirin, mean ribavirin CL_r was ~36% of CL (107 mL/min *vs.* 296 mL/min). Ribavirin CL_r declined with decreasing renal function (Figure 1). Regression of CL_r against CL_{cr} was highly statistically significant ($CL_r = -13.1 + 0.99 \times CL_{cr}$; $r^2 = 0.88$, $p < 0.001$). Ribavirin CL_{nr} also tended to decline with decreased renal function. Compared with control subjects with normal renal function, the degree of decline in mean CL_r was greater in groups II, III, and IV (54%, 23%, and 10%, respectively) than that in mean CL_{nr} (94%, 76%, and 75%).

Following IV administration, ribavirin Vd_{ss} also declined with decreasing renal function. Regression of Vd_{ss} against CL_{cr} was statistically significant ($Vd_{ss} = 414 + 2.55 \times CL_{cr}$; $r^2 = 0.38$, $p < 0.02$).

Absolute bioavailability following oral administration was 35%, 60%, 57%, and 71% for groups I–IV, respectively. Although absolute bioavailability tended to increase with renal dysfunction, regression of bioavailability (%F) against CL_{cr} was not statistically significant ($p = 0.12$).

3.3. *Safety*

Ribavirin was safe and well tolerated when administered as a single oral or IV dose of 400 mg to subjects with renal dysfunction. Eight adverse events of mild to moderate intensity were reported, the most common being headache ($n = 3$), with individual reports of allergy, hyperglycemia, hypoglycemia, pharyngitis, and thrombocytopenia. Apart from the expected laboratory test abnormalities in subjects with renal dysfunction, there were no changes of clinical relevance noted during this study.

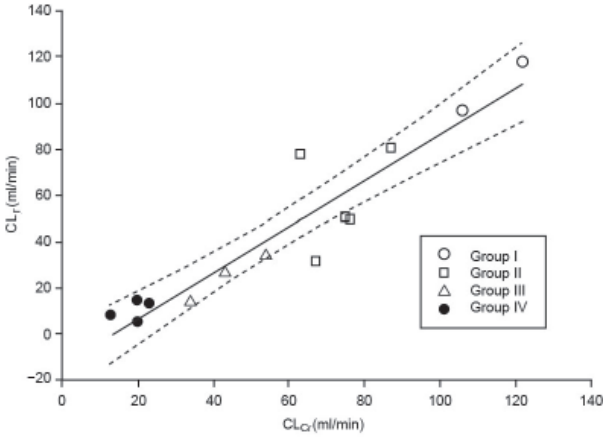

Figure 1. Relationship between individual renal clearance (CL_r) of intravenous ribavirin and creatinine clearance (CL_{cr}) in subjects in groups I to IV. The dotted lines represent the upper and lower bounds of 95% confidence intervals.

4. Discussion

Ribavirin is eliminated by both renal and hepatic routes, with gastrointestinal metabolism accounting for the majority of first-pass elimination of the parent molecule (*24*). Although renal excretion accounts for only 5-15% of the total elimination (*24*), the results of the present study show that the pharmacokinetic properties of ribavirin are significantly altered in subjects with renal dysfunction compared with subjects with normal renal function. We found that ribavirin AUC and C_{max} values were increased, and CL, CL_r, CL_{nr}, Vd_{ss}, and A_e values were reduced in subjects with renal dysfunction compared with controls with normal renal function. The magnitude of these changes increased with the severity of renal dysfunction. We have identified 4 possible mechanisms for the altered pharmacokinetics of ribavirin in subjects with renal dysfunction; these are discussed in the text that follows.

Reduction of renal clearance in absolute and proportional terms

In the present study, mean CL_r after IV dosing in control subjects was 36%, which is similar to previously reported results (*16*). Mean CL_r was 54%, 23%, and 10% relative to subjects with normal renal function in groups II, III, and IV, respectively. In these subjects, the proportion of total CL accounted for by CL_r after IV dosing declined as renal function decreased (25%, 15%, and 7%, in groups II, III, and IV, respectively).

Mean CL_r after oral dosing was 56%, 28%, and 9% compared with controls in groups II, III, and IV, respectively; these values are very similar to those observed after IV dosing. In contrast, in these subjects, the proportion of total CL accounted for by CL_r after oral dosing declined as renal function decreased (14%, 9%, and 5% in groups II, III, and IV, respectively).

Thus, CL_r as a proportion of total CL was always greater after IV dosing compared with oral dosing, both in controls and in subjects with renal dysfunction. This could reflect a reduction in first-pass metabolism, which is substantial after oral dosing (~50%) (*24*). Also, the ratio of oral CL_r:IV CL_r was smallest for subjects with normal renal function (0.42) and was progressively greater as renal function declined (0.56, 0.60, and 0.71 for groups II, III, and IV, respectively). One interpretation of this finding is that the proportion of ribavirin cleared by first-pass metabolism decreases as renal function declines. This would be consistent with the finding that CL_{nr} was reduced and F increased as renal function declined (as follows).

Reduced non-renal clearance in absolute terms

In addition to changes in CL_r, CL_{nr} also decreased as renal function declined. Compared with controls, mean CL_{nr} was 94%, 76%, and 75% in groups II, III, and IV, respectively, after IV dosing. After oral dosing, mean CL_{nr} relative to controls was 54%, 48%, and 27% in groups II, III, and IV, respectively. In a separate study in patients with hepatic dysfunction, there were no changes in single-dose pharmacokinetic parameters in patients with hepatic dysfunction compared with controls (*17*). Thus, the changes in CL_{nr} in the present study may indicate a significant effect of renal dysfunction on nonhepatic sites of ribavirin metabolism. Although the mechanisms underlying changes in nonrenal clearance in subjects with renal dysfunction are poorly understood, this is a common finding with many drugs (*25,26*).

Increased absolute bioavailability

A third possible mechanism is that the higher exposures noted in patients with renal dysfunction could be due to increased absolute bioavailability (F). The present study showed that CL_r as a proportion of total CL was always higher after IV dosing compared with oral dosing, both in control subjects and in patients with renal dysfunction, reflecting the absence of a first-pass metabolism. As previously mentioned, the ratio of oral CL_r to IV CL_r was lowest for subjects with normal renal function and became progressively greater as renal function declined, which may be evidence that the proportion of ribavirin being cleared by first-pass metabolism decreased as renal function declined. This would also be consistent with the reduction in CL_{nr} described earlier. Ribavirin is almost entirely absorbed after oral administration, but undergoes significant first-pass metabolism, and absolute bioavailability is ~50% (*24*). The enzymes responsible for this process and their localization have not yet been identified; however, the site of metabolism is cytosolic and not ribosomal. Hydrolysis to form the carboxamide metabolite is one of the main metabolic pathways for ribavirin (*27*), and hydrolysis reactions are reportedly reduced in chronic renal failure (*28*). Increased absolute bioavailability has been reported in patients with renal dysfunction for a number of other drugs (*26,29-31*), including metoclopramide, erythromycin, propranolol, and other β-blockers.

Reduced volume of distribution

A fourth possible mechanism is that ribavirin Vd is affected by renal dysfunction. In the present study, ribavirin Vd_{ss} declined with decreasing renal function after IV administration, and regression of Vd_{ss} against CLcr was statistically significant. Elimination-phase profiles and $t_{½}$ values after oral administration were similar in patients with declining renal function (in groups II, III, and IV); however, the Vd after oral administration was not determined.

Notable similarities exist between the findings of the present study and the impact of renal impairment on the pharmacokinetics of another purine nucleoside analogue, didanosine (*32*). Both didanosine and ribavirin are substrates for the N1 nucleoside transporter. Renal clearance accounts for ~50% of the total clearance of didanosine in subjects with normal renal function; however, didanosine AUC was increased 4- to 5-fold in patients with end-stage renal disease (*32*). Renal impairment was associated with reductions in didanosine CLr, CLnr, and Vd, but had no effect on absolute bioavailability (*32*). The authors concluded that the changes in didanosine pharmacokinetics observed in patients with renal impairment may be due to altered metabolism of didanosine associated with renal failure.

In the present study, single 400-mg oral and IV doses of ribavirin were generally safe and well tolerated in healthy volunteers with normal renal function and in patients with renal insufficiency. The most commonly reported adverse event, headache, has been reported previously following single- and multiple-dose administration of ribavirin (*15*), and no serious drug-related adverse events were reported.

A possible shortcoming of this study should be acknowledged. Although a relatively small numbers of subjects were enrolled, the pharmacokinetic parameters for each group were similar to those previously reported (*21,22*), and in many analyses renal function was evaluated as a continuous variable.

The present study has identified a number of possible mechanisms (changes in CL_r and CL_{nr}, increased bioavailability, and altered Vd) that might account for the altered single-dose pharmacokinetic profile of ribavirin in patients with renal dysfunction. As ribavirin has a well-established exposure-toxicity profile, it is important to ensure that patients with renal dysfunction are dosed appropriately to avoid excessive hemolysis. Because of the extensive accumulation that occurs with multiple-dose ribavirin pharmacokinetics, this single-dose study cannot provide definitive dosing guidelines for patients with renal impairment (*15,24*). Furthermore, patients with chronic renal dysfunction also appear more likely to develop ribavirin-induced anemia than do control subjects. In addition to ribavirin-induced hemolysis, other factors that may affect ribavirin dosing include inadequate marrow responsiveness to anemia as well as reduced erythrocyte longevity due to uremia.

In conclusion, this study has identified a number of mechanisms to explain the alterations in ribavirin pharmacokinetics in subjects with stable chronic renal impairment, generally similar to those reported previously for didanosine.

Acknowledgments

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Commentary

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Three-dimensional imaging technology offers promise in medicine

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Summary **Medical imaging plays an increasingly important role in the diagnosis and treatment of disease. Currently, medical equipment mainly has two-dimensional (2D) imaging systems. Although this conventional imaging largely satisfies clinical requirements, it cannot depict pathologic changes in 3 dimensions. The development of three-dimensional (3D) imaging technology has encouraged advances in medical imaging. Three-dimensional imaging technology offers doctors much more information on a pathology than 2D imaging, thus significantly improving diagnostic capability and the quality of treatment. Moreover, the combination of 3D imaging with augmented reality significantly improves surgical navigation process. The advantages of 3D imaging technology have made it an important component of technological progress in the field of medical imaging.**

Keywords: Three-dimensional imaging technology, endoscope, augmented reality, surgical navigation

1. Introduction

Medical imaging has played an important role in the diagnosis and treatment of disease in today's world. Medical imaging technology is also needed in medical research and education. Medical equipment currently has two-dimensional (2D) imaging systems that offer 2D slices of whatever or whoever is being examined. Although data of this type can satisfy clinical requirements to a large extent, they poorly depict pathologic changes in 3 dimensions. With the rapid development of three-dimensional (3D) imaging technology, medical imaging is transitioning from 2D patterns to 3D models, thereby providing better visual images and more accurate quantitative results. The advantages of 3D medical imaging, including obvious depiction of spatial locations and logical reasoning, have made it an important component of technological progress in the field of medical imaging.

The use of 3D imaging technology in endoscopy is a typical example. The principle of stereo vision is that different images are observed by the left and right eyes due to parallax and these images are then overlapped on one another by the human brain, thereby resulting in forward and backward, up and down, left and right, and far and near views of an object (*1*). A 2D endoscope has one camera but a 3D endoscope has two cameras. Two separate video signals from slightly different angles are processed by stereo display technology and then shown to observers as stereo images. One advantage of a 3D endoscope is easier surgical navigation. The information gathered by a conventional 2D navigation system cannot be directly interpreted and understood, creating a large gap between conditions in an actual patient and their depiction by an imaging system (*2*). This disadvantage necessitates constant comparisons of the surgical field and a displayed image, thus leading to frequent hand-eye transitions. A 3D endoscope overcomes this drawback in large part and 3D endoscopes have widely been used to perform minimally invasive surgery (*3,4*). A 3D endoscope is a key component of the da Vinci surgical robot (*5*). A surgeon sits at an imaging workstation away from operating table, where he or she operates the system's robotic arms while looking at a monitor that presents 3D images of the surgical site. The use of 3D imaging technology allows the da Vinci surgical robot to perform many complicated procedures that could not be performed with a 2D endoscope.

A feature of 3D imaging technology is its depiction of depth since that technology depicts an object as it

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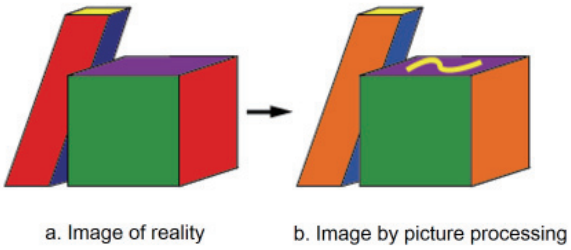

Figure 1. Schematic diagram of 3D imaging technology combined with augmented reality.

would normally appear. This technology has limited use since it is not necessary if an object can be viewed with the naked eye. In the aforementioned example of an endoscope, 3D imaging technology is used to view something that is not visible to the naked eye. Thus, an object can be viewed as a 3D image, which is something that humans are familiar with. Three-dimensional imaging technology is useful for viewing an object that cannot be seen directly by the naked eye, but this technology has limited use in medicine. A more useful approach is to combine 3D imaging technology with augmented reality (*2,6*). Three-dimensional imaging technology produces an image equivalent to that seen with the naked eye, and an actual scene can be depicted virtually using a PC. For example, a surgeon can be shown an image during surgery while keeping his or her eyes on his or her hands. Combining 3D imaging technology with augmented reality allows useful images from that technology to be used during surgery.

Three-dimensional information during surgery can be conveyed by combining 3D imaging technology with augmented reality. For example, a mark can be displayed on an actual object, as shown Figure 1. Color correction can also be done, such as the change in color from red to orange in the same figure. In addition, a surgical procedure can be shown in 3 dimensions. Three-dimensional imaging technology can provide a surgeon with a wealth of information during surgery. Thus, human error during surgery can be reduced. Use of 3D imaging technology in medicine will presumably increase significantly in the future.

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Commentary

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Current clinical uses of the biomarkers for hepatocellular carcinoma

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Summary **Hepatocellular carcinoma (HCC) is a severe condition that is found worldwide. The current methods of HCC screening and diagnosis depend mainly on tumor imaging techniques. Using tumor biomarkers to detect cancer has helped to screen for disease and avoid wasting medical resources. Serum α-fetoprotein (AFP), a glycoform of AFP that reacts with Lens culinaris agglutinin (AFP-L3), and des-gamma carboxyprothrombin (DCP) are biomarkers commonly used to detect HCC in medical practice around the world. However, each of these biomarkers is imperfect when used alone and each has limitations in terms of the sensitivity and specificity with which it detects HCC. Presumably, a combination of these biomarkers is a practical way to improve their performance. That said, novel biomarkers of HCC are being sought to diagnose the disease and also to optimize the treatment modality, to predict prognosis or recurrence, and to discover novel targets for therapeutic interventions.**

Keywords: HCC, biomarkers, AFP, AFP-L3, DCP

Hepatocellular carcinoma (HCC) is one of the most common liver neoplasms in the world, with an estimated global incidence of over 700,000 new cases per year. It is prevalent in Asia and Africa, and its prevalence is increasing in the US, Canada, and Europe (*1*). The molecular pathogenesis of HCC is a complex multistep process in which many signaling cascades are altered, leading to a heterogeneous molecular profile. The management of HCC, including screening, diagnosis, therapy, and preventing recurrence, still poses numerous challenges. Current methods of HCC screening and diagnosis depend mainly on tumor imaging techniques, and especially ultrasonography. Surgical resection and liver transplantation are most promising therapies that offer the best therapeutic outcomes for treatment of HCC, with a 5-year overall survival rate of more than 50%, but they are only feasible for early and intermediate-stage intervention (*2*).

Tumor biomarkers are types of molecules or substances that are objectively measurable in cells, tissues, or body fluids and that indicate the presence or predict the prognosis of tumors. Although the use of serum biomarkers differs in countries in the East and in the West, these biomarkers augment radiological modalities in the current clinical treatment of HCC. The most widely used biomarker of HCC is serum α-fetoprotein (AFP). Serum AFP is commonly regarded as a supplementary parameter for diagnosis and a tool for predicting recurrence and survival. AFP has been found to have a sensitivity of 41-65% and a specificity of 80-90% when detecting HCC, given an AFP cut-off of 20 ng/mL (*3*). A glycoform of AFP that reacts with Lens culinaris agglutinin (AFP-L3) is a second biomarker of HCC. AFP levels are only definitive once a tumor is large in size or in an advanced state, so AFP-L3 is used. Since AFP-L3 is more sensitive and specific for HCC than AFP alone, AFP-L3 is usually assayed with AFP. Des-gamma carboxyprothrombin (DCP) is another well-known biomarker for HCC. Also known as PIVKA II, or protein induced by vitamin K absence/antagonist- II, DCP is an abnormal prothrombin resulting from defective posttranslational carboxylation of the prothrombin precursor. Serum DCP detects HCC with varying levels of success according to studies. DCP is more likely to be elevated in patients with more advanced HCCs (*e.g.* larger tumors, vascular invasion, or metastasis).

Technological advances in genomics, proteomics, and metabolomics have enabled the discovery of novel HCC biomarkers. These newly discovered biomarkers can be roughly divided into the following categories: oncofetal and glycoprotein antigens (including AFP-L3

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Table 1. Clinical uses of the established biomarkers of HCC

Clinical uses	Biomarkers
Screening	AFP, AFP-L3, DCP
Diagnosis	AFP, AFP-L3, DCP, GPC3, AFU, GGT,TSGF, TGF-β
Predicting prognosis	mRNAs, HGF/SF, EGFR, human carbonyl reductase 2

and GPC3), enzymes and isoenzymes (including DCP, GGT, AFU, and Human Carbonyl Reductase 2), growth factors and their receptors (including TGF-β, TSGF, EGFR, and HGF/SF), molecular markers (mRNAs), and pathological biomarkers. Clinical uses of these established HCC biomarkers are shown in Table 1. Unfortunately, no single biomarker can adequately provide a sufficient level of sensitivity and specificity for HCC. A combination of these biomarkers is a practical way to improve their performance. A combined assay of AFP with AFPL3 or des-g-carboxyprothrombin (DCP), which is widely used in Asia and especially in Japan, has better performance than either biomarker alone (*4*). The current study and use of biomarkers is far from comprehensive. Novel biomarkers of HCC could be used to screen for the disease and potentially helpful to optimize the treatment modality, to predict prognosis or recurrence, and to lead to novel targets for therapeutic interventions in the near future.

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Letter

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Suggestions to the media to help us cope with the A/H7N9 crisis in China

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Summary **The A/H7N9 avian influenza virus sparked global concerns about public health. The media published numerous reports about emerging infectious diseases, including their clinical characteristics and genomic information. However, outbreaks of A/H7N9 posed a real challenge to China's emergency management and especially its dealing with the media. This study analyzes ways to deal with the media during A/H7N9 crises and it proposes a shift in public emergency management from an "Impact-Responsive" approach to a "Prevention-Active" approach. A "Prevention-Active" approach should be used when dealing with the media during subsequent outbreaks.**

Keywords: Infectious diseases, avian influenza, media

Since January 2014, some provinces and cities in China have seen continued cases of H7N9 avian influenza. Experts believe that sporadic cases will continue to appear in some cities. This has sparked global public health concerns. Last year, the mass media produced many reports describing this new emerging infectious disease, including its clinical characteristics and genomic information (Figure 1). Outbreaks of A/H7N9 posed a real challenge to China's emergency management, and especially its dealings with the media. Questioners often ask "Will A/H7N9 ultimately prove to be controllable? Will it remain limited to animals? Or will it, like the A/H1N1 virus, adapt to humans and cause a pandemic?" My colleagues and I believe that A/H7N9 has many of the traits that make a new flu virus worrisome. Nevertheless, the fine line between foresight and alarmism can only be accurately drawn in retrospect, and we still have long way to go in terms of developing methods to combat this type of emerging infectious disease. Three aspects should be apparent when assembling information (Figure 2).

China has created a public emergency management system. However, we are mindful that in combating emerging infectious diseases, one of our aims is to gain public confidence through understanding and thus limit public panic. The media play a crucial role in this process. We have shifted from an "Impact-Responsive" approach to mass media, *i.e.* highlighting the response once an incident has occurred, to a "Prevention-Active"

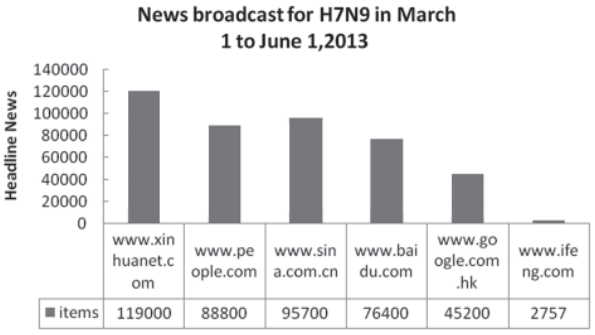

Figure 1. Headline news about H7N9 in major mass media during the crisis from March 1 to June 1, 2013.

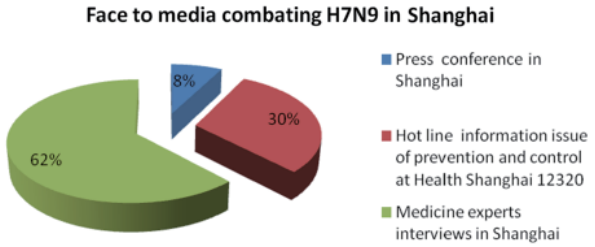

Figure 2. Dealing with the media during the H7N9 crisis in Shanghai in 2013.

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approach, *i.e.* highlighting active prevention and an active response. Emerging infectious diseases (EIDs) are defined as infectious diseases with an incidence that has increased over the past 35 years and that may increase in the near future. These diseases account for about 12% of all human infections due to pathogenic microbes. More emerging infectious diseases will strike in the future. We have a number of tools at our disposal, and one is mass media. The media has a huge impact! The crisis of A/H7N9 avian influenza last year gave us a chance to experience this first-hand. We must capitalize on the media when a similar crisis occurs in the future. Clearly, interaction between the health system and mass media has become more important with the development of modern reporting technology. This prompts suggestions regarding how we deal with the media in the face of the A/H7N9 crisis. We offer suggestions to the media to help us cope with the H7N9 crisis in China.

During the severe acute respiratory syndrome (SARS) epidemic, laboratory scientists and physicians lacked unity, they failed to take advantage of scientific research on SARS, and they lost the confidence of mass media (*1-3*). But substantial progress in this respect has been made during the A/H7N9 crisis, as documented by the New England Journal of Medicine (*4*). Our report was posted as a Morbidity and Mortality Weekly Report (MMWR) and was released early on the US Center for Disease Control's (CDC) website (*http://www.cdc.gov/mmwr*). Epidemiologic notes and reports and updated editorial notes from the US CDC offer constant reminders. The World Health Organization issued a Guidance Document on "Clinical management of severe acute respiratory infections when H7N9 influenza is suspected: What to do and what not to do". Sharing of data on clinical features and treatment of patients is essential to improving understanding of a disease and to refining optimal case management. These steps are termed "Crisis Communication". However, we are always mindful of SARS, for which we paid a very heavy and painful price 10 years ago (*5*). One aim of combating emerging infectious diseases is to gain public confidence and thus limit public panic. The media play an important role in this process. Over the past few years, many more influenza strains that are capable of infecting humans have emerged (*6,7*). So how should we deal with the media? We must be diligent. News management is crucial in the event of a public health crisis. Mass media has greatly affected crises and impacted peoples' everyday lives, the social order, and even the power of government (*8,9*). We have learned lessons from news management during the SARS crisis and so has China's mass media, as shown by its commendable efforts during the recent epidemic.

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