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Standardization of traditional Chinese medicine and evaluation of evidence from its clinical practice

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ABSTRACT: Traditional Chinese medicine (TCM) is a typical traditional medicine (TM) with a long-standing history of preventing and curing diseases in China and other countries in East Asia. Standardization of TCM has been a topic of discussion over the past few decades in China with the goal of promoting advances in TCM in China and elsewhere around the world. Many quality and safety control standards for TCMs have been implemented in China, but systematic standards of efficacy have not been established for TCMs until now because of the absence of evidence from clinical practice. Evidence-based medicine (EBM) is the best way to provide evidence from clinical practice, but the quality of current EBM studies of TCM, and especially randomized controlled trials (RCTs) of TCM, needs to be improved. International registration of clinical trials (CTs) of TCM is a good way to provide quality evidence from clinical practice of TCM because it can improve research transparency and ultimately enhance the validity and value of scientific evidence. This evidence will provide the springboard for efforts to standardize TCM.

Keywords: Traditional medicine, Chinese medicine, evidence, standards system

1. Introduction

In recent decades, a serious situation has developed with the constant appearance of drug side effects and drug tolerance; this situation is compounded by the relatively high cost and length of time needed to develop new drugs. In contrast, the traditional Chinese medicine (TCM), one of the typical traditional medicines (TMs), is low-cost and contains natural products of therapeutic value (*1,2*). Several systematic reviews of rigorous clinical trials (CTs) have shown that many TCM, such as Andrographis, Ginkgo, and Nettle, have therapeutic value in treating certain conditions (*3*). As a result, TM and the TCM in particular have played an increasingly important role in modern health care, with the potential for new or improved clinical protocols and reduced treatment costs (*4*).

The World Health Organization (WHO) stresses that TM can play an important role in achieving the goal of "Health for All" and is dedicated to facilitating the integration of TM and Western medicine worldwide (*5*). TMs have been, and continue to be, used in every country around the world in some capacity. In much of the developing world, 70-95% of the population relies on these TMs for primary care, and the global market for TM was estimated to be US$ 83 billion annually in 2008, with a rate of increase that has been exponential (*6*). In 2000, the WHO proposed that all TMs should be evidence-based (*7*). In order to provide evidence, many countries have promoted CTs of TM in recent years. According to the CTs registry and results database affiliated with U.S. Food and Drug Administration (FDA) and National Institutes of Health (NIH), 739 CTs of TM and 228 CTs of TCM have been registered thus far worldwide (*8*).

2. Standardization of TCM in China

With a long tradition and over 2500 years of continuous practice and refinement through observation, testing, and critical thinking, TCM has played a key role in preventing and curing diseases in China and other countries in East Asia (*9,10*). In order to encourage advances in TCM, the Chinese Government has devised several national standards for TCM over the past few decades (Table 1).

The Chinese Pharmacopoeia is the cornerstone of national standards for TCM. The first edition was compiled by the Ministry of Health of the People's

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Table 1. The National Standards on TCM in China

Years	A series of standards
1953	Chinese Pharmacopoeia
1989	Drug Standards of the Ministry of Health (Finished Herbal Products Volume)
1992	Drug Standards of the Ministry of Health (Herbs Volume)
1999	Regulations for New Drug Approval
1999	Good Clinical Practices
1999	Good Laboratory Practices
2000	Good Supply Practices
2001	Good Manufacturing Practices
2002	Good Agriculture Practices
2005	Clinical Medicine Guidelines (Traditional Chinese Medicines Volume)
2010	Clinical Medicine Guidelines (Herbal Materials Volume)

Republic of China in 1953 and was then updated in 1963, 1977, 1985, 1990, 1995, 2000, 2005, and 2010 with additional records incorporated (*11*). The latest edition (the 2010 edition) describes aspects of 2,136 TCMs in terms of properties, processing, function, *etc*. (*12*). The Drug Standards of the Ministry of Health provides quality control standards for herbs and finished herbal products (*13-15*). In order to guide medical personnel in the scientific use of the Chinese Pharmacopoeia, the Clinical Medicine Guidelines have been published as support of the Chinese Pharmacopoeia since 1990. The Traditional Chinese Medicines Volume was first compiled in the 2005 edition of the Clinical Medicine Guidelines (*16*), which stress the theory and clinical practice of TCM as well as the integration of TCM with modern pharmacology and clinical medicine. The Traditional Chinese Medicines Volume records 1,460 finished herbal products, including all of those in the Chinese Pharmacopoeia, National Essential Drugs List, and Catalog of Drugs for Basic National Medical Insurance. The Herbal Materials Volume was first compiled in the 2010 edition of the Clinical Medicine Guidelines (*17*) and describes aspects of 656 herbal materials in terms of properties, processing, function, *etc*. The Herbal Materials Volume plays an important role in guiding the clinical practice of TCM. The Chinese Government has also devised and implemented several standards to regulate the pharmaceutical industry, such as Regulations for New Drug Approval, Good Laboratory Practices (GLP), Good Clinical Practices (GCP), Good Manufacturing Practices (GMP), Good Supply Practices (GSP), and Good Agricultural Practices (GAP) (*18,19*). The Chinese Government has also promoted the internationalization of TCM in recent years. Announced in 2006, the Development Plan on Standardization of TCM (2006-2010) intends to draft or revise 500 standards (including 50 national standards) implemented in China, sponsor 3-4 international standards, and participate in drafting at least 20 international standards (*20*).

However, the current reality is that the current national standards on TCM focus mainly on identification, processing, and manufacture to control the quality and safety of TCMs. Few standards for the efficacy of TCM have been implemented. The Clinical Medicine Guidelines describe the function of TCMs, the adverse reactions they cause, precautions regarding their use, *etc.*, but evidence regarding usage, dosage, efficacy, and properties of TCMs is mainly based on traditional theories of TCM rather than data from modern scientific research in accordance with evidence-based medicine (EBM). Moreover, there are many limitations in promoting the standards for TCM in Western countries (*21*). This is mainly because a TCM prescription is usually complex and involves a mixture of various bioactive compounds that have diverse mechanisms of action and synergistic/combinational effects. And the prescription emphasizes the overall condition of the individual patient and adopts a holistic approach rather than a particular course of disease or allopathic approach (*19*). Thus, some professionals are skeptical and critical of TCM because they think it is based on inaccurate and mysterious interpretations and experientialism rather than scientific evidence such as definite pharmacokinetic analysis, toxicity testing, and double-blinded clinical trials (*22*).

Given this situation, many measures have been taken in China to further promote the standardization of TCM. In the Chinese Pharmacopoeia (2010 edition) (*12*), the names of Chinese herbal medicine and their Latin names have been corrected in accordance with international standards. Many advanced techniques and methods, such as liquid chromatography/mass spectrometry (LC/MS), DNA molecular identification, have been used to identify components to enhance the sensitivity and specificity of analysis. Measures to control impurities and sterility tests are also required in accordance with pharmacopoeias from Europe and the U.S. With regard to safety standards, inductively coupled plasma mass spectrometry (ICP-MS) is required to test for arsenic, mercury, lead, cadmium and copper in medicines in the Chinese Pharmacopoeia (2010 edition) in order to further control heavy metals, hazardous materials, impurities, residual solvents, and other components. Many Chinese experts are exploring the standardization of TCM, such as standardized verfication (*23*), standardized processing (*24*), and standardized good usage practices (*25*), with using advanced science and technology . Recently, experts built a combination system to predict the properties of medicines in herbals based on their chemical components (*26*). The system had an accuracy of 83.3% for a training set of medicines and 81.0% for a test set. Experts believe that this system will characterize medicines in herbals with TCM properties to help design new prescription for better therapies.

3. Evidence of TCM from clinical practice

Many national standards on TCM have been established in China over the past few decades, and many advanced techniques and methods have also been implemented in recent years to regulate the identification, processing, and manufacture of TCMs, but the established standards focus mainly on quality and safety control of TCMs. Systematic standards for the efficacy of TCMs have not been established until now. The absence of evidence from clinical practice precludes the drafting of standards of efficacy for TCMs. EBM is the best way to provide evidence from clinical practice of TCM and includes evidence from prospective randomized controlled trials (RCTs) of TCMs and evidence from systematic reviews or meta-analysis of RCTs on TCMs.

EBM was introduced in China in 1996. Experts systematically reviewed 3,312 RCTs on TCMs published in 13 journals from mainland China from 1980 to1998 and found that few were RCTs (RCTs accounted for 10.06% of 32,939 clinical research papers in total) and that many were of poor quality (*27,28*). With the development of EBM, experts systematically evaluated 7,422 RCTs on TCM from 26,263 clinical research papers published in mainland China from 1999 to 2004 according to the Cochrane Handbook, Consolidated Standards of Reporting Trials (CONSORT) checklist, Jadad scale, and similar information. They found that the proportion of published RCTs in relation to all types of published CTs increased from 18.6% in 1999 to 35.9% in 2004 ($p < 0.001$) (Figure 1), representing a significant increase. The quality of reported RCTs on TCM also improved, but remains poor overall. Out of a total of 7,422 RCTs, only 587 (7.9%) were randomized or used sequentially generated patient identifiers, 55 (0.7%) were adequately double-blinded, and 823 (11.1%) mentioned drop-outs (*29,30*). A number of other studies have also evaluated the quality of published RCTs on TCM (*31-35*) and found that the poor methodological quality limited the quality of the trial. Flaws included a small sample size, no double-blind testing, lack of long-term outcomes, lack of compliance data, incomplete follow- up data, failure to quantitatively express efficacy, and failure to include data on baseline characteristics or side effects. Experts also assessed the quality of systematic reviews and meta-analysis of TCM published in mainland China from 1994 to 2006 according to the 18 items of the Quality of Reporting of Meta-analyses (QUOROM) Statement and related information. They found that few systematic reviews or reports of meta-analysis met international standards due to insufficiently described methodology or lack of reproducibility (*36*).

Internationally, the trend is to promote multi-center, double-blind CTs with a large sample in order to provide the best evidence from clinical practice. The Declaration of Helsinki states that "Every clinical trial must be registered in a publicly accessible database before recruitment of the first subject." The WHO stresses that international CTs registration will improve research transparency and ultimately enhance the validity and value of scientific evidence. International CTs registration means the publication of an internationally-agreed set of information about the design, conduct and administration of CTs (*37*). Many authoritative international CTs registry platforms have been established worldwide, such as the International Clinical Trials Registry Platform (ICTRP) administrated by the WHO, the Clinical Trials.gov (*www.clinicaltrials.gov*) affiliated with the U.S. FDA and NIH, and the National Research Register (*www.nrr.nhs.uk*) administrated by the U.K. National Health Service (NHS). All of these platforms allow free registration of CTs by investigators from all over the world and numerous aspects, such as study type, study design (allocation, intervention model, masking, *etc.*), the inclusion/exclusion criteria for eligibility, implementation site, are required to register a CT. According to Clinical Trials.gov, 228 studies on CTs of TCM have been registered thus far; of 132 studies sponsored by China (*38*), 83 are from mainland China, 27 are from Hong Kong, and 22 are from Taiwan. Of a total of 132 studies, 50 have been completed, 35 are recruiting, and 47 are not recruiting or have an unknown status. Moreover, 112 studies are randomized and 73 studies are double-blind, respectively accounting for 84.8% and 55.3% of the 132 studies in total.

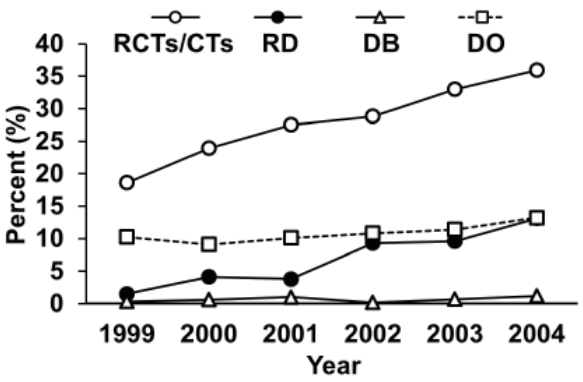

Figure 1. Distribution of RCTs on TCM published in 13 journals from mainland China from 1980 to 2004 and their Jadad scores. RCTs: randomized controlled trials; TCM: traditional Chinese medicine; CTs: clinical trials; RD: randomization; DB: double-blind; DO: dropouts.

In conclusion, the quality of evidence from clinical practice of TCM must be improved and multi-center, double-blind RCTs of TCM with a large sample should be conducted. International registration of CTs on TCM is a good way to provide quality evidence from clinical practice of TCM because it can improve research transparency and ultimately enhance the validity and value of scientific evidence. This evidence will provide the springboard for efforts to standardize TCM.

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Review

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Effective neurofibromatosis therapeutics blocking the oncogenic kinase PAK1

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ABSTRACT: Neurofibromatosis (NF) is a family of genetic diseases which are caused by dysfunction of either *NF1* gene or *NF2* gene. One in 3,000 people suffer from this tumor-carrying NF. *NF1* gene product is a RAS GTPase activating protein (GAP) of 2,818 amino acids, which normally attenuates the GTP-dependent signal transducing activity of the G protein RAS. Dysfunction of this GAP leads to the abnormal activation of RAS, and eventually an oncogenic kinase called PAK1 as well. *NF2* gene product is "Merlin" which directly inactivates PAK1. Thus, dysfunction of Merlin causes the abnormal activation of PAK1. In other words, dysfunction of *NF1* gene (causing type 1 NF) is basically the same as dysfunction of *NF2* gene (causing type 2 NF). In fact the growth of both NF1 and NF2 tumors requires PAK1, and all PAK1 blockers, synthetic chemicals or natural products, suppress the growth of these NF tumor cells both *in vitro* (cell culture) and *in vivo* (mice).

However, until recently, no FDA-approved effective NF therapeutics is available on the market. Here a series of anti-PAK1 products shall be introduced, which would be potentially useful for the life-long treatment of NF patients in the future. These include the most potent HDAC (histone deacetylase) inhibitor FK228 (IC_{50}: around 1 nM), that eventually blocks PAK1, the direct PAK1 inhibitor PF3758309 (IC_{50}: around 10 nM), a CAPE (caffeic acid phenethyl ester)-based propolis extract called "Bio 30" from NZ (New Zealand), and an ARC (artepillin C)-based green propolis extract (GPE) from Brazil. Although the first two drugs are potent, none of them is available on the market as yet. The last two natural (bee-made) products are available on the market, and have been used for the therapy of NF and tuberous sclerosis (TSC) as well as many PAK1-dependent solid cancers such as breast and pancreatic cancers as well as glioma, which altogether represent more than 70% of all human cancers. Since PAK1 is not essential for the normal cell growth, propolis extracts cause no side effects.

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Keywords: NF, cancer, propolis, AMPK, FOXO, lifespan, TSC

1. Introduction

The genetic disease or disorder now called neurofibromatosis (NF in short) was first recognized in 1882 by a German pathologist at Strasbourg University, Friedrich Von Recklinghausen (1883-1910). Later, by the detailed genetic analysis, NF was revealed to have two distinct types, type 1 (NF1) and type 2 (NF2). Dysfunction of *NF1* gene causes NF1, and that of *NF2* gene does NF2. *NF1* gene product has two distinct functional domains. The C-terminal half is a RAS GTPase activating protein (GAP), which attenuates the GTP-dependent signal transducing activity of RAS, a G protein, whose mutation causes malignant transformation (*1*). The N-terminal half is an inactivator of another G protein called RAC (*2*), which is activated by RAS through the PI3-kinase, a unique phospholipid kinase that phosphorylates PIP2 to produce the oncogenic PIP3. Dysfunction of either N or C-terminal half leads to the abnormal activation of the kinase PAK1, a RAC/CDC42-dependent Ser/Thr kinase (*1-4*) (for detail, see Figure 1). *NF2* gene product is Merlin, which turned out to be a direct inhibitor of PAK1 (*4*). Thus, dysfunction of *NF2* gene also leads to the abnormal activation of PAK1. In other words, although NF1 and NF2 are genetically distinguished, phenotypically at the molecular levels, the outcome of these two diseases is basically the same. In fact, both NF1 and NF2 require PAK1 for their growth (*3,4*). NF1 tumors are roughly divided into three types: malignant peripheral nerve sheath tumor (MPNST), plexi-form and dermal neurofibroma (wart-like skin tumor). MPNST is a cancer, but the remaining two are benign, and represent 90% of NF1 tumors. NF2 tumors are divided into two types, meningioma and schwannoma, and both are benign tumors developed in brain and along spine, and lead to loss of eye sight or hearing or both, and in the worst cases cause the total paralysis. Unlike most of cancers, both NF1 and NF2 develop even in the very early stage of life such as 6 months after the birth, and last for the rest of life, increasingly getting worse, without a proper treatment.

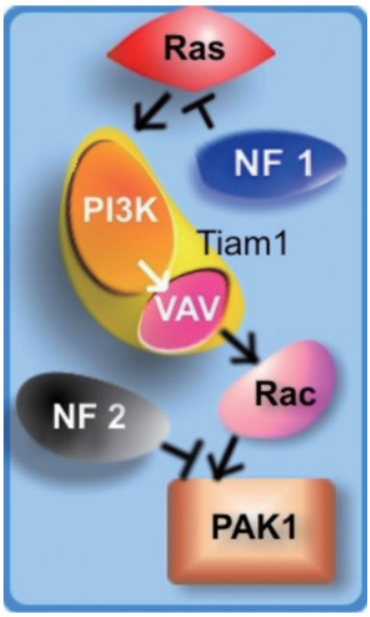

Figure 1. Oncogenic RAS pathways leading to the activation of PAK1. Oncogenic RAS mutants activate the kinase PAK1 through PI3-kinase and VAV or Tiam1 that activate the GTPases RAC/CDC42. This oncogenic signaling is blocked by two tumor suppressors, *NF1* gene product (RAS GAP) and *NF2* gene product (Merlin).

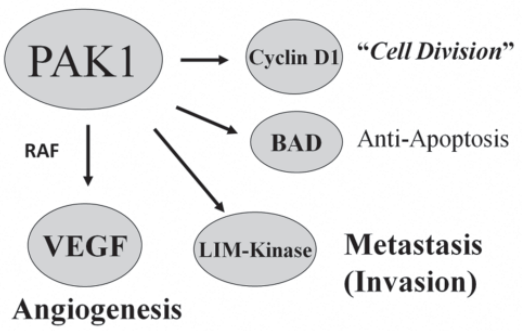

Figure 2. Oncogenic roles of the kinase PAK1.

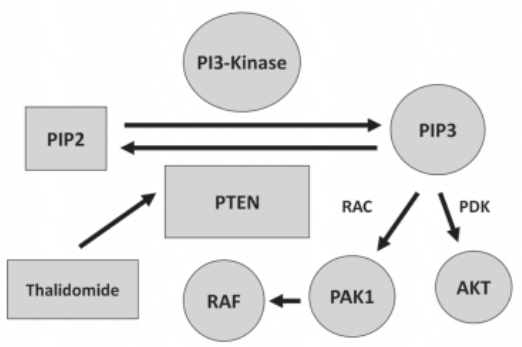

Figure 3. PTEN reverses the oncogenc PI3-kinase signaling.

Thus, unlike the cancer therapy, the systemic treatment of both NF1 and NF2 has to last life-long, and would be certainly desirable to be both safe and inexpensive. Besides, none of conventional anti-cancer drugs such as DNA/RNA/MT (microtubule) poisons is effective on either NF1 or NF2 tumors. Currently the only effective treatment of both NF1 and NF2 are physical treatments, such as surgical removal and gamma knife (a pin-point Cobalt radiation). However, these invasive treatments often cause significant damage on a variety of nerves in the vicinity of tumors, leading to the irreversible loss of the corresponding functions. Furthermore, in the case of NF1, around a half tends to suffer from a learning deficit (LD), although its extent varies from one person to another. This LD cannot be cured by any physical treatment, because its cause is not tumor *per se*, but the abnormal activation of PAK1 in brain which impairs the learning process. In addition, as mentioned before, NF1 patients, in particular NF1 children, have a fragile bone structure, mainly due to vitamin D3 deficiency (*5*). However, this deficiency cannot be eliminated by any physical treatment. Thus, in principle, the ultimate cure of both NF1 and NF2 should owe to either gene therapy using the functional *NF1* or *NF2* gene, or chemotherapy using chemical compounds or natural products which selectively inactivate PAK1. However, in reality, the expression of *NF1* or *NF2* gene in the whole body of each NF patient cannot be achieved by the current level of gene therapy technology. Thus, the most realistic possibility would be the chemotherapy using specific PAK1 blockers.

During past several years since *NF2* gene product "Merlin" was identified as the direct PAK1 inhibitor (*4*), a series of PAK1 blockers were developed or identified by several groups including our own team. In the following sections some of them will be introduced for NF patients and clinicians, which would be useful for the systemic NF therapy at present or in near future.

The oncogenic kinase PAK1 is involved in the following several aspects of oncogenesis (malignant transformation of normal cells) by activating several kinases such as RAF, ERK, and LIM-kinase as well as growth factor genes encoding TGFα and VEGF (see Figure 2): anchorage-independent cell growth, blocking apoptosis (programmed cell death), metastasis/invasion and tumor-induced angiogenesis (blood vessel formation) which is essential for the growth of solid tumors such as NF tumors (*6*,*7*). Thus, unlike the conventional anti-cancer drugs which fail to block both metastasis and angiogenesis of tumors, PAK1 blockers could suppress these malignant aspects of tumors, in addition to killing directly the tumor cells.

By the same token, however, PAK1 blockers cannot be used for the treatment of "pregnant" patients, because like thalidomide these PAK1 blockers might cause the abnormal child birth (teratogenesis) by harming the angiogenesis essential for the normal development of embryos. For thalidomide is now well known to block the PAK1-dependent angiogenesis, and in fact inactivates both PAK1 and another oncogenic kinase called AKT by up-regulating the tumor suppressing phosphatase "PTEN" (*8*), the major antagonist of the oncogenic PI3-kinase, reconverting the oncogenic PIP3 to the inactive PIP2 (see Figure 3). However, it is now clear that the

anti-angiogenesis alone is not sufficient for causing the teratogenesis, because a few thalidomide derivatives such as "CC-5013", which are still anti-angiogenic, are not teratogenic at all (*9*).

In my opinion, AKT blockers including thalidomide and its non-teratogenic derivatives would not be suitable for the life-long treatment of NF, because unlike PAK1, AKT is essential for the growth or survival of normal fast growing cells such as those in bone marrow, and the GLUT-4 dependent glucose uptake by normal cells. In other words, AKT inhibitors cause a series of side effects including immuno-suppression and insulin-resistant diabetes (type 2). PAK1 blockers alone cause basically no side effect.

2. Natural products

2.1. *Propolis*

Propolis is a bee product which has been used for four thousand years since the ancient Egyptian era to treat wounds, infectious and inflammatory diseases, because it has at least anti-bacterial, anti-fungal and anti-viral activities, and boosts the immune system. In other words, it is a traditional multi-functional antibiotic produced by honey bees. Originally back to a million years ago, bees created this resinous material from young buds of poplar trees and a few other plants to protect their larva from any harmful microorganisms or stresses. The wall and bottom of each hexagonal honey comb consists of propolis and fat/wax. In general ethanol-soluble extract (tincture) of propolis is used for topical treatment of wounds. Its long-lasting strong anti-biotic effect is clearly revealed by the fact that propolis was used to preserve the deceased royal family's bodies as mummies under pyramids from the ancient Egyptian era till today. Hippocrates (460-370 BC), the father of medicine in the ancient Greece, recognized its potent healing power, and coined it "Propolis", meaning the protection of city ("polis") or comb. Since he survived around 90 years in the ancient Greece where the average life span of human beings was only 50 years, I tend to believe that he also took propolis regularly, which extends the life span as well, as we shall discuss later.

There is an old German saying: no beekeeper catches cancer. More precisely, only one in 3,000 beekeepers get a cancer, while one in three ordinary people (non-beekeepers) once suffers from a cancer during their life, according to a study by the German Beekeepers' Association (DBB). So the most intrigue question would be why beekeepers are a thousand times more resistant than the ordinary people to cancers. Since neither honey nor royal jelly has any significant anti-cancer activity, it is most likely that a third bee product, namely "propolis", is the major source which makes beekeepers so resistant to cancers. Yes, in 1988, a Jewish-American oncologist at Columbia University, Drizer Grunberger and his colleagues found that propolis from Israel contains an anti-cancer polyphenol called CAPE (caffeic acid phenethyl ester) (see Figure 4) and his colleague Koji Nakanishi succeeded in the chemical synthesis of CAPE (*10*). Since then, propolis became a popular alternative cancer therapeutic, partly because it is rather inexpensive and causes little side effect.

However, it turned out that the CAPE content of propolis vastly varies from one sample to another, depending on where it is produced or harvested. For instance, propolis samples from Far-East, Europe/US, and Oceania are largely the CAPE-based, but those from Egypt, India and Brazil contain no CAPE. Nevertheless, Brazilian green and red propolis showed a strong anti-cancer property. In 1994, a group at Hayashibara Institute in Japan found that the major anti-cancer ingredient in Brazilian green propolis extract (GPE) is another polyphenol called ARC (artepillin C) (*11*), and Hitoshi Hori and his colleagues at Tokushima University in Japan succeeded in the chemical synthesis of ARC in 2002 (*12*) (see Figure 5). Brazilian red propolis extract (RPE) has neither CAPE nor ARC, but contains a triterpene, and suppresses the growth of pancreatic cancer cells *in vitro* (*13*). So an interesting question has arisen: is there any common mechanism underlying the anti-cancer property among these three distinct propolis samples? In 2005, the first clue to the molecular mechanism of CAPE to suppress the growth of cancers was revealed: caffeic acid (CA) down-regulates a G protein called RAC which is the direct activator of the oncogenic kinase PAK1 (*14*). Since CAPE is a hydrophobic derivative of CA, it is most likely that CAPE also down-regulates RAC, and eventually inactivates PAK1. Yes, we confirmed that CAPE indeed blocks PAK1 in a tiny nematode called

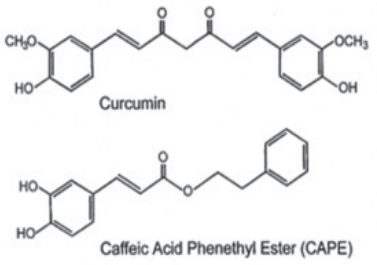

Figure 4. CAPE and curcumin.

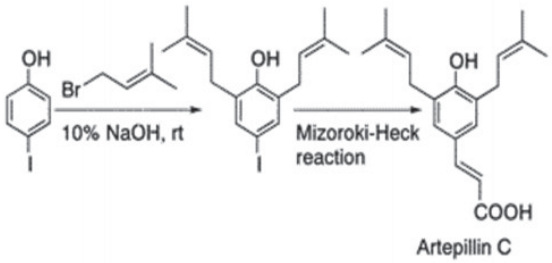

Figure 5. Chemical synthesis of artepillin C.

C. elegans, and activates the *HSP16* gene which is normally inactivated by PAK1 (*6*). Interestingly, ARC also activates the *HSP16* gene in this worm, strongly indicating that ARC also inactivates PAK1 (*6*). In 2007, we confirmed that ARC also indeed inactivates PAK1 selectively, without affecting another oncogenic kinase AKT (*15*). Thus, during 2006-2007, we examined if a CAPE-based propolis extract from NZ (New Zealand) called "Bio 30", and the ARC-based GPE suppress the growth of both NF1 and NF2 tumor xenografts in mice. Yes, both "Bio 30" (see Figure 6) and GPE almost completely block the growth of *NF1*-deficient MPNST and *NF2*-deficient tumor (schwannoma) *in vivo* (*15,16*). Although ARC is the sole anti-cancer ingredient in the GPE, representing 8% of dry weight of this extract, "Bio 30" contains not only CAPE but also several other anti-cancer polyphenols such as pinocembrin, galangin, chrysin, apigenin and CA (*17-21*), representing more than 24% of dry weight of this extract (for detail, see Table 1), and these polyphenols work synergistically with CAPE, and boost the anti-cancer/NF activity of CAPE alone by 600 times *in vitro* (*16*).

For 3-4 years since mid-2007, we have been conducting human trials of "Bio 30" (alcohol-free liquid) containing 250 mg of extract/mL, mainly for NF patients world-wide, and the effective minimum daily dose of "Bio 30" (25 mg/kg = 1 mL/10 kg) has stopped the growth of their tumors in most cases of both NF1 and NF2 patients as well as glioma and pancreatic cancer patients, without any side effect (*22*). Furthermore, in three NF1 (dermal neurofibroma) cases, tumors completely disappeared in a month. Also at least in three cases of NF2 (both schwannoma and meningioma) and one glioma case, these brain tumors shrank by more than 50% in 2-3 years. In two pancreatic cancer cases, both early and metastasized "terminal" cancers completely disappeared in one year (*22*). These preliminary clinical trial data strongly suggest that at least "Bio 30" is effective in suppressing the growth of both NF1 and NF2 tumors as well as glioma and pancreatic cancers for which the conventional anti-cancer drugs are basically useless. However, it should be worth noting that unlike the "Bio 30"-sensitivity of NF/cancer-carrying "cloned" nude mice which are genetically identical, that of these NF/cancer patients turned out to vastly vary from one person to another unsurprisingly, not only depending on the huge difference in their symptom *per se*, but also that in their genetic background. For instance, to cure an early pancreatic cancer needs only 25 mg/kg of "Bio 30" daily for a year, to cure a terminal metastasized pancreatic cancer needs 200 mg/kg of "Bio 30" for a year. The great advantage in "Bio 30" is that even such a massive daily dose causes no side effect. Likewise, shrinking a few wart-like dermal NF1 tumors with 25 mg/kg daily needs only a few weeks, while shrinking either large plexi-form NF1 tumors or NF2-associated brain tumors with the same minimum daily dose needs 2-3 years.

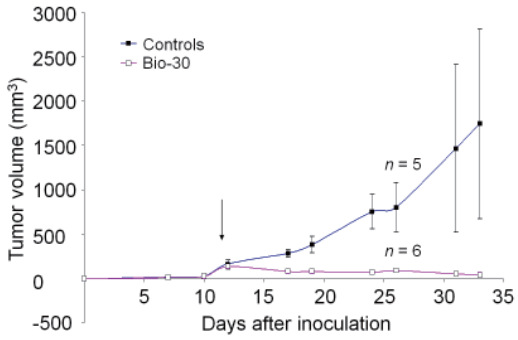

Figure 6. "Bio 30" shrinks the NF2 tumor (NF2-deficient Schwannoma xenograft) in mice.

Table 1. Polyphenol content (mg/g) in "Bio 30" *versus* Chinese red propolis extract (RPE)

Polyphenols	"Bio 30" (*16*)	Chinese RPE (*23*)
CAPE	12	17
CA (caffeic acid)	12	13
Pinocembrin	110	84
Galangin	60	37
Chrysin	30	50
Apigenin	12	ND*

* Not Determined.

Although we have not done any systematic clinical trials of GPE for NF patients, at least in two NF1 (dermal neurofibroma) cases, GPE (8 mg/kg, daily) was sufficient to shrink these skin tumors completely in a month or so, suggesting that GPE is also useful for NF patients. For the last decade, GPE has been used for human trials for a variety of PAK1-dependent cancers such as pancreatic, colon, gastric, breast, prostate, lung, liver, ovarian, cervical, and thyroid cancers as well as glioma, melanoma, and multiple myeloma (MM) which represent more than 70% of cancers, and in many cases, GPE has been proven to be effective in either stopping their growth or shrinking these solid cancers. Thus, I trust that GPE would be useful for both NF1 and NF2 patients as well. The only minor problem with GPE is that it costs several times as much as "Bio 30" which costs only a dollar daily, for a life-long treatment of NF. On the other hand, like any other CAPE-based propolis, "Bio 30" causes an allergic skin reaction only in 1% of population, while GPE causes no allergic reaction.

There are at least two distinct red propolis extracts (RPEs). One is Brazilian RPE, which contains the anti-cancer di-terpene and tri-terpene, and blocks the PAK1-dependent growth of human pancreatic cancer cells with the IC_{50} around 10 μg/mL (*13*), strongly suggesting that these terpenes also block PAK1, and would be potentially useful for NF therapy in the future. However, it remained unavailable on the market until a US company called "JuneBees" recently started selling 30 mL bottles of RPE on-line (junebees.com). Like GPE, this Brazilian RPE is rather expensive, currently costing US$ 70 per bottle. The other RPE is a Chinese brand

from Shandong province, and just like "Bio 30" contains CAPE and several other anti-cancer polyphenols (for detail, see Table 1). This Chinese RPE has the IC_{50} (around 4 μg/mL), similar to that of "Bio 30", to inhibit the PAK1-dependent angiogenesis (*23*), suggesting that the Chinese RPE might be useful for the treatment of NF.

GPE is generally provided in capsules, but "Bio 30" is currently provided as a water-miscible PG (propylene glycol) solution or tincture (75% ethanol solution). Thus, "Bio 30" tastes rather bitter, and when diluted with water, it tends to form a sticky precipitate. In an attempt to improve both the taste and absorption through the gastrointestinal membranes, Manuka Health in NZ and Cyclochem in Japan have jointly developed a new "Bio 30" product which is encapsulated in a natural ring oligo sugar called gamma cyclodextrin (CD). This water-soluble CD (see Figure 7), enzymatically synthesized from starch, has a hydrophobic cavity inside of this nano particle, which is able to capture any hydrophobic small molecules such as those in propolis (*24*). Since the "bitter" polyphenols are inside of CD, propolis extract would be no longer bitter, being suitable in particular for young children, and this CD complex, a mixture of "Bio 30" and CD (1:3 by dry weight), forms a stable and homogeneous emulsion which no longer precipitates. "Bio 30" in this complex could be more efficiently absorbed by intestinal membranes, because bile acids, stored in gallbladder and are secreted into intestine, then replace CD to form a nano micelle of "Bio 30" (*24*). This CD complex of "Bio 30" is currently being tested for its therapeutic efficacy on human cancer xenograft in mice, before the commercialization. However, it is possible to prepare a home-made CD complex, simply by mixing "Bio 30" (alcohol-free liquid) and 20% CD solution at the ratio of 1:5 by volume on a kitchen table of NF patients, and according to a preliminary human trial, the CD complex appears to be more effective than "Bio 30" alone to shrink at least dermal neurofibromas of a few NF1 patients.

The initial reason why we began to focus our research effort on the NZ propolis extract "Bio 30" was a 2002 poster by a University of Sydney group claiming that a NZ propolis shows the highest CAPE content (6-7% of dry extract) among propolis samples around the world. However, we realized that "Bio 30" contains only 12 mg of CAPE per g of dry extract (= 1.2% of extract), and have never found such a CAPE-rich sample in NZ propolis. A few propolis experts suspect that the 2002 report based on a rather low-tech TLC analysis of CAPE content might over-estimate the CAPE content, because often CAPE and a much more abundant polyphenol called pinobanskin 3-acetate overlap each other. Thus, in an attempt to create the "legendary" CAPE-rich propolis extract called "CAPE 60" (containing 60 mg of CAPE per g of extract), we added an extra CAPE (chemically synthesized) to "Bio 30", and compared the anti-cancer efficacy with "Bio 30" alone *in vivo*. As expected, "CAPE 60" is clearly more effective in suppressing both the growth and metastasis of MPNST in mice (*16*), confirming that the higher CAPE content, the more effective the propolis extract. Recently, enzymatic synthesis of CAPE was developed and further improved by a group in Taiwan (*25,26*). A mixture of CA and phenethyl alcohol (PA) can be converted almost 100% to CAPE by the immobilized lipase B called "Novozym 435" (see Figure 8). Thus, in near future, it would be possible for us to create the "CAPE 60" simply by adding a "natural" (enzymatically synthesized) CAPE to "Bio 30" or any other propolis extracts to boost their therapeutic effect for NF patients. Interestingly, coffee beans are rich in CA, while PA is a major fragrance of roses.

2.2. *Chinese (Sichuan) peppepr extract*

Around an early 2005, we started exploring a natural anti-PAK1 source(s) available inexpensively on the market, which would be suitable for a life-long treatment of NF, before "Bio 30" became available on the market.

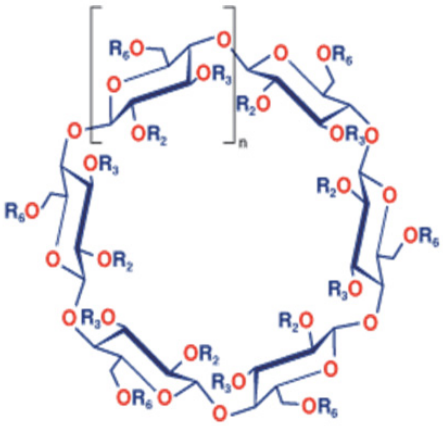

Figure 7. Cyclodextrins (CDs). N = 6, alpha-CD; N = 7, beta-CD; N = 8, gamma CD.

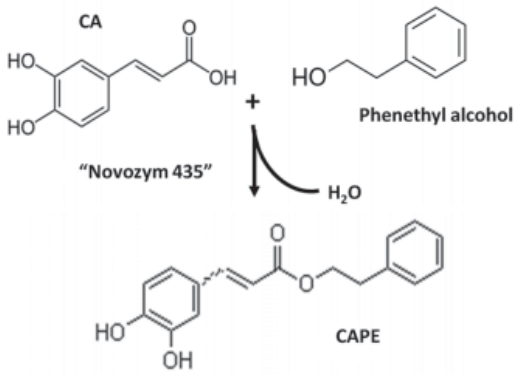

Figure 8. "Enzymatic" synthesis of CAPE.

One of them turned out to be an extract of Chinese (Sichuan) peppercorns, which has been used for thousand years in China as a seasoning for the preparation of a Sichuan cuisine called "Mapo-Tofu", a spicy bean curd dish. This reddish peppercorn is called "Hua Jiao" in Chinese or "Kasho" in Japanese, and related to a Japanese greenish peppercorn called "Sansho" which has been used as a seasoning for "Kabayaki", a grilled eel dish, and a traditional medicine for removing/killing intestinal parasitic nematodes.

We found that 70% ethanol extract of "Hua Jiao" contains an anti-cancer ingredient called "Pepperin" that selectively inactivates PAK1, without affecting another oncogenic kinase AKT (*27*). This extract blocks the growth of MPNST cells with the IC_{50} around 10 μg/mL, without affecting normal cell growth, and at the dose of around 100 mg/kg, twice a week, ***i.p.***, strongly suppresses the growth of *NF1*-deficient breast cancer xenograft in mice (*27*), suggesting that this extract would be useful for NF treatment. However, for the oral administration, ethanol has to be removed from the extract, so that patients would not suffer from the alcoholism. We found that a warm water (around 45°C) can replace 70% ethanol for the extraction of peppercorns. However, this water extract often tends to precipitate the anti-cancer ingredient "Pepperin" when the extract is cooled down to around room temperature. Furthermore, this extract is rather "bitter", although it is not "spicy" at all (the major pungent ingredient "Sanshool" is not water-soluble). Thus, just like the CAPE-based propolis extract, we are currently developing the CD complex of this extract called "Kasui" (water-extract of "Hua Jiao") by adding CD (20-30 g per 200 mL extract) to eliminate the bitter taste, and capture the "Pepperin" inside of CD molecule. According to an NF2 patient who prepared a home-made CD complex of "Kasui", this CD complex is no longer bitter, but apparently "sweet". This CD complex costs only around a dollar daily, and would be suitable for a life-long treatment of NF patients, without any side effect. For a few NF2 patients who carry the "Bio 30"-resistant tumors, we recently started a human trial of this CD complex alone or in combination of CD complex of "Bio 30", in an attempt to overcome this "Bio 30"-resistance.

2.3. *Bitter melon from China or Okinawa*

"Good medicines are bitter." That is an old saying that parents often use to encourage their young children to swallow a bitter medicine. Clearly good NF medicines such as propolis and "Kasui" are not its exceptions. In Okinawa, the southwest island of Japan, people used to live much longer than the rest of Japan, until their eating habit was gradually changed to the so-called American style. Besides Okinawa people are slimmer, less diabetic and more resistant to the summer heat. In an attempt to find the secret recipe of the Okinawa people's longevity and better health, back to 1983, Doroles Takemoto's group at Kansas State University examined the anti-cancer effect of a water-extract from an Okinawa's bitter melon called "Goya". They found that this bitter extract suppresses the development of a chemical induced cancer in mice (*28*). However, the molecular mechanism underlying this anti-cancer property remained unknown. 25 years later, in 2008, a Chinese group in Beijing and Australian group in Sydney jointly found that a similar Chinese bitter melon extract suppresses the diabetes in mice, lowering the blood sugar level, and identified the anti-diabetic tri-terpene in this extract (*29*). Interestingly, this tri-terpene activates the tumor suppressing kinase AMPK, suggesting that this tri-terpene is responsible for suppressing both the insulin-resistant diabetes (type 2) and tumor development. As discussed in detail later, AMPK activates a glucose transport protein called GLUT-4 which is essential for cellular uptake of glucose from blood stream (*30*). Furthermore, another tumor suppressing kinase called "LKB1" activates AMPK, and inactivates PAK1, directly (*31*). Thus, it is most likely that this tri-terpene blocks PAK1 as well as activates AMPK. For more than a dozen anti-PAK1 compounds such as CAPE and curcumin almost invariably activate AMPK (*32*,*33*). In other words, the "bitter" melon extract from Okinawa or China would be a potentially good NF therapeutic, although nobody knows as yet how much we should take daily this bitter extract for NF/cancer therapy.

2.4. *Curcumin (Turmeric)*

As shown in Figure 4, CAPE in propolis and curcumin, the spicy yellow ingredient in Indian curry (Turmeric powder) are structurally very similar, and as expected, both polyphenols were found to block PAK1 and activate AMPK (*6*,*32-34*). Like CAPE, curcumin suppresses the growth of many PAK1-dependent cancer cells *in vitro*, but either CAPE or curcumin alone has never been used clinically, mainly because of their poor bioavailability (water-insolubility). However, around 2005, Razella Kurzrock's group at MD Anderson Cancer Center solubilized curcumin by encapsulating curcumin in liposomes, and successfully started demonstrating its high efficacy in suppressing the PAK1-dependent growth of human colon and pancreatic cancer xenografts in mice: Curcumin in liposomes (20-40 mg/kg) suppresses the growth of these cancers by 50% (*35*,*36*). Furthermore, in 2009 an Indian group led by Sarasija Suresh at Nootan Dental College in Bangalore found that a chemically synthesized beta-dextrin derivative called hydroxypropyl-beta-CD would be the best among CDs to solubilize curcumin effectively and showed both its anti-angiogenic and anti-inflammatory effects *in vivo* (*37*). Thus, in the future (probably in a decade or so) curcumin in either liposomes or CDs would become available on the market for the therapy of these cancers and NF tumors as well.

2.5. *Berberine*

An old Chinese or American Indian herb called "Goldenseal" (or "Yellow Root") contains an anti-cancer and anti-inflammatory yellow-colored bitter-tasting alkaloid called Berberine. Unlike the majority of anti-cancer products, Berberine is relatively water-soluble, and its tannic acid salt (Berberine Tannate) is tasteless and used clinically world-wide as an antibiotic for the therapy of infectious diseases such as malaria. In 1990, an NCI group found that Berberine (0.1 mg/mL) induces a morphological differentiation of RAS-transformed teratocarcinoma cells *in vitro* (*38*). However, the molecular mechanism underlying its anti-cancer action remained unknown until recently. In 2006 a French group of GlaxoSmith Kline (GSK) found that Berberine (100 mg/kg, daily) blocks both the synthesis and accumulation of fats such as cholesterol in mice by activating the tumor suppressive kinase AMPK (*39*). In 2009 a Chinese group at Hong Kong University found that Berberine inactivates both RAC and CDC42, thereby inactivating PAK1 (*40*). Thus, like propolis and other anti-PAK1 products, this alkaloid would be useful for the treatment of PAK1-dependent cancers, NF and inflammatory diseases such as arthritis and asthma as well as type 2 diabetes and obesity. However, its minimum effective daily dose for cancer/NF patients still remains to be determined.

3. Anti-PAK1 drugs

3.1. *The ring peptide FK228*

Around 1993, the most potent anti-cancer antibiotic was developed by a Japanese group of Fujisawa Pharmaceuticals (later renamed "Astellas"). This antibiotic is a unique ring peptide called FK228 which suppresses the growth of RAS cancers such as pancreatic and colon cancer cells with the IC_{50} around 1 nM (*41*). Since these solid cancers require PAK1 for their growth, it has been suspected that FK228 might block the oncogenic PAK1 signaling somehow. In 1998, the direct target of this compound was identified by Minoru Yoshima at Tokyo University and the Fujisawa group jointly. FK228 inhibits histone deacetylase (HDAC) whose zinc metal in the catalytic center is covalently bound to FK228, only when FK228 is reduced by the intracellular glutathione (GSH) which breaks the di-sulfide bond of FK228 molecule (*42*). In other words, FK228 is a prodrug which is activated by GSH. Once histones on chromatin are acetylated, owing FK228, a specific group of genes would be activated. One of them is *p21/WAF1* gene encoding an inhibitor of cyclin-dependent kinases (CDKs). How does FK228 block the oncogenic RAS signaling by activating this gene? Well, RAS activates cyclin D1, an activator of CDKs, through PI3-kinase and PAK1, shifting cell cycle from G1 to S phase. In other words, FK228 blocks the oncogenic RAS-PAK1 signaling by activating *p21* gene which produces the antagonist of RAS-activated cyclin D1. This also means that FK228 blocks downstream of PAK1. However, we suspected another mechanism must be involved in the anti-RAS action of FK228, because we found that FK228 suppresses completely the growth of both *NF1*-deficient (MPNST) and *NF2*-deficient cancer cells even at 100 pM (*43*). In the case of breast cancer cells, the IC_{50} of FK228 is around 5 pM. Around 2005, we found that FK228 inactivates PAK1 with IC_{50} below 1 nM (*44*), indicating that FK228 blocks both upstream and downstream of PAK1. Having been encouraged with these findings, we examined whether FK228 suppresses the growth of human MPNST xenograft in mice. Yes, it shrank MPNST completely at the dose of 2.5 mg/kg, twice a week, *i.p.*, for two months (*43*). Unfortunately, however, FK228 turned out not to pass the blood brain barrier (BBB), so that it would not be effective on brain tumors associated with NF1 or NF2. Nevertheless, in principle, it would be effective in suppressing MPNST, plexi-form and dermal neurofibromas of NF1 patients, when this drug becomes available some days in the future. Currently, a US company called "Celgene" is taking the full responsibility for the on-going clinical trials (phase 2) of this compound mainly for CTCL (cutaneous T-cell lymphoma), a rare cancer which represents less than 0.1% of all cancers.

3.2. *UnPAK309 (PF3758309)*

In 2010, Brion Murray's team at Pfizer Oncology in San Diego developed a new compound called PF3758309 (in short UnPAK 309) that selectively inhibits PAK family kinases including PAK1 with the IC_{50} between 5-15 nM in cells (*45*). *In vivo* (cancer xenografts in nude mice), this compound suppresses the growth of several PAK1-depepndent human cancers such as breast, colon and lung cancers as well as melanoma at daily doses around 20 mg/kg. Since its derivatives pass the BBB, it is likely that UnPAK309 could be potentially useful for the treatment of brain tumors such as glioma, and tuberous sclerosis (NF/TSC) tumors in the future. However, as UnPAK309 clinical trials have just started, in realty it would take several years until this potent PAK inhibitor becomes available on the market for cancer/NF patients.

Another thing worth being pointed out here would be that unlike CAPE and other anti-PAK1 compounds, UnPAK309 directly inhibits AMPK (the IC_{50} in cells around 40 nM) as well, instead of activating AMPK (*45*). Thus, this drug would be the first clear exception for the rule that all anti-PAK1 drugs are AMPK activators.

3.3. *Combination of two Tyr-kinase inhibitors, PP1 and AG 879/GL-2003*

For the following two reasons, we have recently developed our own anti-PAK1 drugs which pass the BBB. FK228 tends to develop the multi-drug resistance

(MDR) by activating MDR genes at least in pancreatic and breast cancers. As mentioned earlier, FK228 fails to pass the BBB. Our best choice was the combination of two distinct Tyr-kinase inhibitors, PP1 and GL-2003 (a water soluble hexylamine derivative of AG 879). This combination blocks two distinct Tyr-kinases, namely SRC family kinases and ETK, essential for the full activation of PAK1 (*46,47*). At dose (20 mg/kg, twice a week, *i.p.*) of each drug, the growth of both FK228-resistant human pancreatic and breast cancer xenografts in mice was almost completely suppressed without any adverse effect (*48*). Since the target kinases of these two drugs are clearly distinct, the chance for cancers to develop the MDR to both drugs would be mathematically null. Furthermore, recently a water-soluble (hexylamine) derivative of PP1 called GL-2010 has also been developed by my former colleagues at WEHI, and a clinical trial of this new combination (GL-2003 and GL-2010) is expected to start in a not-too-distant future.

3.4. *Ivermectin*

In 2004 a Russian group in Moscow reported that an old, safe and inexpensive antibiotic called "Ivermectin" has an anti-cancer property *in vivo* (human cancer xenografts in mice): Ivermectin (3-5 mg/kg, daily) almost completely suppresses the growth of a few distinct human cancers such as melanoma (*49*). However, its anti-cancer mechanism at molecular levels remained entirely unknown. This antibiotic was developed during 1980s jointly by Merck in US and Kitasato Institute in Tokyo as an anti-parasitic drug that kills selectively intestinal nematodes by blocking GABA receptor, but not mammalian counterparts (*50*). A very low dose such as 0.2 mg/kg (taken orally once or twice) is sufficient to eliminate these parasites completely. Since the anti-cancer action works on mammalian cancer cells, it is obvious that its target is not mammalian GABA receptor. Then one day I noticed that this drug has been known for some years to reduce dramatically (by around 90%) the litter size (the number of eggs laid) of *C. elegans*, a non-pathogenic tiny nematode living in nature (woods). This effect happened to be the precisely same as what CAPE or ARC does on the same worm, as discussed later in detail. So I was prompted to examine whether Ivermectin blocks the oncogenic PAK1 signaling or not. Besides it has been known that melanomas require PAK1 for their growth, as the anti-PAK drug FK228 blocks the growth of melanoma. In collaboration with Tamotsu Sudo's group at Hyogo Cancer Center in Japan, we have confirmed that Ivermectin indeed inactivates PAK1, and blocks the PAK1-dependent growth of both human ovarian cancer and NF2 tumor (Schwannoma) cells with the IC_{50} around 5 μM (*51,52*). Thus, this inexpensive old drug could be used as an alternative if cancer/NF patients happen to be among 1% of population who show an allergic skin reaction to the CAPE-based propolis such as "Bio 30", or resistant to any propolis.

4. PAK1 blockers = AMPK activators

As briefly mentioned before, more than a dozen of anti-PAK1 compounds such as CAPE, curcumin, apigenin, berberine, resveratrol, emodin, salidroside, capsaicin/capsiate, OSU-03012, GW2974, metformin, *etc.* are known to activate the tumor suppressing kinase AMPK (see Table 2). AMPK is an AMP-activated kinase that is activated when the AMP/ATP ratio increases. This ratio rises when the cellular glucose/ATP level drops, due to either calorie restriction (CR), fast or extensive physical exercise. Thus, AMPK serves as a sensor of the cellular glucose/ATP level, and is activated in an AMPK-dependent manner. In most cases, these PAK1 blockers activate AMPK through another tumor suppressing kinase called LKB1 which phosphorylates Thr 172 of AMPK (*31*). AMPK phosphorylates several distinct proteins. As mentioned before, one of them is the glucose transporter protein "GLUT-4", which is essential for the cellular uptake of glucose from blood stream. AMPK activates GLUT-4, leading to a transient rise of the cellular glucose level by lowering the blood glucose level. Another target of AMPK is the tumor suppressing transcription factor "FOXO" which is essential for the longevity (*53*). In mammals, AMPK activates FOXO, while PAK1 inactivates FOXO (*54,55*). Thus, most of PAK1 blockers such as CAPE and curcumin activate FOXO through these two distinct routes in a concerted manner. At least two PAK1 blockers = AMPK activators, salidroside and curcumin, were shown to extend the life span of *C. elegans* and the fruit fly Drosophila, respectively (*56,57*). Among the major target genes of FOXO is *HSP16* gene encoding a small heat shock protein, and this "FOXO-HSP16" signaling pathway extends the life span of this

Table 2. Anti-PAK1 and AMPK activating activity of anti-PAK1 products

Products	PAK1 inactivation	AMPK activation	Ref.
CAPE	+	+	*6, 32*
Curcumin	+	+	*33, 34*
Resveratrol	+	+	*95, 96*
Berberine	+	+	*37, 40*
Salidroside	+	+	*31, 94*
Metformin	+	+	*31, 93*
Emodin	+	+	*91, 92*
Capsaicin	+	+	*89, 90*
Apigenin	+	+	*82, 83*
OSU-03012	+	+	*84, 85*
GW2974	+	+	*61*
AG 879	+	+	*86, 87*
AICAR	?	+	*88*
UnPAK309	+	-	*45*

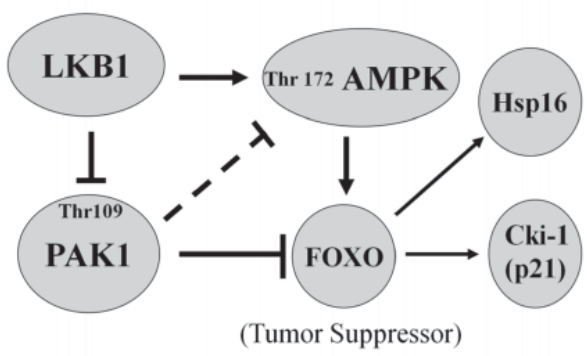

Figure 9. "LKB1" controls both AMPK and PAK1.

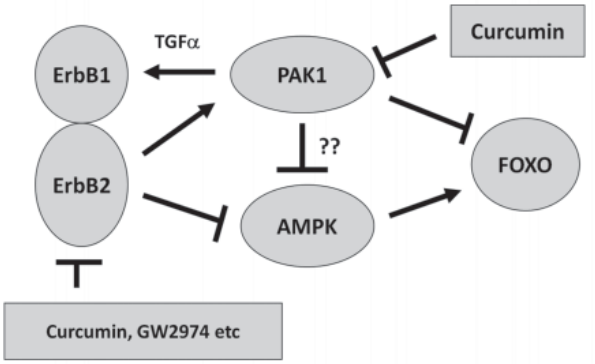

Figure 10. "PAK1-ErbB2" cycle that blocks AMPK.

worm by 50% (*58*). We found that PAK1 blockers from propolis, CAPE and ARC, strongly activate *HSP16* gene in *C. elegans* shortly after the heat shock, and make this worm heat resistant as does the *PAK1*-deficient mutation (*6*). For heat shock proteins in general protect the essential cellular proteins from their denaturation caused by heat (*59*). Thus, it is most likely that both CAPE-based propolis and ARC-based propolis extracts would extend the life span of this worm, and probably all human beings including cancer/NF patients as well, by activating this longevity (FOXO-HSP16) signaling pathway. The higher this signaling, the longer we live.

Why do most of PAK1 blockers turn out to be AMPK activators, or *vice versa*? One of the mechanisms underlying this "mysterious" formula/equation, PAK1 blockers = AMPK activators, is that LKB1 inactivates PAK1 by phosphorylating Thr 109, as well as activating AMPK simultaneously (*31*) (see Figure 9). A second mechanism is based on the vicious oncogenic cycle that PAK1 and the Tyr-kinase ErbB2 form: PAK1 activates another Tyr-kinase called ErbB1 by up-regulating its ligands such as TGFα, which activate ErbB1, and in turn ErbB1 activates ErbB2 by forming a hetero-dimer (*46,60*). Both ErbB1 and ErbB2 are essential for the full activation of PAK1 (*46*). Furthermore, ErbB2 somehow inactivates AMPK (*61*). Thus, if either PAK1 or ErbB2 is blocked, this oncogenic cycle would stop, and AMPK would be reactivated (Figure 10).

5. A "fluorescent" *in vivo* screening for NF therapeutics

Generally speaking, to get the FDA-approval for clinical trials of a given anti-cancer/NF drug under the development, one has to prove its therapeutic effect as well as its safety (non-toxicity) *in vivo*, using a human cancer (or NF1/NF2 tumor) grafted in mice or rats. However, it would cost both time and money to screen for the most effective and safe drug(s) from a huge library of thousands test samples. Here I shall introduce a quick and inexpensive *in vivo* screening system, using a tiny transparent nematode (*C. elegans*) strain called CL2070 which carries the transgenic fusion gene "*HSP16-GFP*" (*62*), in order to screen for a series of PAK1 blockers = AMPK activators which would be useful for NF therapy.

This fusion gene would mass-produce the S65T mutant of green fluorescent protein (GFP) from a jelly fish when the promoter of *C. elegans HSP16* gene is activated by the heat shock treatment (at 35ºC for 2 h) (*62,63*). However, the expression of GFP takes place rather slowly, and reaches the maximum during 12-24 h after the heat shock, in the absence of a PAK1 blocker = AMPK activator, because normally PAK1 suppresses the *HSP16* gene by inactivating the "FOXO" in this worm as in mammals (*6,55*). When this worm is treated with PAK1 blockers such as CAPE, ARC and Ivermectin overnight, the expression of GFP reaches the maximum in a few hours after the heat shock, and the whole transparent worm glows greenly under the blue light (*6*). Of course these PAK1 blockers cause no side (adverse) effect. Instead they make this worm much more heat-tolerant by over-production of heat shock proteins such as HSP16 (*6*). Furthermore, these PAK1 blockers reduce the litter size (the number of eggs laid) of this worm by 85-90%, although all eggs are hatchable (*6*). These phenotypes are precisely the same as those of the *PAK1*-deficient mutant (RB689) of this worm (*6*). Since the more HSP16 is activated, the longer this worm lives (*63*), it is most likely that like salidroside and curcumin (*56,57*), all these PAK1 blockers would extend significantly the life span of this worm. Furthermore, as described earlier, CA is a precursor of CAPE, and blocks PAK1 by down-regulating its activator RAC (*14*). During 2010-2011 a German group at Humboldt University in Berlin found that CA and its covalently-linked dimer called Rosmarinic acid (RA) significantly extend the life span of this worm through "FOXO", and activate heat shock genes such as *HSP12*, inducing the heat-tolerance (*64*). Since RA is a CAPE derivative (3,4-dihydroxyl, 8-carboxyl) as well as CA dimer, it is most likely that CAPE and many other PAK1 blockers would execute such a life-prolonging (so-called 'elixir') effect.

Interestingly, more than two decades ago, Tom Johnson's group at University of Colorado showed that dysfunction of PI3-kinase/Age-1 in this worm lengthens

the life span by 100% and reduces significantly the litter size (*65*). Furthermore, dysfunction of AKT is also known to lengthen the life span of this worm, but just by 50% (*66*). Since both AKT and PAK1 are downstream of PI3-kinase, inactivating "FOXO", it is most likely that dysfunction of PAK1 would contribute to the remaining 50% extension of life span. As described earlier, the "FOXO-HSP16" signaling pathway indeed extends the life span of this worm by 50% (*58*). However, we have not confirmed directly this notion as yet, because unlike the HSP16-GFP/litter size/thermal endurance tests which take only a few days, the longevity test would take 15-30 days, and is technically far more laborious. Of course, if one tries to compare the life span between the *PAK1*-deficient healthy mice (*67*) and the control, the test would take 3-6 years, clearly more than 50 times longer than this worm (RB689 mutant *versus* the control).

In my opinion, the above "fluorescent" GFP assay would be the best approach to the systematic screen for PAK1 blockers (AMPK activators), as this system could be automated by linking a number of 96-well plates containing CL2070 treated with a variety of test compounds to a fluorescence reader for the quantitative analysis. The more fluorescence, the stronger anti-cancer property. Once a few "best" (the most potent) candidates are selected out through this system to make a short list of cancer/NF therapeutics, their anti-cancer/NF property should be re-confirmed by means of the "conventional" xenograft in nude mice, to convince the FDA or cancer/NF patients for their final approval.

6. PAK1-dependent non-cancerous diseases

So far at least 70% of all human cancers, in particular solid tumors, have been identified as PAK1-dependent tumors: Among them are pancreatic, colon, breast, prostate, gastric, lung, cervical, ovarian, and thyroid cancers as well as glioma, melanoma, hepatoma, *NF2*-deficient mesothelioma, MM and MPNST, a malignant NF1 tumor. In addition, the remaining 90% of NF1 tumors, NF2 tumors (meningioma and schwannoma) and TSC tumors also require PAK1 for their growth. TSC is caused by dysfunction of the tumor suppressors TSC1 or TSC2, which leads to the abnormal activation of the oncogenic kinase "TOR" (*68*). Although the "TOR" could be blocked by Rapamycin and its derivatives such as "Afinitor" (*68-71*), they are extremely expensive and cause at least a few rather serious side effects such as immuno-suppression and hypertension. Thus, these TOR inhibitors would not be so suitable for a life-long treatment of TSC. However, since TOR requires PAK1 for the full activation, as curcumin blocks the oncogenic TOR-Raptor interaction (*72*), PAK1 blockers such as propolis could be used for the TSC therapy. In fact, the CAPE-based propolis "Bio 30" stops even the drug-resistant epilepsy often associated with TSC children in our human trials.

In addition, there are several other non-cancerous diseases which also require PAK1 (*67,68,73-81*): Among them are AIDS (HIV-infection), malaria, Alzheimer's (AD), Huntington's (HD), inflammatory diseases (asthma and arthritis), insulin-resistant diabetes (type 2), obesity, hypertension, seizures such as epilepsy, autistic diseases such as Fragile X syndrome, and learning deficit (LD). Thus, propolis and other PAK1 blockers would serve as the effective (and safe) therapeutics for these diseases as well. So it would not be a big surprise if these anti-PAK1 products such as propolis and the "bitter" melons extend our life span in good health by either curing or delaying some of these PAK1-dependent diseases including cancers and NF throughout our entire life.

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Brief Report

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Synthesis and anti-HIV activity of novel 2,4-disubstituted-7-methyl-1,1,3-trioxo-*2H,4H*-pyrazolo[4,5-*e*][1,2,4]thiadiazine derivatives

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ABSTRACT: A series of novel 2,4-disubstituted-7-methyl-1,1,3-trioxo-*2H,4H*-pyrazolo[4,5-*e*][1,2,4]thiadiazines (PTDs) was prepared starting from a ring of pyrazolo[4,5-*e*][1,2,4]thiadiazine nuclei with two different alkyl halides obtained by a facile one-pot reaction. The structures of all synthesized compounds were confirmed by ^{1}H- and ^{13}C-NMR, infrared spectra (IR), and mass spectra (MS) spectroscopic analysis. Anti-HIV activity was evaluated and none of the compounds were found to inhibit HIV replication in human T-lymphocyte (MT-4) cell culture.

Keywords: HIV, pyrazolo[4,5-*e*][1,2,4]thiadiazine, heterocycle, synthesis

1. Introduction

Fused heterocyclic thiadiazine derivatives such as pyridothiadiazines, pyrazinothiadiazines, imidazothiadiazines, triazolothiadiazines, and thienothiadiazines have become of particular interest to chemists and biologists because of their broad spectrum of biological activities and potential pharmacological applications in the treatment of cerebro/cardiovascular disease (*1,2*), cognitive disorders (*3*), cancers, and viral and bacterial infection (*4,5*). Among these derivatives, 2,4-disubstituted-1,1,3-trioxo-*2H,4H*-thieno [3,4-*e*][1,2,4]thiadiazines (TTDs) have been found to be potent non-nucleoside reverse transcriptase inhibitors (NNRTIs) of human immunodeficiency virus type 1 (HIV-1), which selectively block HIV-1 replication at the reverse transcriptase step (*6*). The lead compound QM96625 (Figure 1) displayed highly potent activity and selectivity against HIV-1 replication in human T-lymphocyte (MT-4) cells with an EC_{50} value of 0.10 μM, CC_{50} value > 119.0 μM, and SI > 1,190 (*7,8*).

In order to identify new HIV-1 NNRTIs, the current authors recently undertook a study of a series of 2,4-disubstituted-7-methyl-1,1,3-trioxo-*2H,4H*-pyrazolo[4,5-*e*][1,2,4]thiadiazines (PTDs) because of the known bioisosterism between TTDs and PTDs (*9*). This previous work reported a regioselective method for synthesis of N_2- or N_4-monosubstituted PTDs (*10*) that was used to synthesize N_2,N_4-disubstituted PTDs *via* a one-pot reaction (*11*). This work had also evaluated the anti-HIV-1 activity of PTDs and identified some N_4-monosubstituted and N_2,N_4-disubstituted PTDs that exhibited moderate anti-HIV-1 activity. As a continuation of this research on new anti-HIV-1 agents from PTD analogues, a series of novel 2,4-disubstituted-7-methyl-1,1,3-trioxo-*2H,4H*-pyrazolo[4,5-*e*][1,2,4]thiadiazines were designed and synthesized based on the one-pot reaction that was previously established. The introduction of substituents at N_2 and N_4 moieties of the PTD heterocycle resulted in those substituents having the same anti-HIV activity as a series of TTDs. The current research describes the synthesis of novel disubstituted PTDs and screening of their anti-HIV activity based on cell cultures.

2. Materials and Methods

2.1. *General methods*

All melting points were determined on a micromelting point apparatus and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Bruker Avance-600

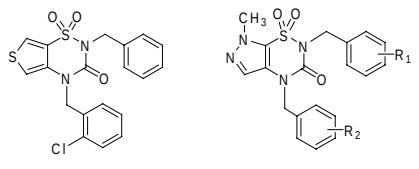

Figure 1. Lead compound in the form of a 2,4-disubstituted thieno[3,4-*e*][1,2,4]thiadiazine (TTD, QM96625) and structures of 2,4-disubstituted-7-methylpyrazolo[4,5-*e*][1,2,4]thiadiazines (PTDs).

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(600 MHz; Bruker BioSpin, Rheinstetten, Germany) in the indicated solvent. Chemical shifts are expressed in δ units with TMS as the internal reference. Infrared spectra (IR) were recorded with a Nexus 470FT-IR spectrometer (Thermo Nicolet, Madison, WI, USA). Mass spectra (MS) were acquired with an LC autosampler device: standard G1313A instrument (Agilent Technologies, Santa Clara, CA, USA) and a AB Sciex API 4000 tandem mass spectrometer (Applied Biosystems, USA) with ESI. All compounds were routinely checked by TLC at 254 nm on pre-coated silica gel G plates with fluorescent indicator, which were prepared in the laboratory. Developed plates were visualized with UV light. Flash column chromatography was performed on a column packed with silica gel 60 (230-400 mesh). Solvents were reagent grade and, when necessary, were purified and dried using standard methods. Concentration of the reaction solutions involved the use of a rotary evaporator at reduced pressure.

2.2. *General procedure for the preparation of N_2,N_4-disubstituted-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazines (3**a-3p**)*

Sodium hydride (60% dispersion in mineral oil, 2 equivalent) was incrementally added to a solution of compound (**1**) (1 equivalent) (Scheme 1) in dry DMF in an inert atmosphere (N_2) and with the temperature below 10°C. After 30 min of stirring, the first alkyl halide (R_2X, 1 equivalent) was added dropwise. The mixture was stirred at room temperature for 20 min and 30-50°C for 12-20 h (checked by TLC). After the addition of the second alkyl halide (R_1X, 1 equivalent), the mixture continued to be stirred at 40-80°C for 12-20 h. After the solvent was evaporated off under reduced pressure, the crude product was purified by recrystallization from ethanol. Specific information on compounds **3a-3p** is indicated in the Appendix.

2.3. *In vitro anti-HIV assay*

The methodology of the anti-HIV assay used here has been previously described (*12,13*). Stock solutions (10× final concentration) of test compounds were added in 25-µL volumes to two sets of triplicate wells so as to allow simultaneous evaluation of their effects on mock- and HIV-infected cells at the beginning of each experiment. Serial five-fold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman Instruments, Fullerton, CA, USA). Untreated control HIV- and mock-infected cell samples were included for each sample.

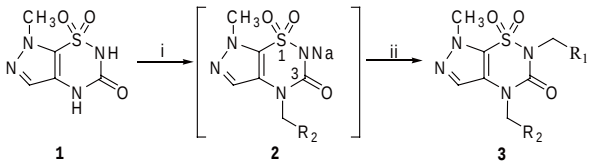
HIV-1 (IIIB) (*14*) or HIV-2 (ROD) (*15*) stock (50 µL) at 100-300 CCID50 (cell culture infectious dose) or culture medium was added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of the test compound on uninfected cells in order to assess the cytotoxicity of the test compound. Exponentially growing MT-4 cells (*16*) were centrifuged for 5 min at 1,000 rpm and the supernatant was discarded. The MT-4 cells were resuspended at 6 × 10^5 cells/mL, and 50-µL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock- and HIV-infected cells was examined spectrophotometrically using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

The MTT assay is based on the reduction of yellow colored MTT (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to a bluish-purple formazan that can be measured spectrophotometrically. Absorbance was read in an eight-channel computer-controlled photometer (Multiscan Ascent Reader, Labsystems, Helsinki, Finland) at two wavelengths (540 and 690 nm). All data were calculated using the median optical density (OD) of three wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduced the absorbance (OD_{540}) of the mock-infected control sample by 50%. The 50% effective concentration (EC_{50}) was defined as the compound concentration required to inhibit virus-induced syncytium formation by 50%.

3. Results and Discussion

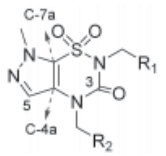
3.1. *Chemistry*

A ring of 7-methyl PTD nuclei (**1**) (Scheme 1) was prepared according to a previously reported procedure (*10*) and was purified by recrystallization from ethanol to yield a white solid in good yield (84%). N_2,N_4-disubstituted hetero[1,2,4]thiadiazines are usually prepared by stepwise alkylation. The first step of alkylation at the N_2 position usually produces a mixture of N_2-, and O_3-monosubstituted products that must be separated by flash column chromatography before the second step of alkylation at the N_4 position. Use of one-pot regioselective alkylation avoids this complicated process (*17*). In brief, the ring of 7-methyl PTD nuclei (**1**) was dissolved in dry DMF (1 mmol/5 mL), which was cooled to under 10°C. Two equimolar NaH was added, followed by 1 equimolar R_2CH_2X, and then 1 equimolar R_1CH_2X was added when the intermediate of the mono N_4-substituted product (**2**) was obtained (checked by TLC). The crude product (**3**) was purified by recrystallization from ethanol to yield a white solid in good yield (Scheme 1). A series of N_2,N_4-disubstituted pyrazolo[4,5-*e*][1,2,4]thiadiazine derivatives (**3a-3p**) were prepared by this method and is listed in Table 1.



Scheme 1. Reagents: (i): NaH/R_2CH_2X (2:1), 30-80°C (ii): R_1CH_2X, 40-80°C.

Table 1. Structures and anti-HIV-1[a] and HIV-2[b] of newly synthesized 2,4-disubstituted-7-methyl-1,1,3-trioxo-4,7-dihydropyrazolo [4,3-*e*][1,2,4]thiadiazines (PTDs 3) *in vitro*



Compound	R_1CH_2	R_2CH_2	EC_{50} (μM)[c]		CC_{50} (μM)[d]	SI[e]	
			HIV-1 IIIB[a]	HIV-2 ROD[b]		HIV-1 III_B	HIV-2 ROD
3a	2-Cl-benzyl	2-Cl-benzyl	> 277.2	> 277.2	> 277.2	× 1	× 1
3b	4-Cl-benzyl	2-Cl-benzyl	> 277.2	> 277.2	> 277.2	× 1	× 1
3c	2,4-Cl_2-benzyl	2-Cl-benzyl	> 253.3	> 198.8	> 251.3	× 1	× 1
3d	2-CN-benzyl	2-Cl-benzyl	> 283.1	> 283.1	≥ 283.1	× 1	× 1
3e	3-CN-benzyl	2-Cl-benzyl	> 283.1	> 283.1	> 283.1	× 1	× 1
3f	4-CN-benzyl	2-Cl-benzyl	> 7.0	> 5.1	7.3	< 1	< 1
3g	4-C_2H_5-benzyl	2-Cl-benzyl	> 281.2	> 276.7	≥ 276.7	× 1	× 1
3h	4-(t-Bu)-benzyl	2-Cl-benzyl	> 264.6	> 264.6	> 264.6	× 1	× 1
3i	4-NO_2-benzyl	2-Cl-benzyl	> 151.7	> 124.8	155.0	< 1	< 1
3j	4-Br-benzyl	2-Cl-benzyl	> 252.3	> 252.3	> 252.3	× 1	× 1
3k	4-NO_2-benzyl	benzyl	> 252.9	> 238.9	265.8	< 1	< 1
3l	2-Br-benzyl	benzyl	> 271.1	> 271.1	> 271.1	× 1	× 1
3m	4-Br-benzyl	2-Br-benzyl	> 231.5	> 231.5	> 231.5	× 1	× 1
3n	4-Cl-benzyl	2-Br-benzyl	> 252.3	> 252.3	> 252.3	× 1	× 1
3o	2-Br-benzyl	3-Cl-benzyl	> 252.3	> 252.3	> 252.3	× 1	× 1
3p	4-Cl-benzyl	2,4-Cl_2-benzyl	> 257.5	> 216.3	≥ 216.3	× 1	× 1
Zidovudine					0.0007		35.6
Nevirapine					0.03		683

[a] Anti-HIV-1 activity measured with strain IIIB.
[b] Anti-HIV-2 activity measured with strain ROD.
[c] Compound concentration required to achieve 50% protection of MT-4 cells from the HIV-1- and HIV-2-induced cytopathogenic effect.
[d] Compound concentration that reduces the viability of mock-infected MT-4 cells by 50%.
[e] SI: selectivity index (CC_{50}/EC_{50}).

3.2. *In vitro anti-HIV activity*

Compounds **3a-3p** were evaluated for their *in vitro* anti-HIV-1 activity by using the IIIB strain of HIV-1 and the ROD strain of HIV-2. Compounds were monitored for their inhibition of the virus-induced cytopathic effect in MT-4 cells. The results are summarized in Table 1, where the data for zidovudine and nevirapine have been included for comparison purposes. However, none of compounds were found to inhibit HIV replication in MT-4 cell culture. Results revealed that the five-member ring moiety of the heterothiadiazines had a major role in affecting anti-HIV activity. As a result, PTDs had much lower activity than TTDs despite having very similar structures. This might be due to the fact that the pyrazole moiety of pyrazolothiadiazines is more hydrophobic than the thiophene ring of thienothiadiazines, which is ill-suited to the structural requirement for a hydrophobic body as is assumed in the "butterfly" model (*17*).

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Appendix

*Synthesis of compounds **3a-3p***

*2,4-Di(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine (**3a**).* Compound (**1**) and 2-chlorobenzyl chloride were allowed to react at 40-50°C for 8 h. After the addition of 2-chlorobenzyl chloride, the mixture was allowed to react at 50-60°C for 20 h. Purification by recrystallization from ethanol yielded compound **3a** as a white solid (80%), mp: 116-118°C. IR (KBr, cm^{-1}): 1,699 (C=O), 1,331, 1,195 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.80 (s, 1H, PyH), 7.09 (dd, 1H, J = 7.58 Hz, J = 1.52 Hz, PhH), 7.26-7.51 (m, 7H, PhH), 5.17 (s, 2H, NCH_2), 5.11 (s, 2H, NCH_2), 4.08 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 148.7 (C=O), 132.9, 133.2, 132.1, 131.6, 129.9, 129.5, 129.4, 129.3, 127.8, 127.7, 127.6, 127.5, 126.4 (C-5), 125.9 (C-4a), 122.2 (C-7a), 47.3 (N_2-CH_2), 41.7 (N_4-CH_2), 39.1 (CH_3). MS (EI): m/z 451.4 $[M + H]^+$ (calcd for $C_{19}H_{16}Cl_2N_4O_3S$: 450.03).

*2-(p-Chlorobenzyl)-4-(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine (**3b**).* Compound (**1**) and 2-chlorobenzyl chloride were allowed to react at 40-50°C for 8 h. After the addition of 4-chlorobenzyl chloride, the mixture was allowed to react at 50-60°C for 12 h. Purification by recrystallization from ethanol yielded compound **3b** as a white solid (65%), mp: 128-130°C. IR (KBr, cm^{-1}): 1,693 (C=O), 1,331, 1,193 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.75 (s, 1H, PyH), 7.50 (dd, 1H, J = 7.86 Hz, J = 1.23 Hz, PhH), 7.01 (dd, 1H, J = 7.63 Hz, J = 1.25 Hz, PhH), 7.26-7.41 (m, 6H, PhH), 5.15 (s, 2H, NCH_2), 5.02 (s, 2H, NCH_2), 4.08 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 148.8 (C=O), 133.0, 135.2, 132.1, 132.4, 129.9, 129.8, 129.5, 128.5, 127.7, 127.4, 126.3 (C-5), 125.8 (C-4a), 122.2 (C-7a), 47.2 (N_2-CH_2), 43.5 (N_4-CH_2), 39.1 (CH_3). MS (EI): m/z 451.4 $[M + H]^+$ (calcd for $C_{19}H_{16}Cl_2N_4O_3S$: 450.03).

*2-(2,4-Dichlorobenzyl)-4-(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine (**3c**).* Compound (**1**) and 2-chlorobenzyl chloride were allowed to react at 40-50°C for 8 h. After the addition of 2,4-dichlorobenzyl chloride, the mixture was allowed to react at 50-60°C for 20 h. Purification

by recrystallization from ethanol yielded compound **3c** as a white solid (55%), mp: 138-140°C. IR (KBr, cm^{-1}): 1,698 (C=O), 1,330, 1,196 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.25 (s, 1H, Pyr-H), 7.02 (d, 1H, *J* = 7.26 Hz, PhH), 7.20-7.43 (m, 6H, PhH), 5.24 (s, 2H, NCH_2), 5.22 (s, 2H, NCH_2), 4.15 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 149.1 (C=O), 133.3, 133.7, 132.5, 131.7, 131.6, 129.7, 129.2, 129.1, 128.6, 128.5, 127.2, 126.0, 125.2, 125.1, 122.6 (C-7a), 46.7 (N_2-CH_2), 41.4 (N_4-CH_2), 38.9 (CH_3). MS (EI): m/z 487.3 $[M + 3]^+$ (calcd for $C_{19}H_{15}Cl_3N_4O_3S$: 483.99).

2-(o-Cyanobenzyl)-4-(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3d)***. Compound (**1**) and 2-chlorobenzyl chloride were allowed to react at 40-50°C for 8 h. After the addition of 2-cyanobenzyl chloride, the mixture was allowed to react at 70-80°C for 20 h. Purification by recrystallization from ethanol yielded compound **3d** as a white solid (60%), mp: 130-132°C. IR (KBr, cm^{-1}): 2,226 (CN), 1,695 (C=O), 1,317, 1,192 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.77 (s, 1H, PyH), 7.84 (dd, 1H, *J* = 7.73 Hz, *J* = 1.31 Hz, PhH), 7.04 (dd, 1H, *J* = 7.57 Hz, *J* = 1.27 Hz, PhH), 7.25-7.71 (m, 6H, PhH), 5.22 (s, 2H, NCH_2), 5.15 (s, 2H, NCH_2), 4.09 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 48.8 (C=O), 133.7, 139.6, 133.1, 132.8, 132.0, 129.9, 129.5, 128.6, 127.8, 127.7, 127.5, 126.3 (C-5), 125.8 (C-4a), 122.1 (C-7a), 117.2, 110.9 (CN), 47.3 (N_2-CH_2), 42.4 (N4-CH_2), 39.1 (CH_3). MS (EI): m/z 442.4 $[M + H]^+$ (calcd for $C_{20}H_{16}ClN_5O_3S$: 441.07).

2-(m-Cyanobenzyl)-4-(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3e)***. Compound (**1**) and 2-chlorobenzyl chloride were allowed to react at 40-50°C for 8 h. After the addition of 3-cyanobenzyl chloride, the mixture was allowed to react at 70-80°C for 20 h. Purification by recrystallization from ethanol yielded compound **3e** as a white solid (61%), mp: 116-118°C. IR (KBr, cm^{-1}): 2,233 (CN), 1,693 (C=O), 1,329, 1,192 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.70 (s, 1H, PyH), 7.79 (s, 1H, PhH), 7.50 (dd, 1H, *J* = 7.86 Hz, *J* = 1.23 Hz, PhH), 7.06 (dd, 1H, *J* = 7.63 Hz, *J* = 1.28 Hz, PhH), 7.27-7.76 (m, 5H, PhH), 5.15 (s, 2H, NCH_2), 5.10 (s, 2H, NCH_2), 4.08 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 148.8 (C=O), 133.0, 138.0, 132.6, 132.1, 131.6, 131.2, 129.9, 129.8, 129.5, 127.6, 127.5, 126.3 (C-5), 125.9 (C-4a), 112.0 (C-7a), 118.8, 111.5 (CN), 47.3 (N_2-CH_2), 43.2 (N_4-CH_2), 39.1 (CH_3). MS (EI): m/z 442.4 $[M + H]^+$ (calcd for $C_{20}H_{16}ClN_5O_3S$: 441.07).

2-(p-Cyanobenzyl)-4-(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3f)***. Compound (**1**) and 2-chlorobenzyl chloride were allowed to react at 40-50°C for 8 h. After the addition of 4-cyanobenzyl chloride, the mixture was allowed to react at 70-80°C for 20 h. Purification by recrystallization from ethanol yielded compound **3f** as a white solid (63%), mp: 164-166°C. IR (KBr, cm^{-1}): 2,227 (CN); 1,690 (C=O); 1,334, 1,193 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.78 (s, 1H, PyH), 7.79 (s, 1H, PhH), 7.04 (d, 1H, *J* = 7.61 Hz, PhH), 7.27-7.85 (m, 7H, PhH), 5.15 (s, 2H, NCH_2), 5.12 (s, 2H, NCH_2), 4.08 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 148.9 (C=O), 132.9, 141.9, 132.8, 132.1, 129.9, 129.5, 128.4, 127.9, 127.7, 127.5, 126.3, 126.2 (C-5), 125.9 (C-4a), 122.0 (C-7a), 118.8, 110.5 (CN), 47.3 (N_2-CH_2), 43.7 (N_4-CH_2), 39.1 (CH_3). MS (EI): m/z 442.5 $[M + H]^+$ (calcd for $C_{20}H_{16}ClN_5O_3S$: 441.07).

2-(p-Ethylbenzyl)-4-(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3g)***. Compound (**1**) and 2-chlorobenzyl chloride were allowed to react at 40-50°C for 8 h. After the addition of 4-ethylbenzyl chloride, the mixture was allowed to react at 70-80°C for 20 h. Purification by recrystallization from ethanol yielded compound **3g** as a white solid (59%), mp: 112-114°C. IR (KBr, cm^{-1}): 1,699 (C=O), 1,338, 1,192 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.74 (s, 1H, PyH), 7.50 (dd, 1H, *J* = 7.75 Hz, *J* = 0.75 Hz, PhH), 6.99 (d, 1H, *J* = 7.25 Hz, PhH), 6.16-7.32 (m, 6H, PhH), 5.16 (s, 2H, NCH_2), 4.99 (s, 2H, NCH_2), 4.08 (s, 3H, CH_3), 2.54-2.58 (q, 2H, *J* = 7.59 Hz, CH_2), 1.13-1.16 (t, 3H, *J* = 7.59 Hz, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 148.9 (C=O), 133.0, 133.4, 143.4, 132.1, 129.9, 129.5, 128.0, 127.9, 127.7, 127.4, 126.2 (C-5), 125.8 (C-4a), 122.4 (C-7a), 47.1 (N_2-CH_2), 44.0 (N_4-CH_2), 39.1 (Py-CH_3), 28.0 (1C, CH_2), 15.8 (1C, CH_3). MS (EI): m/z 445.5 $[M + H]^+$ (calcd for $C_{21}H_{21}ClN_4O_3S$: 444.1).

2-(p-(t-Bu)benzyl)-4-(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3h)***. Compound (**1**) and 2-chlorobenzyl chloride were allowed to react at 40-50°C for 8 h. After the addition of 4-(*t*-Bu)benzyl chloride, the mixture was allowed to react at 70-80°C for 20 h. Purification by recrystallization from ethanol yielded compound **3h** as a white solid (55%), mp: 140-142°C. IR (KBr, cm^{-1}): 1,700 (C=O), 1,344, 1,189 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.75 (s, 1H, PyH), 7.50 (dd, 1H, *J* = 7.90 Hz, *J* = 1.08 Hz, PhH), 7.00 (dd, 1H, *J* = 7.52 Hz, *J* = 1.02 Hz, PhH), 7.26-7.35 (m, 6H, PhH), 5.16 (s, 2H, NCH_2), 4.99 (s, 2H, NCH_2), 4.08 (s, 3H, CH_3), 1.25 (s, 9H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 150.2 (C=O), 133.0, 133.1, 148.9, 132.1, 129.9, 129.5, 127.7, 127.6, 127.4, 126.3, 125.8 (C-5), 125.3 (C-4a), 122.4 (C-7a), 47.1 (N_2-CH_2), 43.9 (N_4-CH_2), 39.1 (CH_3), 34.4 (C), 31.2 (3C, CH_3). MS (EI): m/z 473.3 $[M + H]^+$ (calcd for $C_{23}H_{25}ClN_4O_3S$: 472.13).

2-(p-Nitrobenzyl)-4-(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine

(3i). Compound (**1**) and 2-chlorobenzyl chloride were allowed to react at 40-50°C for 8 h. After the addition of 4-nitrobenzyl bromide, the mixture was allowed to react at 50-55°C for 12 h. Purification by recrystallization from ethanol yielded compound **3i** as a white solid (70%), mp: 210-212°C. IR (KBr, cm^{-1}): 1,689 (C=O), 1,331, 1,193 (SO_2), 1,520, 1,278 (NO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.78 (s, 1H, PyH), 7.49 (dd, 1H, *J* = 7.34 Hz, *J* = 1.28 Hz, PhH), 7.05 (dd, 1H, *J* = 7.68 Hz, *J* = 2.10 Hz, PhH), 7.29-8.21 (m, 6H, PhH), 5.18 (s, 2H, NCH_2), 5.15 (s, 2H, NCH_2), 4.09 (s, 3H, CH_3). MS (EI): m/z 462.4 $[M + H]^+$ (calcd for $C_{19}H_{16}ClN_5O_5S$: 461.06).

2-(p-Bromobenzyl)-4-(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3j)***. Compound (**1**) and 2-chlorobenzyl chloride were allowed to react at 40-50°C for 8 h. After the addition of 4-bromobenzyl bromide, the mixture was allowed to react at 50-55°C for 12 h. Purification by recrystallization from ethanol gave compound **3j** as a white solid (68%), mp: 148-150°C. IR (KBr, cm^{-1}): 1,696 (C=O), 1,330, 1,192 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.75 (s, 1H, PyH), 7.53 (d, 2H, *J* = 8.39 Hz, PhH), 7.50 (d, 1H, *J* = 7.76Hz, PhH), 7.00 (d, 1H, *J* = 7.34 Hz, PhH), 7.28-7.32 (m, 4H, PhH), 5.15 (s, 2H, NCH_2), 5.00 (s, 2H, NCH_2), 4.08 (s, 3H, CH_3). MS (EI): m/z 495.2 $[M + H]^+$, 497.2 $[M + 3]^+$ (calcd for $C_{19}H_{16}BrClN_4O_3S$: 493.98).

2-(p-Nitrobenzyl)-4-benzyl-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3k)***. Compound (**1**) and bromobenzyl bromide were allowed to react at 30-40°C for 12 h. After the addition of 4-nitrobenzyl bromide, the mixture was allowed to react at 40-50°C for 12 h. Purification by recrystallization from ethanol yielded compound **3k** as a white solid (75%), mp: 202-204°C. IR (KBr, cm^{-1}): 1,692 (C=O), 1,327, 1,190 (SO_2), 1,514, 1,214 (NO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.84 (s, 1H, PyH), 8.21 (d, 2H, *J* = 8.72 Hz, PhH), 7.63 (d, 2H, *J* = 8.70 Hz, PhH), 7.26-7.34 (m, 5H, PhH), 5.19 (s, 2H, NCH_2), 5.10 (s, 2H, NCH_2), 4.06 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 149.0 (C=O), 135.8, 144.0, 128.8, 128.7, 127.1, 123.7, 147.1, 127.8, 126.3 (C-5), 125.6 (C-4a), 122.0 (C-7a), 48.8 (N_2-CH_2), 43.4 (N_4-CH_2), 39.0 (CH_3). MS (EI): m/z 428.4 $[M + H]^+$ (calcd for $C_{19}H_{17}N_5O_5S$: 427.1).

2-(o-Bromobenzyl)-4-benzyl-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3l)***. Compound (**1**) and bromobenzyl bromide were allowed to react at 30-40°C for 12 h. After the addition of 2-bromobenzyl bromide, the mixture was allowed to react at 40-50°C for 12 h. Purification by recrystallization from ethanol yielded compound **3l** as a white solid (74%), mp: 182-184°C. IR (KBr, cm^{-1}): 1,701 (C=O), 1,337, 1,192 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.88 (s, 1H, PyH), 7.66 (dd, 1H, *J* = 7.93 Hz, *J* = 1.08 Hz, PhH), 7.19 (dd, 1H, *J* = 7.72 Hz, *J* = 1.10Hz, PhH), 7.25-7.37 (m, 7H, PhH), 5.12 (s, 2H, NCH_2), 5.08 (s, 2H, NCH_2), 4.06 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 148.9 (C=O), 134.7, 135.9, 132.7, 129.6, 128.9, 128.2, 127.9, 127.6, 127.2, 126.4, 125.7 (C-5), 122.0 (C-4a), 121.7 (C-7a), 48.5 (N_2-CH_2), 44.4 (N_4-CH_2), 38.9 (CH_3). MS (EI): m/z 461.4 $[M + H]^+$, 463.3 $[M + 3]^+$ (calcd for $C_{19}H_{17}BrN_4O_3S$: 460.02).

2-(p-Bromobenzyl)-4-(o-bromobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3m)***. Compound (**1**) and 2-bromobenzyl bromide were allowed to react at 30-40°C for 12 h. After the addition of 4-bromobenzyl bromide, the mixture was allowed to react at 40-50°C for 12 h. Purification by recrystallization from ethanol yielded compound **3m** as a white solid (76%), mp: 146-148°C. IR (KBr, cm^{-1}): 1,696 (C=O), 1,330, 1,192 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.74 (s, 1H, PyH), 7.67 (d, 1H, *J* = 7.81 Hz, PhH), 7.53 (d, 2H, *J* = 8.42 Hz, PhH), 6.93 (d, 1H, *J* = 7.58 Hz, PhH), 7.24-7.32 (m, 4H, PhH), 5.10 (s, 2H, NCH_2), 5.01 (s, 2H, NCH_2), 4.08 (s, 3H, CH_3). MS (EI): m/z 541.2 $[M + H]^+$ (calcd for $C_{19}H_{16}Br_2N_4O_3S$: 537.93).

2-(p-Chlorobenzyl)-4-(o-bromobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3n)***. Compound (**1**) and 2-bromobenzyl bromide were allowed to react at 30-40°C for 12 h. After the addition of 4-chlorobenzyl chloride, the mixture was allowed to react at 50-60°C for 20 h. Purification by recrystallization from ethanol yielded compound **3n** as a white solid (70%), mp: 128-130°C. IR (KBr, cm^{-1}): 1,695 (C=O), 1,331, 1,192 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.12 (s, 1H, PyH), 7.60 (dd, 1H, *J* = 7.83 Hz, *J* = 1.15 Hz, PhH), 6.88-7.47 (m, 7H, PhH), 5.15 (s, 2H, NCH_2), 5.08 (s, 2H, NCH_2), 4.14 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz,) δ: 149.2 (C=O), 134.1 (C-1'), 137.3 (C-1"), 132.5, 131.6, 129.6, 129.5, 129.0 128.4, 128.1, 127.2, 126.9, 126.7, 125.2 (C-4a), 125.0 (C-5), 122.6 (C-7a), 46.6 (N4-CH_2), 43.7 (N_2-CH_2), 38.9 (CH_3). MS (EI): m/z 495.2 $[M + H]^+$, 497.2 $[M + 3]^+$ (calcd for $C_{19}H_{16}BrClN_4O_3S$: 493.98).

2-(o-Bromobenzyl)-4-(m-chlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine ***(3o)***. Compound (**1**) and 3-chlorobenzyl chloride were allowed to react at 40-50°C for 20 h. After the addition of 2-Br benzyl bromide, the mixture was allowed to react at 40-50°C for 12 h. Purification by recrystallization from ethanol yielded compound **3o** as a white solid (70%), mp: 108-110°C. IR (KBr, cm^{-1}): 1,680 (C=O), 1,327, 1,192 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.91 (s, 1H, PyH), 7.66 (dd, 1H, *J* = 7.40

Hz, *J* = 0.92 Hz, PhH), 7.20 (d, 1H, *J* = 7.70 Hz, PhH), 7.23-7.38 (m, 6H, PhH), 5.16 (s, 2H, NCH_2), 5.07 (s, 2H, NCH_2), 4.07 (s, 3H, CH_3). MS (EI): m/z 495.2 $[M + H]^+$, 497.2 $[M + 3]^+$ (calcd for $C_{19}H_{16}BrClN_4O_3S$: 493.98).

*2-(p-Chlorobenzyl)-4-(2,4-dichlorobenzyl)-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazine (**3p**).* Compound (**1**) and 2,4-dichlorobenzyl chloride were allowed to react at 40-50°C for 20 h. After the addition of 4-chlorobenzyl chloride, the mixture was allowed to react at 50-60°C for 20 h. Purification by recrystallization from ethanol yielded compound **3p** as a white solid (67%), mp: 150-152°C. IR (KBr, cm^{-1}): 1,694 (C=O), 1,333, 1,192 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.78 (s, 1H, PyH), 7.68 (d, 1H, *J* = 2.15 Hz, PhH), 7.05 (d, 1H, *J* = 8.39 Hz, PhH), 7.36-7.41 (m, 5H, PhH), 5.12 (s, 2H, NCH_2), 5.01 (s, 2H, NCH_2), 4.08 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 148.8 (C=O), 133.2, 135.2, 133.1, 132.4, 132.3, 129.8, 129.4, 129.2, 129.0, 128.9, 128.5, 127.8, 126.3 (C-5), 125.7 (C-4a), 122.2 (C-7a), 47.0 (N_2-CH_2), 43.5 (N_4-CH_2), 39.1 (CH_3). MS (EI): m/z 485.3 $[M + H]^+$ (calcd for $C_{19}H_{15}Cl_3N_4O_3S$: 483.99).

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A new cell-based reporter system for sensitive screening of nuclear export inhibitors

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ABSTRACT: Nucleocytoplasmic transport of proteins across the nuclear pore complex (NPC), mediated by the nuclear localization signal (NLS) and the nuclear export signal (NES), is a vital homeostatic process in eukaryotic cells and also in mitogen-activated protein kinase (MEK) signaling molecule in tumor cell proliferation. Some viruses, including the influenza virus and HIV-1, also employ this nuclear export mechanism during their life cycle. Hence, drugs that control nucleocytoplasmic transport of proteins are putative candidate antivirals or anti-cancer agents. Thus, we previously developed a GFP/NES-MDCK reporter cell system for screening novel nuclear export inhibitors. NES signal-conjugated GFP accumulates in the nucleus in the presence of the nuclear export inhibitor leptomycin B (LMB). In this study, a stable GFP/NLS/NES fusion protein-expressing cell line was established, and its potential as a reporter was evaluated. The GFP/NLS/NES-MDCK cell line demonstrates improved nuclear accumulation of GFP in a time-course treatment with LMB. In addition, the dose-response data demonstrated superior sensitivity of GFP/NLS/NES-MDCK over GFP/NES-MDCK cells. As low as 0.01 ng/mL LMB is sufficient to cause accumulation of the GFP fusion protein in the nucleus in GFP/NLS/NES-MDCK cells, while at least 1 ng/mL of LMB is needed for the accumulation of GFP fusion protein in the nucleus of GFP/NES-MDCK cells. These results indicate that the newly established GFP/NLS/NES-MDCK cell line is a potentially powerful tool to screen for novel nuclear export inhibitors.

Keywords: Reporter system, nuclear export, antiviral drugs

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1. Introduction

In eukaryotic cells, proteins and other macromolecules constantly move in and out of the nucleus. The nuclear envelope separates the translational and metabolic machinery of the cytoplasm from the genetic material and transcriptional machinery of the nucleus. The nuclear pore complex (NPC), a 125-MDa macromolecular complex of polypeptides collectively referred to as nucleoporins, with a central diameter of about 10 nm (*1*), is a conduit for the bi-directional movement of molecules between the nucleus and the cytoplasm. Some of these nucleoporins contain arrays of hydrophobic peptide repeats containing phenylalanine and glycine residues, which form the permeability barrier of the NPC. Small molecules (for instance, ions and proteins) up to 50 kDa in size, or less than 10 nm in diameter, can freely diffuse through the NPC without consuming energy. Nucleocytoplasmic shuttling of macromolecules larger than 50 kDa is an active, energy-dependent, and essential process that is mediated by selective sequence-specific motifs, the nuclear localization signal (NLS) and the nuclear export signal (NES) (*2*). Molecules that have to enter the nucleus are carried in by nuclear import receptors called importins (karyopherins) (*3*). Importin α proteins recognize the NLS on cargo proteins (*4*). Once the cargo is bound by importin α, the complex is recognized and bound by importin β that subsequently binds to the fibrils of the NPC and is responsible for the actual translocation. Besides cargo and transport receptors, the signaling protein Ran, which hydrolyzes GTP, is responsible for regulating the interaction of transport receptor and cargo (*5*), and Ran-GDP/Ran-GTP concentration gradients across the nuclear envelope drive nuclear import and export (*6*). Once inside the nucleus, binding of Ran-GTP to importin β elicits a conformational change resulting in cargo release in the nucleus (*7*). By contrast, proteins that transfer cargo out of the nucleus are called exportins. Exportin1, also known as chromosome region maintenance protein 1 (CRM1), mediates the export of the numerous proteins bearing a NES from the nucleus (*8,9*). Ran-GTP stimulates binding of the CRM1 to NES cargo in

the nucleus, and the complex is then exported to the cytoplasm where Ran-GTP is hydrolyzed to Ran-GDP. Thereafter, Ran undergoes a conformational change, causing the complex to dissociate and the exportins and Ran-GDP to shuttle back to the nucleus leaving the cargo behind in the cytoplasm. CRM1 is the normal exportin for proteins, RNAs, and ribonucleoproteins complexes (*10*). Leptomycin B (LMB), an antibiotic with antifungal and antitumor activity, has recently become an important tool for elucidating nuclear localization and trafficking in eukaryotic cells. The export substrates of CRM1 possess a leucine-rich NES. A protein containing both NES and NLS is supposed to shuttle between nucleus and cytoplasm, but instead accumulates preferentially in the nucleus of cells treated with LMB (*11*). LMB specifically and irreversibly inhibits CRM1 by covalently binding to a cysteine residue in a central domain of CRM1 (*12*), confirming that CRM1 is a crucial factor for nuclear export of proteins in eukaryotes. The NES has also been identified in viral proteins such as type A influenza virus NS2 (*13*), HIV-1 Rev (*14*), and in host factors like cAMP-dependent protein kinase A inhibitor (PKI-α) (*15*), heat shock cognate protein 70 (Hsc70) (*16*), and MAP kinase kinase (MEK) (*17*).

A virus is a unique pathogen that utilizes host cell environment and proteins for its propagation. Therefore, nuclear export proteins can serve as targets for new and potent antiviral drugs since some viruses, including HIV-1 and influenza virus utilize the nuclear export machinery of the host cell. Development of antiviral drugs is one of the best ways to treat these infectious diseases. However, newly developed antiviral drugs are likely to be ineffective on some viruses in short time, especially RNA viruses, which develop resistance through mutation. For instance, a globally high prevalence of more than 90% was reported for oseltamivir-resistant influenza A (H1N1) in 2009 compared to a lower rate in 2008 (*18*). Similarly, HIV can also develop drug resistance, and therefore, a multi-drug combination therapy protocol termed HAART (highly active anti-retroviral therapy) is now used to treat HIV patients (*19*).

We have focused on identifying novel nuclear export inhibitors that confer selective inhibition of viral propagation with minimal toxicity to host cells. To achieve this, a MDCK cell-based reporter system based on an enhanced GFP fused with a NES domain was established (*20*). GFP-fused protein accumulates in the nucleus in the presence of LMB in this cell line. Using this cell line, we previously reported 2 possible nuclear export inhibitors, namely, ACA and valtrate (*21*). Nonetheless, the cell line was not sufficiently sensitive to screen for nuclear export inhibition activity, possibly because of the absence of NLS in its construct. In this report, we describe a newly established stable cell line that expresses a GFP/NLS/NES fusion protein, and thus, is more sensitive to nuclear export inhibitors as evidenced by the significant nuclear GFP fluorescence.

2. Materials and Methods

2.1. *Chemicals*

Mardin-Darby canine kidney cells (MDCK) were grown in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (unless otherwise stated) at 37°C in a 5% CO_2 incubator. Geneticin (G418) was dissolved in water at 10 mg/mL and stored at −20°C. LMB was purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA, USA) and was dissolved in 100% ethanol at a concentration of 10 µg/mL and stored at −20°C. Paraformaldehyde (Wako, Osaka, Japan) was prepared as 4% (w/v) in PBS and stored at 4°C. Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) was stored as a 10 mM stock solution in water at −20°C.

2.2. *Construction of pEGFP-NLS-NES plasmid*

To generate a plasmid harboring GFP/NLS/NES, the previously established pEGFP-NES (*22*) was modified to add the NLS coding region from the SV40 large T antigen (Figure 1). First, 2 oligonucleotides, 5'-GATCTCCAAAAAAGAAGAGAAAGGTACA-3' and 5'-AGCTTGTACCTTTCTCTTCTTTTTTGGA-3' encoding the NLS sequence (PKKKRKV), were annealed by incubation at 90°C for 15 min, followed by gradual cooling at room temperature. The resulting oligonucleotide segment was subsequently ligated between the GFP and NES coding sequence of pEGFP-NES by using *Hin*dIII and *Bgl*II restriction sites with a ligation mix (Nippon Gene, Tokyo, Japan). The ligation product was transformed into ultra-competent *Escherichia coli* Mach1 cells and grown on a Luria-Bertani agar plate containing 30 µg/mL kanamycin. Plasmids were isolated from a single cultured positive colony by the alkaline lysis method. Verification of NLS sequence in the plasmid was confirmed with an Applied Bio 3100-Avant Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). To increase the amount of pEGFP-NLS-NES plasmid, a 100-mL culture suspension of *E. coli* cells harboring the plasmid was grown, and the plasmid was purified by Nucleobond® Xtra Midi-prep kit (Machery-Nagel, Duren, Germany).

2.3. *Establishment of GFP/NLS/NES fusion protein expressing cells*

MDCK cells were seeded into 12-well plate at a density of 1×10^5 cells/well in MEM medium supplemented with 10% FBS without antibiotics and incubated for 24 h at 37°C in a 5% CO_2 incubator. After 1 day, plasmid DNA was introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Briefly, 1.6 µg of

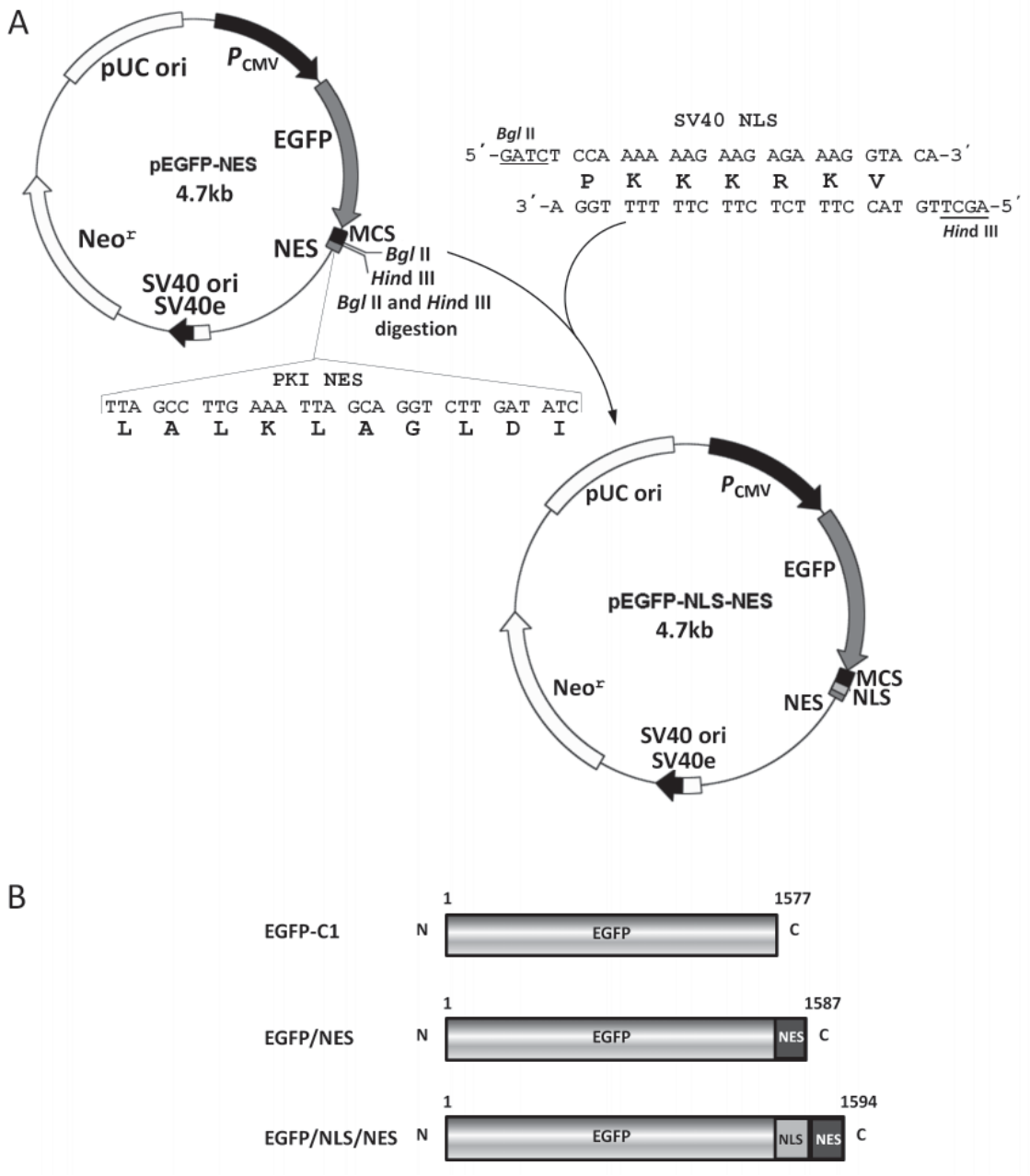

Figure 1. Schematic depiction of GFP-NLS-NES plasmid construction. (A) An insert DNA fragment encoding a PKKKRKV polypeptide from the SV 40 large T antigen was cloned into a *Hin*dIII and *Bgl*II digested pEGFP-NES plasmid. **(B)** Schematic representations of wild-type GFP-C1 and its derivatives.

plasmid DNA was mixed with 8 μL of Lipofectamine 2000 and incubated for 20 min at room temperature to allow DNA-Lipofectamine 2000 complexes to form. The transfection mixture was added to each well containing cells and medium, mixed gently by rocking the plate and incubated for 5 h after which the growth medium was replaced. Twenty-four hours after transfection, the cells were passaged onto a 90-mm dish and incubated at 37°C in a 5% CO_2 incubator. Cells transfected with pEGFP-NLS-NES were selected for neomycin resistance by culturing in a selection medium containing 600 μg/mL of G418 for 2 weeks. The G418-resistant clones (GFP/NLS/NES-MDCK) were then collected and expanded.

2.4. *Characterization of established cell line*

GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK cells were separately cultured on 15-mm glass coverslips in a 24-well plate at a density of 6×10^4 cells/well in 500 μL of MEM medium supplemented with 5% FBS and 1% penicillin/streptomycin. After 24 h, the cells were fixed for 10 min with 4% (w/v) paraformaldehyde in 1× PBS at room temperature, followed by staining with 10 μM Hoechst 33342 for 10 min at room temperature. The coverslips were then inverted onto microscope slides, and the morphology and the GFP fluorescence distribution pattern of each cell line was observed using an Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany).

2.5. *Time-course nuclear translocation assay*

GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK cells were independently seeded on glass coverslips in a 24-well plate at a concentration of 6×10^4 cells/well in 500 µL of MEM medium containing 5% FBS and 1% penicillin/streptomycin and incubated at 37°C in 5% CO_2 incubator. After 24 h, each cell line was treated with 10 ng/mL LMB and further incubated for 1 h and 3 h. Cells at representative timepoints were washed twice with 1× PBS and fixed with 4% (w/v) paraformaldehyde in 1× PBS for 10 min, and subsequently stained with 10 µM Hoechst 33342 for 10 min. The coverslips were then mounted onto glass slides, and the nucleocytoplasmic distribution of GFP-fusion protein was observed under an Axiophot microscope (Carl Zeiss).

2.6. *Dose-course nuclear translocation assay*

The 3 different cell lines, namely, GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK were separately cultured for 24 h on 15-mm glass coverslips in a 24-well plate at a concentration of 6×10^4 cells/well in 500 µL of MEM medium containing 5% FBS and 1% penicillin/streptomycin and incubated at 37°C in a 5% CO_2 incubator. After 24 h, each cell line was subjected to a varying concentration of LMB (10, 1, 0.1, 0.03, and 0.01 ng/mL) for 3 h. After removing the LMB-containing MEM, cells were rinsed twice with 1× PBS, fixed with 4% paraformaldehyde in 1× PBS for 10 min, and stained with 10 µM Hoechst 33342 for 10 min. The coverslips were then mounted onto glass slides, and the nucleocytoplasmic distribution of GFP-fusion protein was observed under Axiophot microscope (Carl Zeiss).

3. Results

3.1. *Establishment of a cell line expressing a GFP/NLS/NES fusion protein*

To establish a cell line stably expressing the GFP/NLS/NES fusion protein, MDCK cells were transfected with pEGFP-NLS-NES (Figure 1), and colonies were selected in the presence of geneticin (G418) sulfate. One clone out of the 3 individual clones isolated demonstrated relatively high fluorescence and was preferentially selected and designated GFP/NLS/NES-MDCK.

3.2. *General properties of the established cell line*

To evaluate the general characteristics of the cell lines established, GFP-MDCK, GFP/NES-MDCK (GES5) (*20*), and GFP/NLS/NES-MDCK cell lines were cultured for 24 h, and the nucleocytoplasmic distribution of the GFP-fusion protein was observed under a fluorescence microscope (Figure 2). GFP fluorescence is detected in both the nucleus and the cytoplasm of GFP-MDCK cells, although the fluorescence is slightly higher in the nucleus. GFP/NES-MDCK cells demonstrate significantly higher GFP fluorescence accumulation in the cytoplasm, while the nucleus appears dark due to the lack of GFP fluorescence. By contrast, GFP fluorescence in GFP/NLS/NES-MDCK cells is distributed in both the nucleus and cytoplasm, but the fluorescence intensity is slightly higher in the nucleus than in the cytoplasm. This GFP fluorescence distribution in both the nucleus and the cytoplasm indicates that the NLS in the GFP/NLS/NES-MDCK is functional in the cell. All GFP/NLS/NES-MDCK cells had extensive bright green fluorescence that facilitates microscopic observation. Although these cell lines were fundamentally MDCK, the observed size of the nucleus in GFP/NLS/NES-MDCK cells was slightly smaller compared to other counterparts.

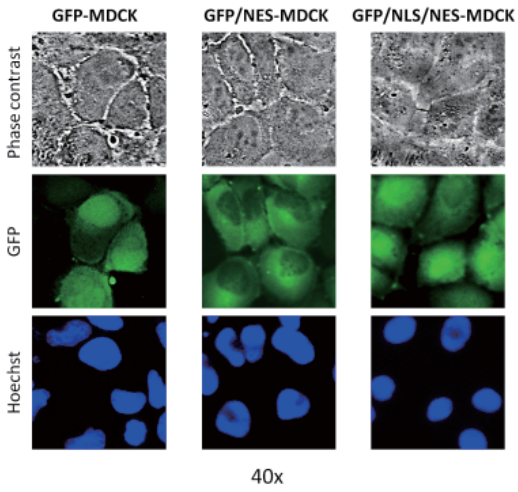

Figure 2. Distribution of sub-cellular fluorescence in established cell lines. Three different cell lines, namely, GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK were independently cultured, fixed with 4% paraformaldehyde and successively stained with 10 µM Hoechst 33342. The nucleocytoplasmic distribution of GFP fusion protein was observed using a fluorescence microscope (40×).

3.3. *Effect of LMB on nuclear accumulation of GFP in stable cell lines*

To compare the distribution and intensity of GFP fluorescence in the presence of nuclear export inhibitor, the 3 cell lines were treated with 10 ng/mL of LMB treatment for specified length of time and observed under fluorescence microscope (Figure 3). As previously reported, the GFP fusion protein is primarily detected in the nucleus of the GFP/NES-MDCK cell line, whereas the GFP fluorescence in GFP-MDCK did not change after 1 or 3 h of LMB treatment (*20*). By contrast, at both 1 h and 3 h timepoints, fluorescence accumulation was significantly higher in the nucleus of GFP/NLS/NES-MDCK cells when compared to the fluorescence of GFP/NES-MDCK cells. These results demonstrate a more prominent response to LMB treatment in the newly established GFP/NLS/NES-MDCK cells than in the GFP/NES-MDCK cells.

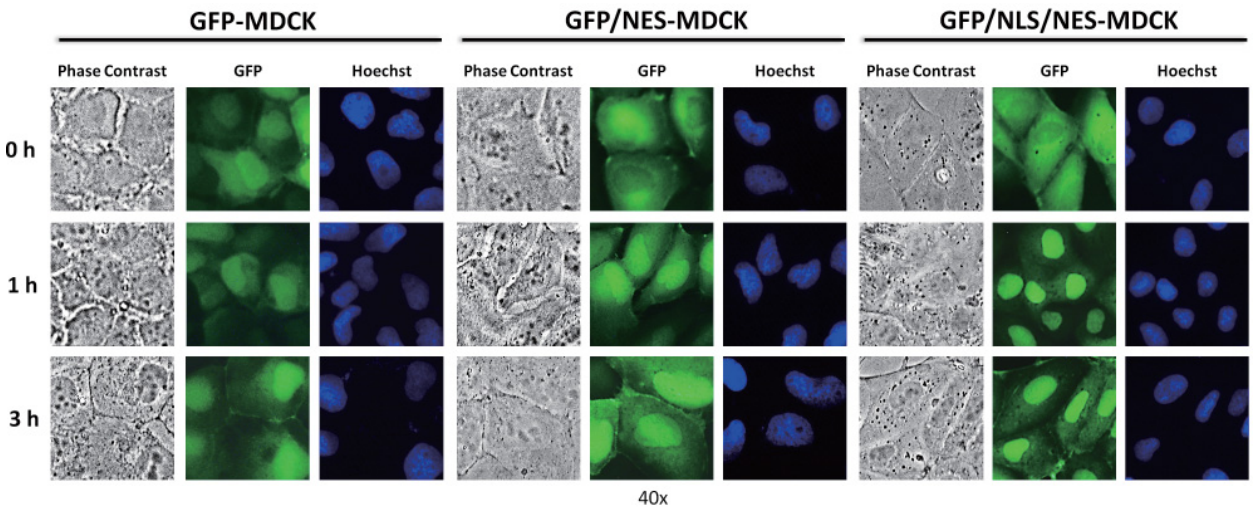

Figure 3. Time-course of nuclear translocation. GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK cells were independently cultured for 24 h, and each cell line was treated with 10 ng/mL LMB for 1 and 3 h. Cells from representative timepoints were then fixed with 4% paraformaldehyde and stained with 10 μM Hoechst 33342 and observed under a fluorescence microscope (40×).

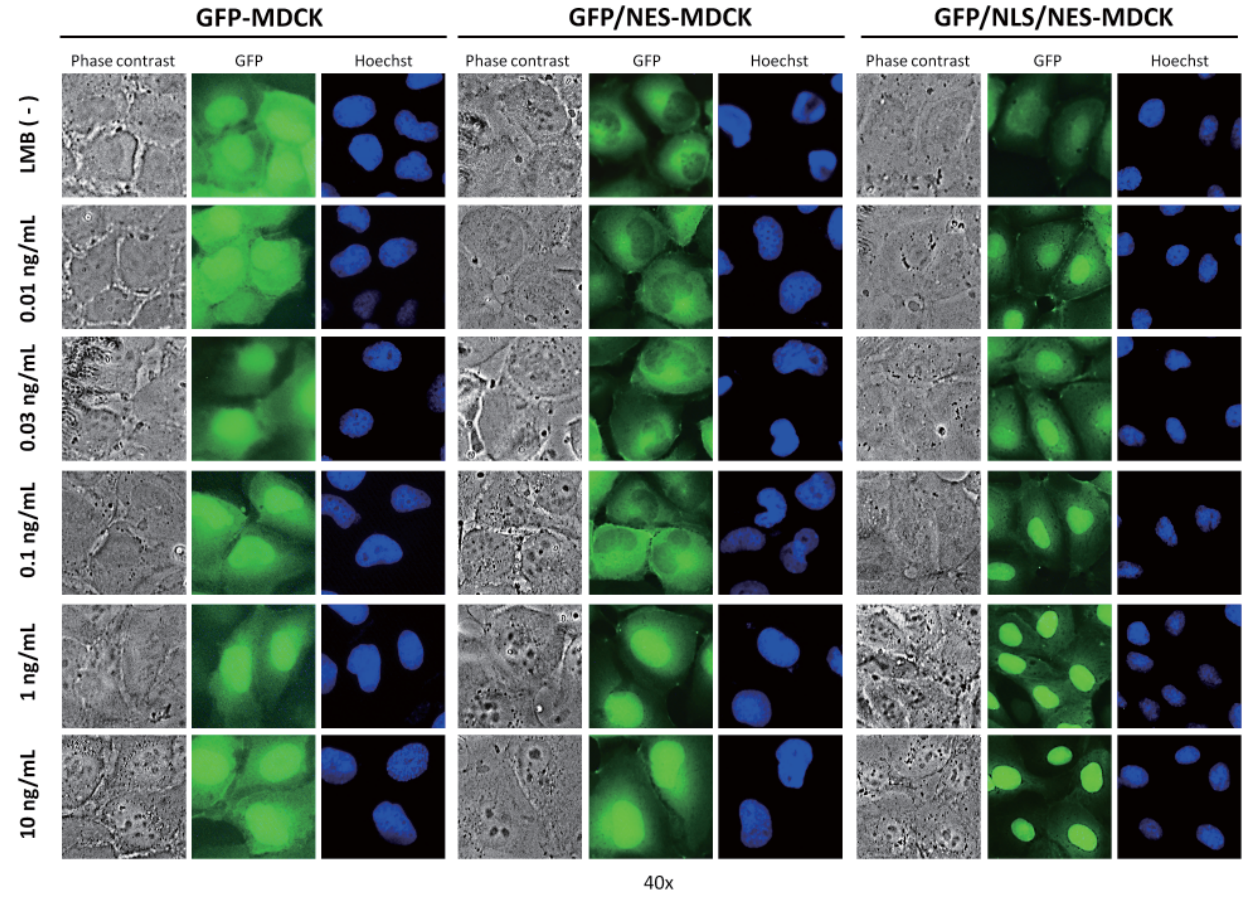

Figure 4. Titration of LMB. GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK cells were cultured independently for 24 h before each cell line was treated with varying concentrations of LMB for 3 h. The cells were fixed with 4% paraformaldehyde, stained with 10 μM Hoechst 33342, and the nucleocytoplasmic fluorescence distribution and intensity was observed under a fluorescence microscope (40×).

3.4. *Sensitivity of the GFP/NLS/NES-MDCK cells to the nuclear export inhibitor*

To demonstrate the sensitivity of GFP-MDCK, GFP/NES-MDCK, and GFP/NLS/NES-MDCK cells to different concentrations of LMB, these cells were treated with varying concentrations of LMB. As shown in Figure 4, a steady dose-dependent increase in nuclear fluorescence accumulation is observed in both GFP/NES-MDCK and GFP/NLS/NES-MDCK cells.

Furthermore, at 3-h post-LMB treatment, significant nuclear localization of GFP fluorescence is observed in GFP/NLS/NES-MDCK at an LMB dose as low as 0.01 ng/mL. By contrast, GFP/NES-MDCK does not appear to respond to LMB doses below 1 ng/mL. These results suggest that the newly established GFP/NLS/NES-MDCK cell line, but not the GFP/NES-MDCK cell line, is hypersensitive to LMB.

4. Discussion

The aim of this study was to establish a cell line with improved sensitivity of GFP distribution to nuclear export inhibitors as a tool for preliminary screening of nuclear export inhibitors as antiviral lead compounds. To achieve this goal, we developed a cell line stably expressing a GFP/NLS/NES fusion protein to monitor the localization of GFP fluorescence in the nucleus in the presence of putative nuclear export inhibitors. In general, the GFP/NLS/NES fusion protein is distributed in both the nucleus and the cytoplasm of GFP/NLS/NES-MDCK cells; however, the fluorescence intensity in the nucleus was slightly higher than that in the cytoplasm of these cells (Figure 2). GFP fluorescence is observed in both the nucleus and cytoplasm of GFP/NLS/NES-MDCK cells because of the action of NLS and NES signals that enable the GFP-fusion protein to be transported to and from the nucleus. Nuclear fluorescence intensity is slightly higher than the cytoplasmic fluorescence intensity of GFP/NLS/NES-MDCK cells possibly because the NES of PKI is slightly weaker than the NLS of SV40 large T antigen (*15*). By contrast, GFP/NES-MDCK cells demonstrate substantially higher GFP fluorescence in the cytoplasm, while the nucleus is devoid of green fluorescence.

The LMB time-course response data (Figure 3) demonstrate that the GFP-fused protein localizes mainly in the nucleus of GFP/NES-MDCK cells, as previously reported (*20*). Despite the addition of LMB, no variation in sub-cellular GFP fluorescence distribution was observed in GFP-MDCK cells. By contrast, a GFP with both NLS and NES – expected to shuttle between the nucleus and cytoplasm – extensively accumulates in the nucleus of the LMB-treated cells. At 1-h and 3-h timepoints, GFP fluorescence was significantly more conspicuous in the nucleus of GFP/NLS/NES-MDCK cells than in the nucleus of GFP/NES-MDCK cells. This remarkable nuclear accumulation response of the newly established GFP/NLS/NES-MDCK cell line in the presence of nuclear export inhibitor is crucial in the efficient detection of novel nuclear export inhibitors.

Dose-response assays demonstrate considerable nuclear fluorescence accumulation in GFP/NLS/NES-MDCK cells at LMB concentrations as low as 0.01 ng/mL, whereas previously established GFP/NES-MDCK cells (*20*) do not exhibit nuclear accumulation of GFP-fusion protein at less than 1 ng/mL of LMB. Thus, there is almost a hundred-fold increase in LMB sensitivity in the GFP/NLS/NES-MDCK cell line when compared to the GFP/NES-MDCK cell line. Moreover, the effective working concentration of 0.01 ng/mL LMB in GFP/NLS/NES-MDCK cells is significantly lower than the working concentrations (5-10 ng/mL) employed in other nuclear export inhibition studies (*22-24*). This improved sensitivity underscores the utility of this new cell line as a tool for screening potentially novel nuclear export inhibitors even at very low concentrations. The newly established cell line is also safer because it eliminates the need for the use of live influenza or HIV-1 viruses when screening for antivirals with nuclear export inhibition activity. Furthermore, recent studies have demonstrated that MEK contains NES (*17*) and it shuttles between the cytoplasm and nucleus (*25*) and thus inhibitors of nuclear transport may also present novel opportunity for the development of anti-cancer drugs. This cell line is easily maintained with standard media and serum, and hence, it is fairly cost-effective for high-throughput screening. We are currently using this cell line to screen for novel nuclear export inhibitors.

Acknowledgments

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Levels of effectiveness of gene therapies targeting survivin and its splice variants in human breast cancer cells

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ABSTRACT: In order to develop an effective strategy of breast cancer therapy targeting survivin and its splice variants survivin-ΔEx3 and survivin-2B, the present study constructed four expression vectors by fusing the survivin antisense gene, the survivin (T34A) gene, the survivin-ΔEx3 antisense gene, and the survivin-2B gene with the enhanced green fluorescent protein (eGFP) gene. Each of these vectors was transiently transfected into the B-Cap-37 human breast cancer cell line. The effects of these four vectors with diverse genes on the proliferation and apoptosis of B-Cap-37 breast cancer cells were examined and compared *in vitro* using MTT and flow cytometry assays. Results of the MTT assay indicated that all four gene therapy plasmids were most effective at inhibiting the proliferation of B-Cap-37 cells 72 h after transfection. However, the four gene therapies had different rates of cell inhibition. pcDNA3.1(+)-egfp-anti-survivin and pcDNA3.1(+)-survivin (T34A)-egfp had almost equivalent or better effectiveness at suppressing cell growth. pcDNA3.1(+)-egfp-anti-survivin-ΔEx3 moderately inhibited the growth of B-Cap-37 cells. In contrast, pcDNA3.1(+)-survivin-2B-egfp had limited inhibition of cell growth. Similar profile of effectiveness of four gene therapies in soliciting cell apoptosis was also observed. These results suggest the relative importance of targeting survivin and its splice variant survivin-ΔEx3 in breast cancer treatment.

Keywords: Survivin, splice variant, growth inhibition, apoptosis, breast cancer

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1. Introduction

Breast cancer is one of the most frequent female malignancies worldwide. Existing treatment modalities such as chemotherapy, radiotherapy, and chemotherapy are not always effective and curative and even cause significant side effects. In recent years, gene therapy has been incorporated in breast carcinoma treatment as a novel anti-tumor strategy and has had considerable success (*1,2*). Selecting the appropriate target and therapeutic strategy are key factors for an effective gene therapy.

Survivin, a member of the inhibitor of apoptosis (IAP) family, has attracted considerable attention as an ideal target for cancer treatment because it is highly and uniquely expressed in most human tumors and plays a critical role in both control of cell division and inhibition of apoptosis (*3-7*). A high level of survivin expression has been observed in breast carcinoma and has been found to be strongly associated with poor patient prognosis (*8-10*). This fact suggests that survivin may represent a promising target for breast cancer gene therapy. However, several survivin splice variants, such as survivin-ΔEx3 and survivin-2B, have also been identified in breast cancer thus far. Survivin-ΔEx3 is presumed to play a positive role in inhibiting apoptosis while survivin-2B might act as a proapoptotic factor in breast cancer and a high level of its expression is thought to be associated with a good prognosis (*11*). Targeting either, whether by decreasing the expression of survivin-ΔEx3 or increasing the expression of survivin-2B, may induce apoptosis and suppress proliferation in breast cancer cells, but their potential importance, especially when compared to survivin, to novel gene drug research and development remains unclear.

Several studies reported that inhibiting or blocking survivin activity by strategies like siRNA, antisense RNA, and dominant negative mutants promoted cell apoptosis in many cancers (*12-16*). In order to examine the effectiveness of targeting survivin and its splice variants and different gene therapy strategies in treating

breast cancer, four gene therapy plasmids expressing survivin (T34A) (the dominant negative mutant of survivin), antisense survivin, antisense survivin-ΔEx3, and survivin-2B were constructed and transiently transfected into the B-Cap-37 breast cancer cell line. The present study examined their inhibition of proliferation and induction of apoptosis in breast cancer cells.

2. Materials and Methods

2.1. *Cell line and cell culture*

Human breast carcinoma cell line B-Cap-37 is kindly provided by Dr. Yanhong Zhang (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). They were cultivated in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and penicillin-streptomycin (100 IU/mL-100 μg/mL) and incubated at 37°C in a humid atmosphere (5% CO_2, 95% air).

2.2. *Vector construction and identification*

Total RNA was isolated from the B-Cap-37 breast carcinoma cell line with Trizol reagent (Promega, Madison, WI, USA). The cDNAs of survivin and two splice variants were amplified by RT-PCR using 5 μg total RNA with the RT-PCR kit (Promega) and primers P1 and P2 (Table 1). The amplified PCR products of survivin and its splice variants survivin-2B and survivin-ΔEx3 were introduced into a pGEM-T vector (Promega) after clean-up and A-tailing reaction with Pfu/An Amp Tailing Kit (Takara Bio, Otsu, Shiga, Japan), and the resultant plasmid was used to transform *E. coli* DH5α cells (EMD Chemicals Inc., Darmstadt, Germany). The transformants were cultured on an Luria broth (LB) plate with IPTG/X-gal and ampicillin and white clones were selected. The recombinant plasmids pGEM-T-survivin, pGEM-T-survivin-2B, and pGEM-T-survivin-ΔEx3 were identified with restriction endonuclease cleavage of *Eco*R I and *Xho* I. PCR amplification and sequence confirmation were performed (Invitrogen). Similarly, a fluorescent reported gene in the form of the enhanced green fluorescent protein (eGFP) gene was cloned from the pcDNA-eGFP vector (Clontech, Mountain View, CA, USA) by PCR with Pfu (a thermostable DNA Polymerase isolated from *Pyrococcus furiosus*) (Takara Bio) and then inserted into the pGEM-T vector. The primer pairs used were Pegf1a/Pegf1b and Pegf1a/Pegf2 (Table 1). pGEM-T-egfp was then selected and sequenced for confirmation.

2.3. *Gene therapy vectors construction and identification*

Survivin (T34A), a nonphosphorylated mimic of survivin generated though site-directed mutagenesis (Thr34→Ala), was obtained by overlapping PCR as described in a previous report (*17*). Survivin (T34A), antisense survivin, antisense survivin-ΔEx3, and survivin-2B were each subcloned with eGFP into the eukaryotic expression vector pcDNA3.1(+) (Invitrogen) through multiple cloning sites. These vectors were then subjected to DNA sequence analysis for sequence confirmation.

2.4. *Establishment of cancer cells containing constructed vectors*

B-Cap-37 cells were seeded in 24-well plates (1×10^5 cells/mL, 500 μL per well) and grown to 50-60% confluence after growing overnight. Cells were transiently transfected with the four gene therapy vectors of pcDNA3.1(+)-survivin (T34A)-egfp, pcDNA3.1(+)-egfp-anti-survivin, pcDNA3.1(+)-egfp-anti-survivin-ΔEx3, and pcDNA3.1(+)-survivin-2B-egfp with FuGENE® 6 (Roche, Basel, Switzerland) under suitable conditions according to respective manuals. The pcDNA-eGFP vector was transfected into cells to serve as the control. Transfection efficiency was determined by fluorescence microscopy.

2.5. *MTT assay*

The established cells containing different vectors were seeded into 96-well plates and incubated normally. At the indicated times, cells were treated with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/mL and continuously incubated for 4 h at 37°C in a 5% CO_2 incubator. After the medium was carefully removed and DMSO 500 μL was added to each well, the cells were incubated at 37°C for 10 min. The cell absorbance of each well was determined at 570 nm with an Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) based on absorbance intensity at 485 nm. All experiments were repeated at least three times.

Table 1. Primers for plasmid design and construction

Target	Primer	Sequence (5'-3')	Restriction enzyme cutting site
Subcloning of survivin and its splice variants	P1	gaattcggccggctgcggggcattcgc	*Eco*R I
	P2	gtcgaattctcacaggtctgagcagcgatcctgcttgct	*Xho* I
Subcloning and identification of eGFP	Pegf1a	gacctcgagatggtgagcaagggcgaggagctg	*Xho* I
	Pegf1b	cgaggatccatggtgagcaagggcgaggagctg	*Bam*H I
	Pegf2	atgcggccgcgggccccttgtacagctcgtccatgccgag	*Not* I

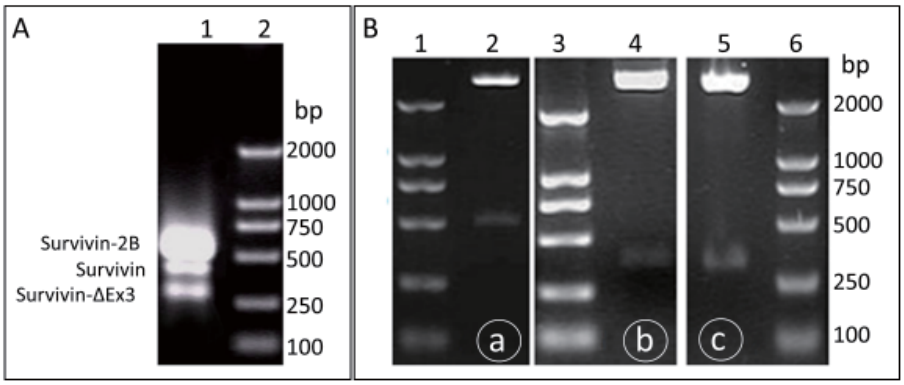

Figure 1. Agarose-gel electrophoresis of PCR products of survivin and splice variants gene (A) and target fragments in pGEM-T cleaved by restriction endonuclease *Eco*R I and *Xho* (B). (A) Lane 1: survivin-ΔEx3 with an MW of 320 bp, survivin with an MW of 429 bp, and survivin-2B with an MW of 510 bp. **Lane 2**: marker. **(B) a**, pGEM-T-survivin-2B; **b**, pGEM-T-survivin, **c**, pGEM-T-survivin-ΔEx3.

2.6. *Flow cytometry assay*

Flow cytometry was performed to examine the induction of apoptosis by various gene therapies in B-Cap-37 cells. Cells stained with propidium iodide (PI) (CycleTEST™ PLUS DNA Reagent Kit, Becton, Dickinson and Company, San Jose, CA, USA) were analyzed with a FACScan (Becton, Dickinson and Company) using Cell Fit software. All experiments were repeated at least three times. Apoptotic hypodiploid cells have less DNA than diploid cells in the G1 phase due to apoptosis-induced DNA fragmentation.

2.7. *Statistical analysis*

Data are shown as the mean ± S.D. and were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test (DMCT). The level of statistical significance was $p < 0.05$. Statistical analysis was done with SPSS/Win11.0 software (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. *Cloning and sequencing of cDNA of survivin, its splice variants, and eGFP*

cDNA of survivin and its splice variants survivin-2B and survivin-ΔEx3 was obtained from the B-Cap-37 breast carcinoma cell line using RT-PCR with Pyrobest DNA polymerase and total RNA as a template. Three amplified fragments of different sizes, approximately 500 bp, 400 bp, and 300 bp, are shown in Figure 1A. The PCR products were ligated into pGEM-T after clean-up and A-tailing reaction. The results of restriction enzyme digestion are shown in Figure 1B. The resulting pGEM-T were sequenced and the results revealed that the DNA fragment of about 500 bp was survivin-2B (510 bp), the fragment of about 400 bp was survivin (429 bp, GenBank accession No. CR541740), and the fragment of about 300 bp was survivin-ΔEx3 (320 bp). The nucleotide sequences were consistent with those published in the literature (*18*).

The eGFP gene with *Eco*R I/*Xho* I or *Not* I/*Xho* I restriction site was amplified by PCR and inserted into the pGEM-T vector. The process of identification was similar to that for survivin and its splice variants. Sequence analysis indicated that the 717 bp of eGFP were consistent with the known sequence and indicated that eGFP was ready for use in subsequent steps.

3.2. *Construction and verification of expression vectors*

To examine the effectiveness of targeting survivin and its splice variants in cancer treatment, four gene therapy plasmids, *i.e.* pcDNA3.1(+)-survivin (T34A)-egfp, pcDNA3.1(+)-egfp-anti-survivin, pcDNA3.1(+)-egfp-anti-survivin-ΔEx3, and pcDNA3.1(+)-survivin-2B-egfp, were successfully constructed *via* different connection strategies following a series of steps as shown in Figure 2. Restriction cleavage and sequencing revealed that the recombinant expression plasmids had the correct sequences and reading frames.

3.3. *Growth inhibition by four gene therapy plasmids in B-Cap-37 cells*

Four gene therapy vectors were transiently transfected into B-Cap-37 cells using the cationic liposome method. The expression of the target genes and reporter gene in the cells had already begun after 48 h of transfection. An MTT assay revealed almost no inhibition of cell growth 24 h after transfection, slight inhibition of cell growth 48 h after transfection, and maximum inhibition 72 h after transfection (Figure 3). However, the growth inhibition rates had decreased at 96 h compared to those at 72 h. This may be due to decreasing activity of the transfected expression vectors while untransfected cells continued to proliferate. The ratio of liposomes to DNA had no evident effect on transfection efficiency, regardless of whether it was 3:1 or 6:1.

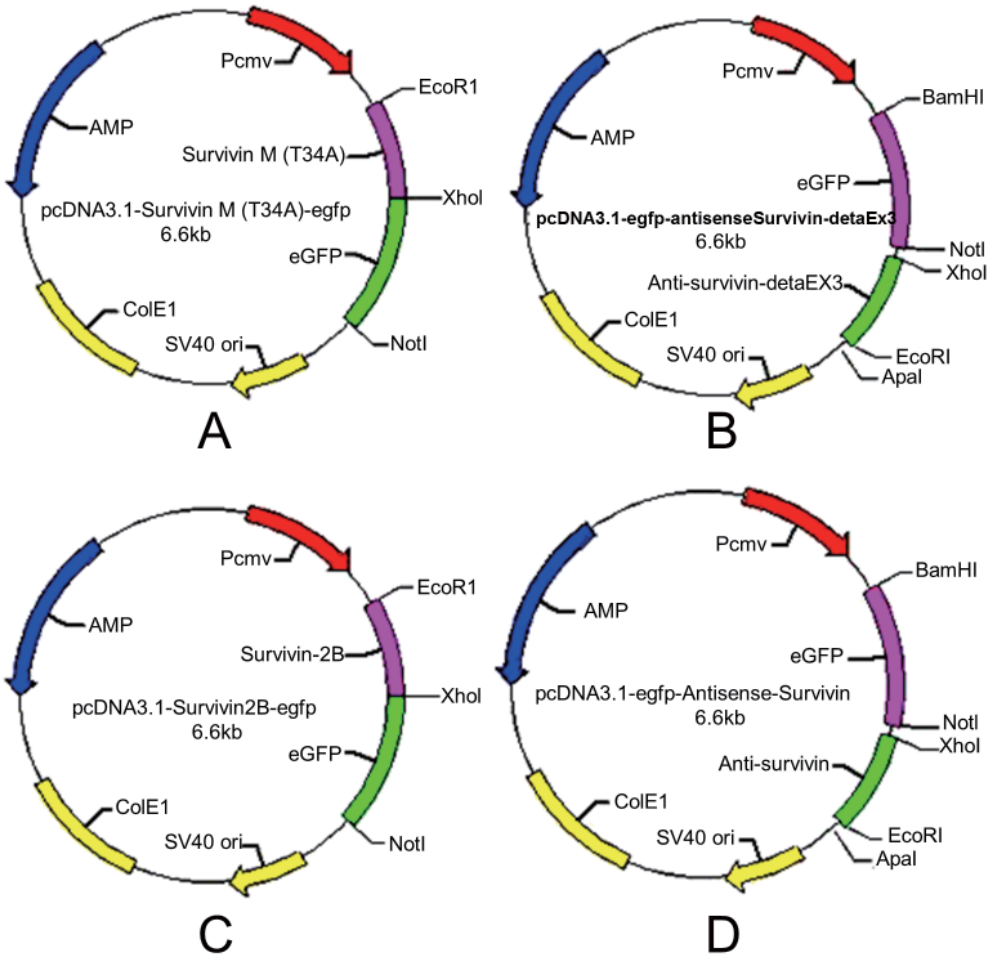

Figure 2. Construction of four gene therapy vectors simultaneously expressing target genes and the eGFP gene. (A) pcDNA3.1(+)-survivin (T34A)-egfp; **(B)** pcDNA3.1(+)-egfp-anti-survivin-ΔEx3; **(C)** pcDNA3.1(+)-survivin-2B-egfp; **(D)** pcDNA3.1(+)-egfp-anti-survivin.

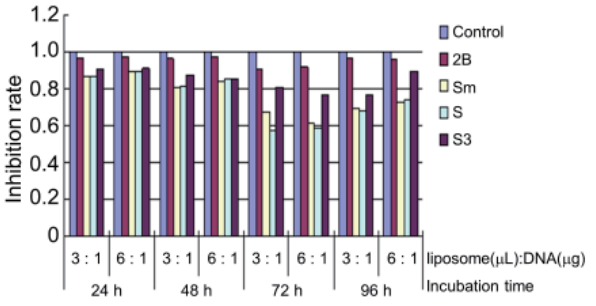

Figure 3. Effects of four gene therapy plasmids on cell proliferation. Cells were transiently transfected with four gene therapy plasmids and incubated for 24 h, 48 h, 72 h, and 96 h. Cell growth was evaluated with an MTT assay and the inhibition rate was calculated by comparison to the control in which cells were transfected with pcDNA-egfp.

Results of the MTT assay revealed that the four gene therapy plasmids had different levels of effectiveness at suppressing the growth of B-Cap-37 cells. pcDNA3.1(+)-egfp-anti-survivin and pcDNA3.1(+)-survivin (T34A)-egfp were most effective at inhibiting cell growth, with cell inhibition rates of 42% ($p < 0.01$ *vs.* control) and 39% ($p < 0.01$ *vs.* control), as were determined 72 h after transfection. pcDNA3.1(+)-egfp-anti-survivin-ΔEx3 moderately inhibited growth in B-Cap-37 cells, as indicated by an inhibition rate of 34% ($p < 0.05$ *vs.* control). In contrast, pcDNA3.1(+)-survivin-2B-egfp had an inhibition rate of only 9.5% ($p > 0.05$) in B-Cap-37 cells 72 h after transfection (Figure 3).

3.4. *Apoptosis induction by four gene therapy plasmids in B-Cap-37 cells*

Cells transfected with various constructed plasmids were incubated for 72 h. Then, flow cytometric analysis was performed to determine the proportion of apoptotic cells by detecting hypodiploid cells. As shown in Figure 4, the sub-G0/1 population of B-Cap-37 cells transfected with pcDNA3.1(+)-egfp-anti-survivin increased significantly in comparison to the control and the percentage of hypodiploid cells reached 30.06 ± 3.25%. Similar results were obtained in B-Cap-37 cells transfected with pcDNA3.1(+)-survivin (T34A)-egfp, as indicated by an inhibition rate of 28.68 ± 3.25%. When

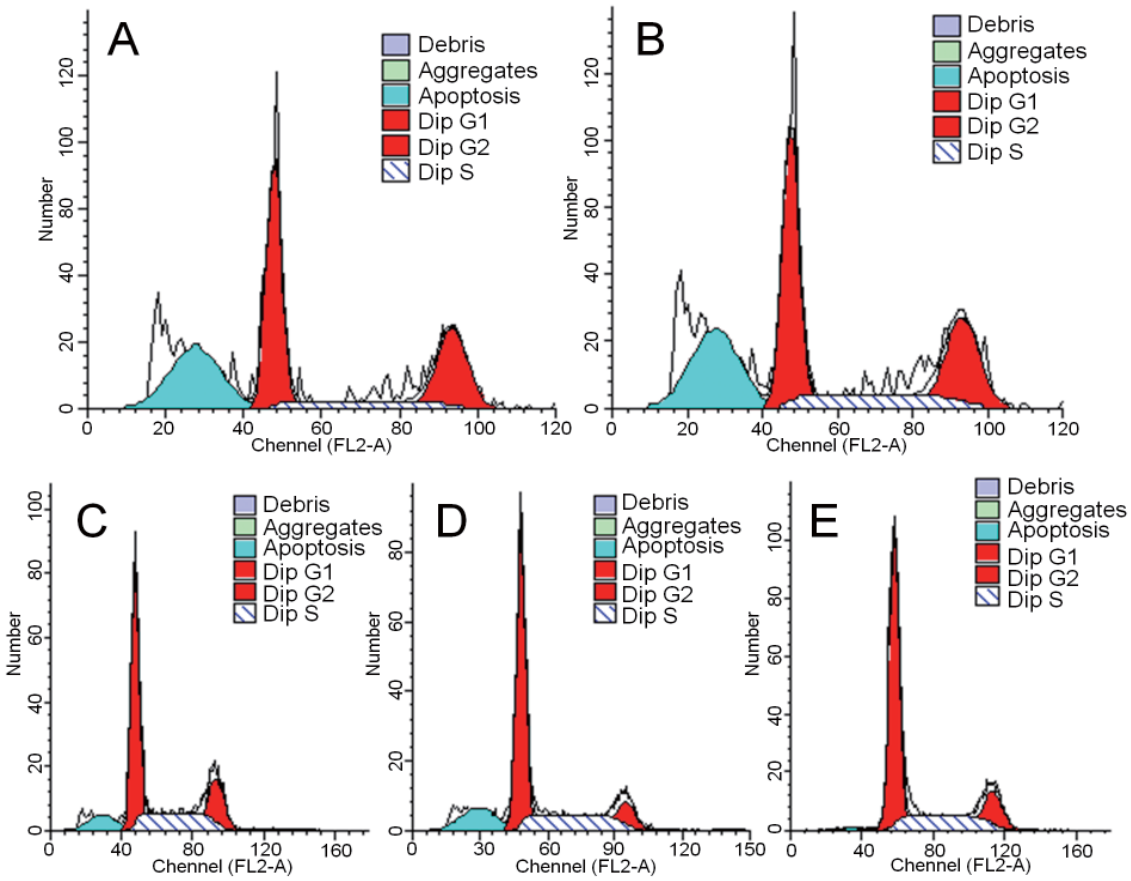

Figure 4. Induction of B-Cap-37 cell apoptosis by four gene therapy plasmids. Cells were transfected with four gene therapy plasmids and incubated for 72 h. The ratio of liposomes to DNA is 3:1. **(A)** pcDNA3.1(+)-survivin (T34A)-egfp; **(B)** pcDNA3.1(+)-egfp-anti-survivin-ΔEx3; **(C)** pcDNA3.1(+)-survivin-2B-egfp; **(D)** pcDNA3.1(+)-egfp-anti-survivin; **(E)** pcDNA-egfp.

cells were transfected with pcDNA3.1(+)-egfp-anti-survivin-ΔEx3 and pcDNA3.1(+)-survivin-2B-egfp, the percentage of hypodiploid cells was found to be 14.66 ± 3.25% and 9.37 ± 3.25%. These results suggest that antisense survivin and survivin (T34A) were most effective at inducing cell apoptosis in B-Cap-37 cells.

4. Discussion

The present study constructed four plasmids expressing survivin (T34A), antisense survivin, antisense survivin-ΔEx3, and survivin-2B and transiently transfected each of these plasmids into the B-Cap-37 human breast cancer cell line. The inhibition of growth and induction of apoptosis by these gene therapy strategies was then investigated in this cell line. Results indicated that targeting survivin, by using either antisense survivin or the dominant negative mutant survivin (T34A), was most effective at suppressing cell growth and inducing cell apoptosis. Targeting survivin-ΔEx3 *via* use of its antisense RNA resulted in moderate anti-tumor action. In contrast, transfecting cells with plasmids expressing survivin-2B resulted in limited inhibition of growth and induction of apoptosis. These results suggest the feasibility of targeting survivin and/or its splice variant survivin-ΔEx3 in treating breast carcinoma.

Survivin and its splice variants, which are involved in multiple signal transduction pathways, have potential value in cancer diagnosis and treatment (*6,19*). In recent years, many strategies of targeting survivin for therapeutic purposes have been explored. The present research revealed that targeting survivin by using antisense RNA or its dominant negative mutant survivin (T34A) was almost equally effective at inhibiting cell growth and inducing cell apoptosis in B-Cap-37 cells. Like antisense survivin, the survivin mutant survivin (T34A) is also clearly a potential agent for breast cancer treatment. The effectiveness of survivin (T34A) may be because the dominant negative mutant competed with survivin, thus leading to phosphorylation-defective survivin (*14*). These results indicate that inhibiting or blocking survivin may be the first step for drugs designed to treat breast carcinoma.

In order to further define the roles of survivin splice variants survivin-ΔEx3 and survivin-2B and their biological significance in breast cancer, the present study constructed plasmids that expressed antisense survivin-ΔEx3 and survivin-2B for use as possible gene therapy agents. Compared to the control, antisense survivin-ΔEx3 significantly inhibited the proliferation and promoted the apoptosis of breast cancer cells *in vitro*. That said, splice variant survivin-2B had limited

anti-tumor action in B-Cap-37 cells, which was consistent with previous reports (*19-22*). These results suggest that survivin-ΔEx3 may also serve as a target for drugs to treat breast cancer.

In conclusion, this research has provided clues on the effectiveness of targeting survivin and its splice variants in treating breast carcinoma. Although the best strategy appears to be blocking survivin, designing treatments that inhibit both survivin and survivin-ΔEx3 may be a rational and comprehensive strategy for treating breast carcinoma.

Acknowledgements

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Original Article

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Ferulic acid induces heme oxygenase-1 *via* activation of ERK and Nrf2

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ABSTRACT: This study investigated the effect of ferulic acid (FA) on the up-regulation of heme oxygenase-1 (HO-1) in lymphocytes and the molecular mechanisms involved. Lymphocytes were treated with FA (0.001-0.1 μM) for certain times. Cell viability, the activity and level of expression of HO-1, and signal pathways were analyzed. FA significantly up-regulated HO-1 expression both at the level of mRNA and protein in lymphocytes. Moreover, FA induced NF-E2-related factor (Nrf2) nuclear translocation and transcriptional activity, which is upstream of FA-induced HO-1 expression. In addition, lymphocytes treated with FA exhibited activation of extracellular regulated kinase (ERK) and treatments with U0126 (an ERK kinase inhibitor) attenuated the FA-induced activation of Nrf2, resulting in a decrease in HO-1 expression. Zinc protoporphyrin (ZnPP, a HO-1 inhibitor) markedly suppressed cytoprotection from radiation-induced cell damage by FA. Results suggested that the ERK signaling pathway controlled the anti-oxidation of FA by regulating the expression of the antioxidant enzyme HO-1.

Keywords: Ferulic acid, radiation, oxidation, heme oxygenase-1

1. Introduction

Ionizing radiation is known to induce oxidative stress through generation of reactive oxygen species (ROS), resulting in an imbalance of pro-oxidant and anti-oxidant activity and ultimately resulting in cell death (*1,2*). There has been considerable public and scientific interest in the use of phytochemicals derived from dietary components to combat oxidative stress (*3,4*). Ferulic acid (FA) is a phytochemical commonly found in many herbs used in traditional Chinese medicine (*5*). FA exhibits a wide range of therapeutic effects against various diseases like cancer, diabetes, and cardiovascular and neurodegenerative diseases (*6,7*). A wide spectrum of benefits to human health has been attributed to this phenolic compound, at least in part, because of its strong antioxidant activity (*8*). FA effectively scavenges superoxide anion radicals and inhibits lipid peroxidation (*9*). It possesses antioxidant properties by virtue of the phenolic hydroxyl group in its structure (*10*). The hydroxy and phenoxy groups of FA donate electrons to quench free radicals (*10*). The phenolic radical in turn forms a quinone methide intermediate, which is excreted *via* bile (*7*).

Recent studies have highlighted the important biological effects of heme oxygenase-1 (HO-1) reaction products that have antioxidant functions (*11*). HO-1 is a crucial factor in the response to oxidative injury, a major result of which is the degradation of heme to biliverdin, iron, and carbon monoxide (*12*). Upregulation of many phase-2 detoxifying and antioxidant enzymes, including HO-1, is mediated by antioxidant response elements (AREs) (*13*). NF-E2-related factor (Nrf2) is responsible for the activation of ARE-driven antioxidant gene expression (*14*). Recent studies have demonstrated that the activation of the mitogen-activated protein kinases (MAPKs) contributes to the induction of HO-1 and the modulation of ARE-driven gene expression *via* Nrf2 activation (*15*).

A previous report by the current authors showed FA was a nontoxic and effective radioprotectant in animal studies (*16*). FA has been found to protect lymphocytes from radiation-induced early damage (*17*), and this effect may be mediated by scavenging ROS (*18*). However, the detailed mechanism of FA's role in protection from radiation-induced cellular damage needs to be clarified. This study investigated the effect of FA on the up-regulation of HO-1 in lymphocytes and the molecular mechanisms involved.

2. Materials and Methods

2.1. *Drugs and reagents*

FA (purity > 99%) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological

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Products (Beijing, China). [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium]bromide (MTT), zinc protoporphyrin (ZnPP), and U0126 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against Nrf2, extracellular regulated kinase (ERK), phospho-ERK, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Nrf2 and HO-1 were purchased from BioTek (Winooski, VT, USA). A reverse transcription (RT) system and polymerase chain reaction (PCR) system were purchased from Takara Biotechnology (Dalian) (Dalian, Liaoning, China). Polyvinylidene difluoride membranes, and enhanced chemiluminescence kits were purchased from Amersham (Arlington Heights, IL, USA). RPMI 1640 medium and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). Trizol was purchased from Invitrogen (Carlsbad, CA, USA). Nuclear and cytoplasmic extraction kits and electrophoretic mobility shift assay (EMSA) kits were from Pierce (Rockford, IL, USA). Other analytically pure reagents were from Promega (Madison, WI, USA).

2.2.*Cell culture and treatment*

AHH-1 cells, kindly donated by Dr. Ping-Kun Zhou (Beijing Institute of Radiation Medicine, Beijing, China), were cultured routinely in RPMI 1640 medium, supplemented with 10% heat inactivated fetal bovine serum, in a humidified atmosphere of 5% CO_2 at 37°C. The cells were then uniformly irradiated at room temperature with a ^{60}Co γ source (Beijing Institute of Radiation Medicine, Beijing, China) at a dose rate of 2.51 Gy/min.

2.3. *Cell viability analysis*

An MTT assay was used to determine the effect of FA on the viability of AHH-1 cells. Cells were seeded at a density of 1×10^5 cells/well in 96-well plates, with the indicated concentrations of FA, and then subjected to γ-irradiation at 3 Gy 12 h later. Cells were then cultured for 48 h, 20 μL of MTT was added to each well, and cells were further incubated for 4 h allowing the conversion of MTT into formazan crystals. After centrifugation, the supernatant was aspirated, 200 μL dimethyl sulfoxide was added to each well to dissolve the formazan crystals, and the absorbance was read at 540 nm with a Victor 1420 Multilabel Counter (Wallac, Turku, Finland). The reduction in optical density (OD) caused by radiation and drug treatment was used as a measurement of cell viability, normalized to cells incubated in control medium, that was considered to be 100%.

2.4. *HO-1 assay*

An HO-1 assay was performed with an ELISA Kit by adding 0.1 mL volume of culture medium to a 96-well micro-titration plate coated with HO-1 antibody and incubating those plates for 90 min at 37°C. The liquid in the wells was discarded and plates were treated with 0.1 mL of biotinylated antibody for HO-1 for 60 min at 37°C. Plates were washed with PBS three times, 0.1 mL of streptavidin-peroxidase solution was added, and plates were incubated for 30 min at 37°C. After plates were washed with PBS five times, 0.09 mL tetramethylbenzidine was added, and plates were incubated for 18 min at 37°C. The reaction was then terminated with stop buffer. The absorbance was measured at 450 nm with a microplate reader after incubation for 5 min at room temperature, and wells without cells served as blanks. The amount of HO-1 secreted from cells was expressed as the mean concentration quantified by the binding activity of antibodies.

2.5. *Semiquantitative RT-PCR analysis*

RT-PCR analysis was performed by modifying a previously described technique (*19*). At certain timepoints, cells were collected and total RNA was extracted according to the manufacturer's instructions. For RT-PCR analysis, 1 μg total RNA was reverse-transcribed using RT-PCR kits. PCR was used to amplify target cDNA with the following conditions: 32 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 45 sec. The following primers and predicted sizes of PCR products were used: 5-GCAGAGGGTGATAGAAGAGG-3(sense) and 5-GTAAGGACCCATCGGAGAA-3(antisense) for HO-1; 5-TCATTGACCTCAACTACATG-3(sense) and 5-CAAAGTTGTCATGGATGACC-3(antisense) for GAPDH.

2.6. *Western blotting assay*

Western blotting analysis was performed by modifying a previously described technique (*20*). After treatment, the cells were washed twice in PBS and suspended in a lysis buffer and then placed on ice for 30 min. The supernatant was collected after centrifugation at 15,000 × g for 20 min at 4°C. Whole lysates (50 μg) were resolved on a 10% sodium dodecyl sulphate-polyacrylamide gel. The fractionated proteins were electrophoretically transferred to an immobilon polyvinylidene difluoride membrane and probed with the appropriate antibodies. The blots were developed using an enhanced chemiluminescence kit. In all immunoblotting experiments, the blots were reprobed with anti-actin antibody, which served as a control for protein loading.

2.7. *EMSA assay*

Nuclear extracts were prepared using a nuclear and cytoplasmic extraction kit, and EMSAs were carried out using a lightshift chemiluminescent gel shift kit in accordance with the manufacturer's instructions. Briefly, oligonucleotide 5'-TTTTCTGCTGACTCAAG GTCCG-3' containing the ARE binding site was biotin-

labeled using a 3'-end labeling kit and the DNA probe was incubated with 2 μg of the nuclear extract at room temperature for 20 min. The protein-DNA complexes were then separated on a 6% polyacrylamide gel and electrically transferred to a nylon membrane for chemiluminescence band detection. The specificity of the binding was examined using competition experiments, in which a 200-fold excess of the unlabeled oligonucleotide with the same sequence was added to the reaction mixture prior to the addition of the biotin-labeled oligonucleotide.

2.8. *Plasmid construction*

A plasmid to harbor the ARE-promoter was constructed by subcloning the sequence "CCG CTC GAG GTG ACA AAG CAC CCG TGA CAA AGC ACC CGT GAC AAA GCA CCC GTG ACA AAG CAC CCG AAG CTT GGG" to PGL4 vector *via* restriction enzyme sites of *Xho* I and *Hin*d III. The orientation of all constructs was verified using sequencing and restriction analysis.

2.9. *Transient transfection of ARE promoter and luciferase assay*

Cells were sub-cultured at a density of 1×10^5 cells in 96-well plates to maintain approximately 60-80% confluence. The cells were transiently transfected with the plasmid harboring the ARE-promoter using the transfection reagent in accordance with the manufacturer's instructions. After transfection for 6 h at 37°C, cells were supplied with new culture media with FA (0.001-0.1 μM) and then incubated overnight. Cells were washed three times with PBS and lysed with reporter lysis buffer. Twenty μL of cell extract was mixed with 100 μL of the luciferase assay reagent at room temperature, and the mixture was placed in a luminometer to measure the light produced.

2.10. *Statistical analysis*

All experiments were performed at least three times. The data were presented as means ± S.E.M. (standard error of the mean). Statistical comparison of groups was done with one-way variance analysis and a least significant difference *t* test using SPSS 16.0. The significance level was defined as $p < 0.05$.

3. Results

3.1. *Effect of FA on HO-1 expression and activity*

HO-1 plays an important role in the course of the cellular defense against oxidative stress, so this study investigated whether the antioxidant activity of FA is related to HO-1 expression. AHH-1 cells exposed to FA for 24 h showed a concentration-dependent increase in HO-1 protein expression (Figure 1A). The enhanced HO-1 activity correlated with increased HO-1 expression (Figure 1E). Treatment of cells with 0.1 μM FA resulted in a time-dependent enhancement of HO-1 mRNA and protein expression (Figures 1B and 1D) with a similar increase in HO-1 activity (Figure 1F).

3.2. *FA increased the transcriptional activity of Nrf2, nuclear translocation, and ARE-binding*

Several investigators have defined Nrf2 as a major transcription factor regulating ARE-driven phase 2 gene expression. Therefore, this study attempted to determine whether FA activated Nrf2 in association with its HO-1 up-regulation. Activation of Nrf2 was determined by Western analysis of nuclear extracts from AHH-1 cells treated with different doses of FA. As shown in Figure 2A, FA treatment caused increased Nrf2 accumulation in the nuclear fraction. To elucidate the role of Nrf2 in transcriptional activation of ARE, EMSA was performed using the oligonucleotide harboring the HO-1 specific ARE sequence. Incubation of the nuclear extract from AHH-1 cells after FA treatment (0.1 μM) with biotin-labeled ARE oligonucleotide resulted in the enhanced ARE-binding activity of Nrf2 (Figure 2B). To verify the functional relevance of Nrf2 binding to the ARE sequence of HO-1, an ARE-promoter construct containing the Nrf2 binding DNA consensus site linked to a luciferase reporter gene was used. As illustrated in Figure 2C, FA increased the transcriptional activity of Nrf2. These results further suggested that Nrf2 mediates the FA-induced activation of the HO-1 promoter.

3.3. *FA activates Nrf2 via phosphorylation of ERK*

To further elucidate the upstream signaling pathway involved in FA mediated Nrf2 activation and induction of HO-1, this study examined the activation of ERK, a major signal molecule involved in cell survival despite oxidative stress. Results showed that FA enhanced ERK phosphorylation, and U0126, a specific ERK kinase inhibitor, inhibited this activation (Figure 3A). U0126 treatment resulted in marked suppression of FA, causing HO-1 induction (Figure 3B) and the nuclear accumulation of Nrf2 (Figure 3C). Similarly, U0126 inhibited the transcriptional activity of Nrf2 (Figure 3D).

3.4. *Effect of FA on cell damage induced by oxidative stress*

The protective effect of FA from oxidative stress was examined in AHH-1 cells irradiated with γ-rays. Cells were pretreated with FA for 24 h before being irradiated with 3 Gy of radiation. Cell viability was determined with an MTT assay 48 h later. FA increased cell survival in a dose-dependent manner (Figure 4A). In order to ascertain whether increased HO-1 activity due to FA is

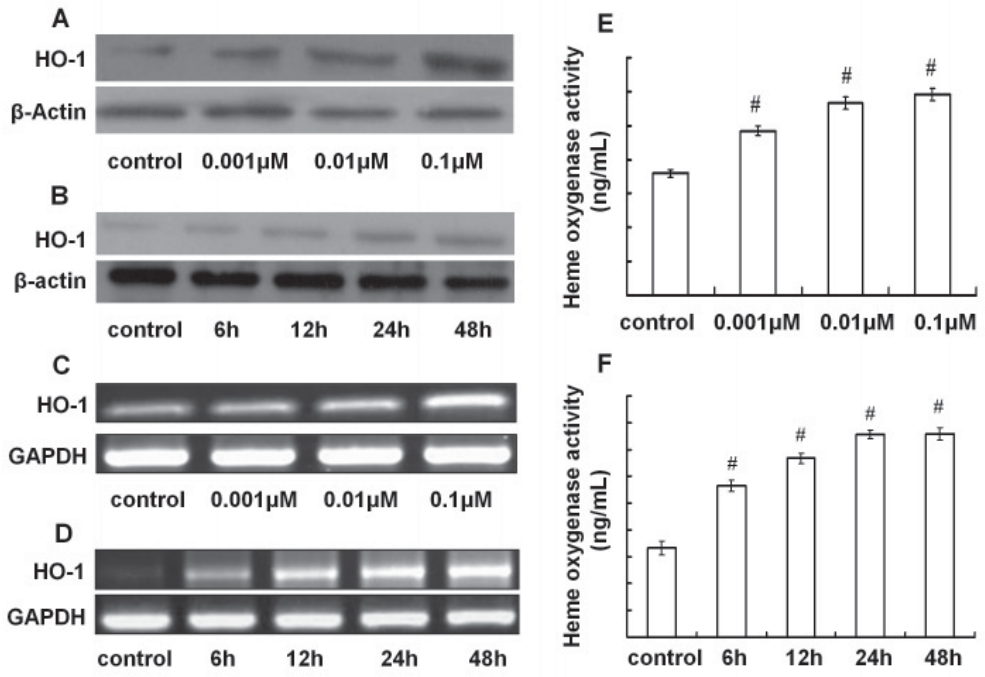

Figure 1. Effect of FA on HO-1 mRNA, protein expression, and activity in AHH-1 cells. (A) Effect of FA on HO-1 protein expression in a concentration-dependent manner. AHH-1 cells were treated for 24 h with the indicated concentrations of FA and the expression of HO-1 was detected with specific antibody. **(B)** Effect of FA on HO-1 protein expression at the times indicated. AHH-1 cells were treated with FA at a concentration of 0.1 μM and the expression of HO-1 protein was detected with specific antibody at the times indicated. **(C)** Effect of FA on mRNA of HO-1 in a concentration-dependent manner. AHH-1 cells were treated for 24 h with the indicated concentrations of FA and the mRNA of HO-1 was detected by RT-PCR. **(D**) Effect of FA on mRNA of HO-1 at the times indicated. AHH-1 cells were treated with FA at a concentration of 0.1 μM and the mRNA of HO-1 was detected at the times indicated. **(E)** Effect of FA on HO-1 activity in a concentration-dependent manner. HO-1 activity was measured at 24 h after exposure to various concentrations of FA. Each bar represents the mean ± S.E.M. in three experiments. # Significantly different from control ($p < 0.05$). **(F)** Effect of FA on HO-1 activity at the times indicated. Cells were treated with 0.1 μM FA and HO-1 activity was measured at the times indicated. Data are expressed as the means ± S.E.M. in triplicate experiments. # Significantly different from control ($p < 0.05$).

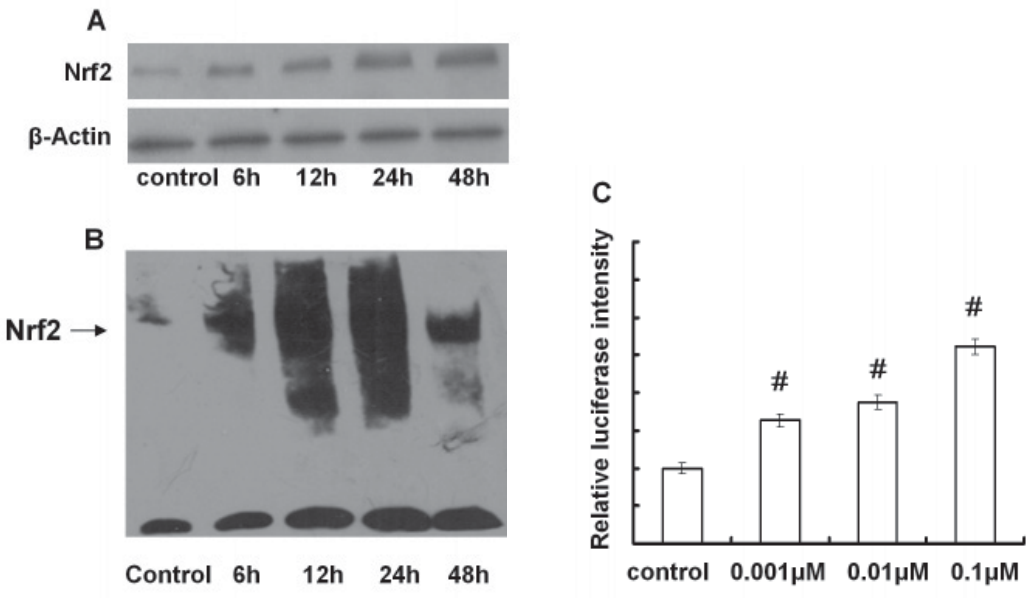

Figure 2. Effect of FA on Nrf2 expression, its translocalization into the nucleus, and its transcriptional activity in AHH-1 cells. (A) Effect of FA on the protein levels of nuclear Nrf2. Nuclear extract was prepared from AHH-1 cells treated with 0.1 μM FA for the times indicated. **(B)** Effect of FA on the ARE-binding activity of Nrf2 in AHH-1 cells. Nuclear extract was prepared from AHH-1 cells treatment with 0.1 μM FA for the times indicated. **(C)** Effect of FA on the transcriptional activity of ARE in AHH-1 cells. AHH-1 cells were transfected with an ARE-luciferase construct or control vector. After standing overnight, cells were treated with FA, cell lysates were mixed with a luciferase substrate, and luciferase activity was measured with a luminometer. The control group transfected with control vector and treated without FA. Data are expressed as the means ± S.E.M. in triplicate experiments. # Significantly different from control ($p < 0.05$).

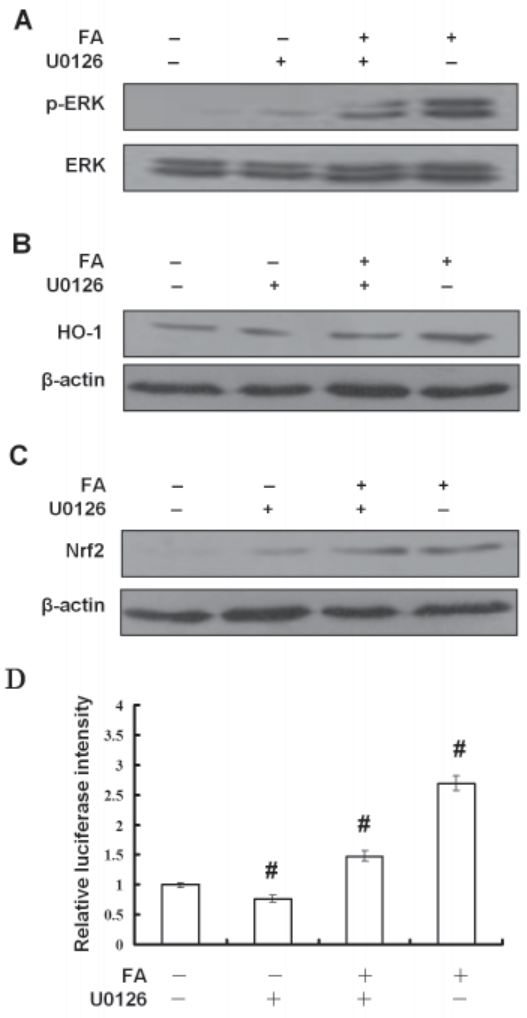

Figure 3. Induction of HO-1 and activation of Nrf2 by FA *via* phosphorylation of ERK. (A) Effect of U0126 on FA-induced phospho-ERK expression. After treatment with U0126, cell lysates were electrophoresed, and ERK and phospho-ERK were detected with specific antibodies. **(B)** Effect of U0126 on FA-induced HO-1 expression. Cells were incubated with 0.1 μM FA in the absence or presence of U0126 for 4 h. Cell lysates were electrophoresed and HO-1 was detected with specific antibody. **(C)** Effect of U0126 on FA-induced Nrf2 expression. Nuclear extract was prepared from AHH-1 cells treated with 0.1 μM of FA in the absence or presence of U0126 for 4 h and subjected to Western blotting to measure Nrf2 protein expression. **(D)** Effect of U0126 on FA-induced transcriptional activity of ARE in AHH-1 cells. AHH-1 cells were transfected with the ARE-luciferase construct. After standing overnight, cells were treated with FA in the absence or presence of U0126, cell lysates were mixed with a luciferase substrate, and luciferase activity was measured with a luminometer. Data are expressed as the means ± S.E.M for three experiments. # Significantly different from untreated cells ($p < 0.05$).

related to cytoprotection from oxidative stress, AHH-1 cells were pretreated with ZnPP, the inhibitor of HO-1. The protective effect of FA was attenuated (Figure 4B), illustrating that HO-1 induction may be partly determined by the protective effect of FA. Furthermore, cytoprotection from radiation-induced cytotoxicity by FA was reduced by U0126 (Figure 4B), suggesting the ERK signaling plays a role in FA-mediated HO-1 gene induction and cytoprotection.

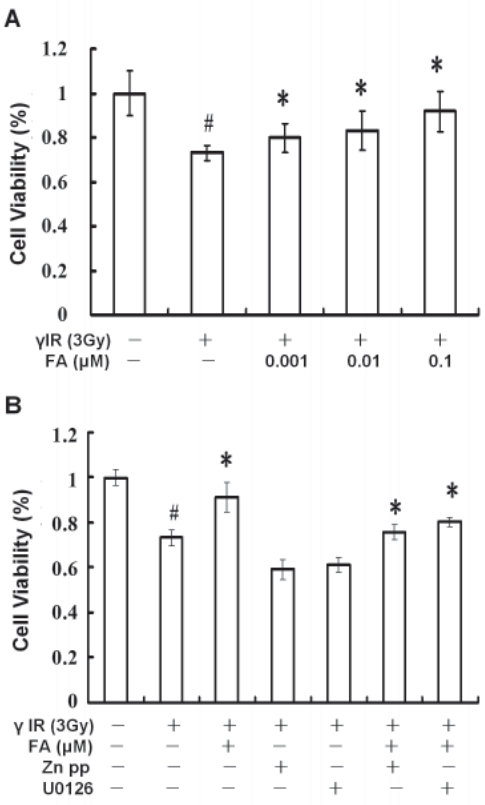

Figure 4. Protective effect of FA against radiation-induced cell damage. (A) Effect of FA on cell damage induced by γ-ray irradiation. AHH-1 cells were treated with 0.001, 0.01, and 0.1 μM FA, followed 24 h later by γ-irradiation at 3 Gy. After 48 h, cell viability was assessed by the MTT assay. # $p < 0.05$ compared to the value with the control, * $p < 0.05$ compared to the value with 3 Gy radiation alone. **(B)** AHH-1 cells were pre-incubated with 0.1 μM FA for 24 h and HO-1 ZnPP or U0126 for 4 h, followed by exposure to 3 Gy irradiation. Cell viability was assessed by the MTT assay. # $p < 0.05$ compared to the value with the control, * $p < 0.05$ compared to the value with 3 Gy radiation alone.

4. Discussion

Heme oxygenase is a rate-limiting catalyst in the degradation of heme to biliverdin, which is further converted to the antioxidant bilirubin by biliverdin reductase, free iron, and carbon monoxide (*12*). Three HO isozymes have been identified with distinct genes. Of these, HO-1, a stress-response protein, can be induced by various oxidative-inducing agents. Numerous *in vitro* and *in vivo* studies have shown that the induction of HO-1 is an important mechanism of cellular protection from oxidative injury (*21*). HO-1-mediated cytoprotection has been shown to be critical for tissues that were vulnerable to oxidative stress (*12*). Therefore, HO-1 is considered an important target of a number of chemopreventive and cytoprotective agents (*11*). Growing evidence suggests that HO-1 provides cytoprotection, so modulation of HO-1 expression by a pharmacological agent may represent a novel method of therapeutic intervention. In particular, the identification of a non-cytotoxic inducer of HO-1 may maximize the intrinsic antioxidant potential of cells. The current study demonstrated that

FA significantly up-regulated HO-1 expression both at the level of mRNA and protein in lymphocytes and that this increased expression was accompanied by a gradual increase in HO-1 activity.

Nrf2 is an important transcription factor that regulates ARE-driven gene expression. Nrf2 plays a key role in the transcriptional regulation of *HO-1* gene expression through interaction with ARE (*22*). Increase nuclear accumulation of Nrf2 plays a key role in the transcriptional regulation of the *HO-1* gene expression through interaction with ARE (*23*,*24*). Under normal physiologic conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with its repressor Keap 1 (*25*). Upon stimulation by inducers, however, Nrf2 dissociates from Keap 1 and translocates into the nucleus, where it dimerizes with cofactors like small Maf protein and binds to ARE. This leads to mass activation of highly specialized proteins, including HO-1 and other antioxidant enzymes (*13*). The current study found that FA induced nuclear translocation of Nrf2 and its ARE binding and transcriptional activity in AHH-1 cells. This study is the first to demonstrate that FA induced the expression of HO-1 in AHH-1 cells, at least in part, through activation of Nrf2 signaling.

Numerous studies have demonstrated that protein phosphorylation is a potential mechanism for the activation of Nrf2-ARE-mediated pathways. Several cytosolic kinases, including PKC, PI3K, and MAPK, have shown to modify Nrf2 and to be potentially involved in Nrf2-mediated signal transduction at AREs (*25*). Furthermore, the MAPK pathway activated by ERK is reported to be a signaling pathway for Nrf2 activation. ERK is reported to phosphorylate Nrf2, which may facilitate the release of Nrf2 from the Keap1-Nrf2 complex and trigger nuclear translocation of Nrf2. There, it forms a heterodimer with small Maf protein (*25*). Treatment with U0126 prior to exposure to FA reduced Nrf2 nuclear translocation and transcriptional activity of Nrf2, suggesting that Nrf2 might be a direct downstream target of ERK. The present results indicate that the ERK pathway may be a critical component for the activation of Nrf2 in the cellular signaling network, which mediates the transcriptional regulation of *HO-1* gene expression in AHH-1 cells.

In summary, the present results suggest that FA up-regulates HO-1 expression and activity. In addition, evidence indicates that ERK kinases act to mediate the up-regulation of HO-1 by FA. These results offer new insights into the antioxidative mechanisms of FA. Further studies are needed to clarify the molecular mechanisms underlying FA-induced activation of Nrf2 and subsequent induction of HO-1.

Acknowledgements

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Original Article

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In vitro bactericidal activity against periodontopathic bacteria by electrolyzed ion-reduced water

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ABSTRACT: As typical periodontopathic bacteria, *Porphyromonas gingivalis* (*P. gingivalis*) and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) were exposed to electrolyzed ion-reduced water (ERI) and ERI containing 1% sodium carboxymethylcellulose (CMC-Na) (ERI-1% CMC-Na), and the time course of their bactericidal action was evaluated. More than 99% of each bacteria species were killed after exposure to each solution for 15 sec. In addition, 1% CMC-Na, which was added to prolong bactericidal action, did not affect the bactericidal action of ERI. Its bactericidal action was concentration-dependent. No viable *P. gingivalis* bacteria were observed at a concentration of 15% of the undiluted solution and no viable *A. actinomycetemcomitans* bacteria were observed at a concentration of 50%, indicating differences in the bactericidal action of ERI for the two bacteria species. These results suggest that ERI may be extremely useful in preventing and treating periodontal diseases.

Keywords: Electrolyzed ion-reduced water, functional electrolyzed alkaline water, functional electrolyzed water, periodontopathic bacteria, bactericidal activity

1. Introduction

The electrolysis of water containing a small amount of salt or tap water produces a form of water that is useful and is generally known as functional electrolyzed water. Various types of functional water can be produced depending on the conditions of electrolysis, and these types of water are classified into two major categories. One is functional electrolyzed acidic water, which is generated at the anode and is used mainly for sterilization. The other is functional electrolyzed alkaline water, which is generated at the cathode and is used mainly for drinking and washing (*1,2*). Many studies have examined electrolyzed acid water, revealing that this water has marked bactericidal activity and is safe and environmentally friendly. Therefore, this water has been used in various fields (*3,4*) such as washing food (*5,6*) and medicine (*7,8*). In dentistry in particular, functional electrolyzed acidic water is used to wash dental materials and as a mouthwash (*4,9*). However, there are problems with using functional electrolyzed acidic water in dentistry, such as the decrease in bactericidal strength due to organic matter such as blood and saliva and the corrosion of dental instruments (*10*).

Functional electrolyzed alkaline water is used as drinking water and as a supplement to sustain/promote health and treatment. This water also has cleaning action and has been reported to be useful in washing dental cutting instruments and the oral cavity (*4*). However, few studies have examined functional electrolyzed alkaline water, and its usefulness has not been adequately confirmed.

The current authors have conducted several studies on electrolyzed ion-reduced water (ERI) (*11-19*). The resulting ERI, which was produced by using the general method of producing functional electrolyzed water, is physically electron-rich water obtained after the application of electric current/voltage to water containing a small amount of salt using a special diaphragm system. This water has cleansing, deodorizing, antimicrobial, and anti-dust action because its potent alkalinity and negatively-charged ions detach and remove dirt and bacteria that cause odor (*11*). ERI prevents oxidation, and, therefore, also has rust-preventing and anti-septic action. In addition, stable emulsions of various types of oil can be prepared using ERI with no emulsifiers, demonstrating its emulsifying action (*12*). Capitalizing on these properties, ERI is widely used as a cleansing agent in various industrial products (*11*). It is also reported to have burn-healing action and an effect on atopic dermatitis (*16-19*).

To investigate the clinical use of this strong bactericidal action of ERI in dentistry, the current study evaluated the bactericidal action of ERI on periodontopathic bacteria.

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2. Materials and Methods

2.1. *Materials*

Porphyromonas gingivalis (*P. gingivalis*) JCM8528 and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) JCM12985 were purchased from Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama, Japan (*20*). Modified general anaerobic medium (GAM) broth and agar media were purchased from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. AnaeroPack® Kenki (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) was used for anaerobic culture. S-100® (A. I. System Product Corp., Kasugai, Aichi, Japan) was used as the ERI. Sodium carboxymethylcellulose (CMC-Na) was purchased from Kanto Chemical Co., Inc., Tokyo, Japan. All reagents were of special grade.

2.2. *Preparation of ERI and ERI containing 1% CMC-Na (ERI-1% CMC-Na)*

ERI was produced using an ERI generator (A. I. System Product Corp.), and ERI was used in experiments immediately after its production. At 20°C, this ERI had a pH of 12.0-12.4, oxidation reduction potential of −344 mV, specific gravity of 1.002, and an osmolarity of 100 mOsm (corresponding to a 0.3% NaCl solution). An ERI solution containing 1% CMC-Na was prepared. Controls were a 0.3% NaCl solution with the same osmolarity as that of the ERI and a 0.3% NaCl solution containing 1% CMC-Na.

2.3. *Bacteria strains*

The bacteria strains used were *P. gingivalis* JCM8528 and *A. actinomycetemcomitans* JCM12985.

2.4. *Bacterial culture and preparation*

P. gingivalis and *A. actinomycetemcomitans* were cultured in GAM broth medium, and an AnaeroPack® Kenki was added as a disposable O_2-absorbing and CO_2-generating agent to an anaerobic jar kept under anaerobic conditions at 37°C for 4 days.

After culture, bacteria were collected by centrifugation at 10,000 × g for 10 min, washed with phosphate-buffered saline (PBS) and diluted with PBS, and subjected to measurement with a UV-visible spectrophotometer V-630 (JASCO Corp., Tokyo, Japan) at 540 nm. A bacterial suspension with an optical density of about 0.3 (viable cell count: 10^8/mL) was prepared.

2.5. *Bactericidal action of ERI and ERI-1% CMC-Na*

With the *P. gingivalis* samples, 0.1 mL of bacterial suspension (10^8/mL) was added to 10 mL of ERI or ERI-1% CMC-Na and stirred. Then, 10 μL aliquots were added to 1 mL of GAM broth medium after 15, 30, and 60 sec. In addition, 0.1 mL of the mixture was added to GAM agar medium. These procedures were performed aerobically at room temperature, followed by anaerobic culture in an anaerobic jar using an AnaeroPack® Kenki at 37°C for 2-5 days. Colony-forming units/mL (CFU/mL; viable cell count) were determined and the CFU/mL was compared to the value after bacterial exposure to 0.3% NaCl solution or that containing 1% CMC-Na as the controls.

With the *A. actinomycetemcomitans* samples, a similar experiment was performed using GAM broth and agar medium.

2.6. *Bactericidal action of diluted ERI*

With the *P. gingivalis* samples, a bacterial suspension (10^8/mL) was added to 10 mL of ERI diluted to concentrations of 50, 25, 15, 10, and 5% (undiluted ERI-1% CMC-Na), and 10 μL was added to 1 mL of GAM broth medium. In addition, 0.1 mL of this was added to GAM agar. All of these procedures were performed aerobically at room temperature. Subsequently, culture was continued in an anaerobic box using an AnaeroPack® Kenki at 37°C for 2-5 days, and CFU/mL was determined.

With the *A. actinomycetemcomitans* samples, a similar experiment was performed using GAM broth and agar medium.

2.7. *Dependence on the pH of ERI*

The pH of ERI was adjusted with hydrochloric acid using the pH/Ion Meter F-53 (Horiba, Ltd., Japan) as a combination electrode. Each bacteria species was cultured using the above methods, and CFU/mL was determined.

2.8. *Influence of the pH of ERI or NaCl solution*

The pH of ERI was adjusted using the above method. The pH of NaCl solution was adjusted using a pH meter after the addition of NaOH solution to 0.3% NaCl. Each bacteria species was cultured using the above method, and CFU/mL was determined.

3. Results

Two typical periodontopathic bacteria species (*P. gingivalis* and *A. actinomycetemcomitans*) were added to ERI and ERI-1% CMC-Na, and the time course of their bactericidal action was evaluated. The results are shown in Figure 1. As shown in Figures 1A and 1B, the viable cell count (CFU/mL) did not change after exposure to the controls of 0.3% NaCl or 1% CMC-Na even after 60 sec, whereas more than 99 and 100% of each bacteria species were killed after exposure to ERI or ERI-1% CMC-Na for 15 and 30 sec, respectively.

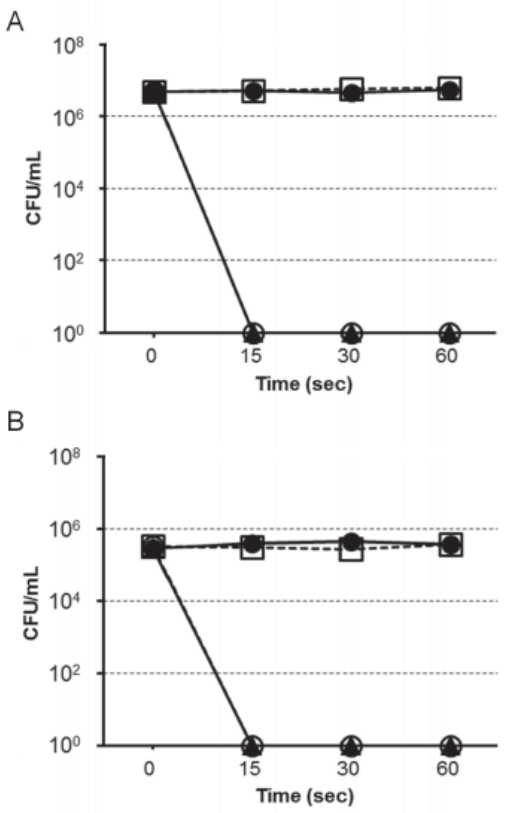

Figure 1. Bactericidal action of ERI or ERI-1% CMC-Na on *P. gingivalis* (A) and *A. actinomycetemcomitans* (B). Control (0.3% NaCl): solid line (●), ERI: solid line (▲), Control-1 (0.3% NaCl + 1% CMC-Na): dashed line (□), ERI-1% CMC-Na (ERI + 1% CMC-Na): dashed line (○). ERI: electrolyzed ion-reduced water; CMC-Na: sodium carboxymethylcellulose.

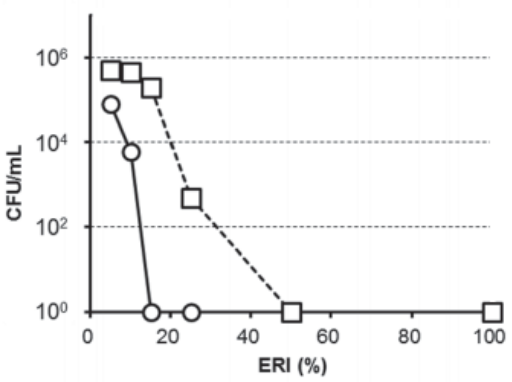

Figure 2. Bactericidal action on *P. gingivalis* and *A. actinomycetemcomitans* by ERI concentration. *P. gingivalis*: solid line (○), *A. actinomycetemcomitans*: dashed line (□).

The results of evaluating the concentration dependence of the effects of ERI on the two periodontopathic bacteria species are shown in Figure 2. The bactericidal action of ERI was concentration-dependent. Viable *P. gingivalis* bacteria were observed after exposure to ERI diluted with 0.3% NaCl to a concentration of 10%, but no viable bacteria were observed at an ERI concentration of 15%. Similarly, viable *A. actinomycetemcomitans* bacteria were present at an ERI concentration of 25% but absent at a concentration of 50%.

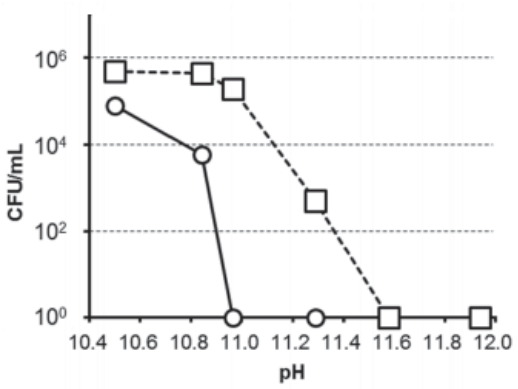

Figure 3. Bactericidal action of ERI on *P. gingivalis* and *A. actinomycetemcomitans* by dependence on pH. *P. gingivalis*: solid line (○), *A. actinomycetemcomitans*: dashed line (□).

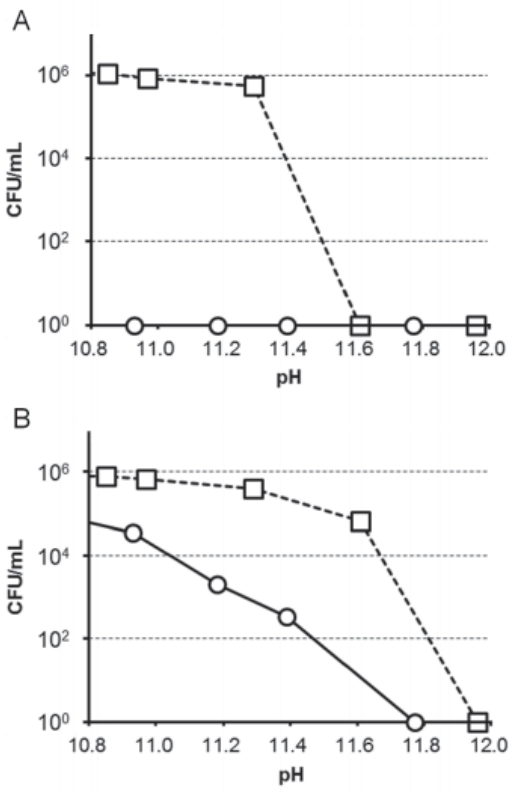

Figure 4. Bactericidal action on *P. gingivalis* (A) and *A. actinomycetemcomitans* (B) in terms of pH changes. ERI + HCl: solid line (○), 0.3% NaCl + NaOH: dashed line (□).

Results regarding the effect that a decrease in the pH of ERI had on its bactericidal action are shown in Figure 3. No viable *P. gingivalis* bacteria were observed at a pH ≥ 11.0. Bactericidal action on *A. actinomycetemcomitans* bacteria was observed at a pH ≥ 11.6.

Bactericidal action was compared when the pH of 0.3% NaCl was adjusted by adding dilute NaOH solution and that of ERI was adjusted by adding dilute HCl solution. The results are shown in Figure 4. With ERI, viable *P. gingivalis* bacteria were observed at a pH ≥ 10.8. With 0.3% NaCl, viable *P. gingivalis* bacteria were present at a pH of 11.3 but absent at a pH of 11.6 (Figure 4A). With ERI, viable *A. actinomycetemcomitans*

bacteria were present at a pH of 11.4 but absent at a pH of 11.8. With 0.3% NaCl, viable bacteria were present at a pH of 11.6 but absent at a pH of 12.0 (Figure 4B).

4. Discussion

Two periodontopathic bacteria species were exposed to ERI and ERI-1% CMC-Na, and the time course of their bactericidal action was evaluated (Figure 1). Results indicated no difference in the bactericidal action of ERI and ERI-1% CMC-Na, confirming that the addition of 1% CMC-Na had no effect on the bactericidal action of ERI. In this study, CMC-Na was added based on a study on electrolyzed acidic gels by Kase *et al.* (*21*). In their study, a glycerin solution containing salt was electrolyzed, and electrolyzed acidic water in gel form was produced to prolong the bactericidal action of electrolyzed strongly acidic water. Since ERI does not have prolonged bactericidal action when used by itself in the oral cavity, CMC-Na was added to provide viscosity in order to prolong bactericidal action. As shown in Figures 1A and 1B, ERI and ERI-1% CMC-Na had bactericidal action persisting for several dozen seconds, so the bactericidal action of mouthwashes with antimicrobial agents such as chlorine dioxide, cetylpyridinium chloride, chlorhexidine, and triclosan (*9*) or toothpastes containing those agents should help with treatment to prevent periodontal disease.

Evaluation of the concentration dependence of the bactericidal action of ERI indicated that ERI had bactericidal action on *P. gingivalis* at a concentration of 10% of undiluted ERI and on *A. actinomycetemcomitans* at a concentration of 25% (Figure 2). These results suggest differences in the bactericidal action of ERI for these bacteria species.

Evaluation of the effect that a decrease in the pH of ERI had on bactericidal action indicated that ERI had decreased bactericidal action on *P. gingivalis* at a pH ≤ 11.0 and on *A. actinomycetemcomitans* at a pH ≤ 11.6 (Figure 3). Therefore, bactericidal action of ERI and a dilute NaOH solution (Figure 4) was compared in a more limited pH range (pH 10.8-12.0). No viable *P. gingivalis* bacteria were observed with ERI at a pH ≥ 10.9 and dilute NaOH solution at a pH ≥ 11.6 (Figure 4A). No viable *A. actinomycetemcomitans* bacteria were observed with ERI at a pH of 11.8 and dilute NaOH solution at a pH of 12.0 (Figure 4B). These results suggest that the bactericidal action of ERI is due to not only to hydroxide ions (OH^-) but also to its low oxidation reduction potential (−344 mV).

Lee and Choi (*9*) reported that the antibacterial activity of electrolyzed water (pH 8.4) is presumably due to the combined action of short-lived reactive oxygen species (ROS) such as singlet oxygen, superoxide free radicals (O_2^-), and hydroxyl radicals (OH^-), and free chlorine. The aforementioned results and the current results suggest that the antibacterial activity of ERI on two types of bacteria is due to the synergistic effect of a very high negative oxidation-reduction potential (−344 mV) and hydroxyl radicals (OH^-). This may prove extremely useful to the prevention and treatment of periodontal diseases through daily oral care, such as rinsing the mouth out with ERI and/or brushing the teeth with ERI-1% CMC-Na.

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Original Article

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Preparation and evaluation of fenoterol hydrobromide suppositories

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ABSTRACT: Fenoterol HBr is a bronchodilator known to be subject to first pass effect after oral administration. The aim of this study was to prepare and evaluate fenoterol HBr suppositories. Suppositories were prepared by a fusion method using different fatty bases, *viz*. Witepsol H15, Witepsol E75, Suppocire AP, and Suppocire BM, as well as different hydrophilic bases, *viz.* polyethylene glycol and poloxamer bases. *In vitro* release studies revealed a greater release of the drug from hydrophilic bases than from fatty bases. The effect of incorporating different types and concentrations of non-ionic surfactants (Tween 60 and Span 20) on the release rate of the drug from Witepsol H15, as a model fatty base, was investigated. Results showed an enhanced release at low surfactant concentrations. A very fast 100% drug release was achieved when the drug was incorporated as an aqueous solution in Witepsol H15 (F17). This formula was selected to test the effect of fenoterol HBr suppositories on histamine-induced bronchospasms in Guinea pigs. No dyspnea of the animals was recorded for up to 30 min. In addition, thermogel liquid suppositories of different poloxamer 188 and poloxamer 407 proportions in the presence of sodium alginate as a mucoadhesive polymer were prepared. The different formulations behaved similarly concerning sustainment of drug release, however, only the formula containing 15% poloxamer 188 and 25% poloxamer 407 (F20) showed optimal gelation at body temperature. In conclusion, among the studied suppository bases there are bases suitable for fast release of the drug like F17 and hydrophilic bases especially polyethylene glycol, as well as other bases for sustained release applications of fenoterol HBr like fatty and thermogel bases.

Keywords: Suppositories, fenoterol, histamine induced bronchospasm, surfactants, drug in solution form, mucoadhesive liquid suppositories

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1. Introduction

Fenoterol is a direct acting sympathomimetic agent with β-adrenoreceptor stimulant activity. It is used to treat symptoms of asthma, chronic bronchitis, emphysema, and other lung diseases (*1*). Fenoterol is available in the market in the form of syrup, tablet, and inhaler. Fenoterol acts rapidly on inhalation, but is incompletely absorbed from the gastrointestinal tract and is also subject to extensive first-pass metabolism by sulfate conjugation (*2*). Literature review lacks any information concerning the availability of fenoterol HBr in the form of suppositories. The rectal route for drug administration was proven to be advantageous over other routes because of the reduced side effects such as gastrointestinal irritation and the avoidance of both disagreeable taste and first pass effect (*3*). Consequently, rectal administration of fenoterol HBr in suppository form may offer an advantage over its oral administration to increase its bioavailability. Moreover, it offers an advantage over inhalation for pediatrics, which probably finds difficulty in inhaling the drug. Studies have shown that the release properties of many suppositories depend considerably on the physicochemical properties of the drug, suppository base and formulation adjuvants (*4-7*) and a lot of formulation work is normally required to optimize the properties of suppository preparations. Thus, the objective of this investigation was to prepare fenoterol suppositories and to optimize its release characteristics from different suppository bases.

2. Materials and Methods

2.1. *Materials*

The following materials were used: fenoterol HBr (Boehringer Ingelheim, Basel, Switzerland), Polyethylene glycol 400, 1500, and 6000 (Union Carbide, New York, NY, USA), Witepsol H15 and Witepsol E75 (Dynamit Nobel, Leverkusen, Germany), Suppocire BM and Suppocire AP (Gattefossé, Gennevilliers, France), Tween 60 and Span 20 (Atlas Chemical Industries, Wilmington, DE, USA), poloxamer 188 and poloxamer 407 (BASF, Ludwigshafen, Germany), and sodium alginate (Hayashi Pure Chemicals, Tokyo, Japan). All other chemicals were

analytical grade.

2.2. *Preparation of fenoterol HBr suppositories*

Adult 2 g suppositories containing 10 mg of fenoterol HBr were prepared by a cream melting technique (*8*) using different fatty and hydrophilic bases. The suppository base was melted and then the drug was added. Homogeneous dispersions were formed in melted base and then molded in a metal mold (2 g capacity). The selected fatty bases were Witepsol H15, Witepsol E75, Suppocire BM, and Suppocire AP. Hydrophilic bases composed of polyethylene glycol (PEG) mixtures such as PEG 1500/PEG 4000 (3:1) (F1), PEG 1500/PEG 4000 (9:1) (F2), and PEG 1500/PEG 6000 (2:1) (F3) as well as hydrophilic bases consisting of mixtures of poloxamer 188 and propylene glycol in the ratios of 10:0 (F4), 9:1 (F5), and 8:2 (F6) were prepared.

Moreover, suppositories containing additionally 2, 5, and 10% (w/w) Tween 60 and Span 20 were prepared using Witepsol H15 as suppository base. Furthermore, the incorporation of the drug in Witepsol H15 in the form of an aqueous solution (0.2 mL/suppository) instead of the powdered drug form using 2% Span 20 for emulsification was investigated. All suppositories were kept in the refrigerator and were stored in a desiccator at room temperature for 24 h before use.

2.3. *Preparation of in situ gelling mucoadhesive liquid suppositories*

Liquid suppositories were prepared as previously described (*9*). In brief, fenoterol HBr and sodium alginate (0.6%) were solubilized in distilled water and the solution was cooled down to 4°C. Poloxamer 188 (P188) and poloxamer 407 (P407) in different proportions were then slowly added to the solution with continuous agitation. The liquid suppositories were left at 4°C until a clear solution was obtained.

2.4. *Evaluation of the prepared fenoterol HBr suppositories*

2.4.1. *Weight variation*

The weight variation test was determined according to the British Pharmacopoeia. Briefly, twenty suppositories were weighed individually and the average weights were determined. No suppositories should deviate from average weight by more than 5% except two, which may deviate by not more than 7.5%.

2.4.2. *Content uniformity*

Five suppositories were randomly selected from each base and assayed individually for drug content. The suppository was melted with gentle heating in a water bath in the presence of 25 mL of Sorensen's phosphate buffer solution, pH 7.4. The volume was adjusted to 250 mL with phosphate buffer. The flask was agitated on a shaking water bath (Gallenkamp, Loughborough, UK) at 37°C for 4 h. After centrifugation and filtration, the UV absorbance of the solution was measured spectrophotometrically (Shimadzu UV double beam spectrophtometer, Kyoto, Japan) at λ_{max} 276 nm against a blank solution prepared by treating plain suppositories in the same manner.

2.4.3. *Hardness*

The hardness test was carried out on plain, as well as medicated suppositories. Hardness was determined at room temperature (about 25°C) using a hardness tester (Erweka hardness tester, SBT, Heusenstamm, Germany).

2.4.4. *Measurement of gelation temperature of liquid suppositories*

The gelation temperature was measured according to the method reported (*10*). Briefly, 10 g of each gel were placed in a transparent glass vial with a magnetic stirring bar (15 mm × 6 mm). The preparation was heated starting from 20°C, and was increased 1°C/min with constant stirring at 100 rpm. The temperature at which the magnetic bar stopped moving was taken as the gelation temperature. The evaluation was repeated three times for each formulation.

2.4.5. *In vitro release of fenoterol HBr from solid suppository bases*

The USP rotating basket dissolution apparatus (Pharma Test, Hainburg, Germany) was used at 37°C and 50 rpm for the *in vitro* release study of fenoterol HBr from solid suppository bases. The release was done for 120 min in 300 mL Sorensen's phosphate buffer, pH 7.4. Samples, each of 3 mL, were withdrawn from the release medium at specified time intervals and replaced by fresh buffer. The samples were filtered through Millipore filter (pore size 0.22 μm; Millipore, Billerica, MA, USA) and analyzed spectrophotometrically at 276 nm against a blank of plain suppositories treated by the same method as tested fenoterol HBr suppositories. Each release experiment was performed in triplicate. The extent of drug release was assessed from the total amount of drug present in the dissolution medium at the end of the release experiment.

2.4.6. *In vitro release of fenoterol HBr from liquid suppository bases*

Drug release study of liquid suppositories was performed for 360 min in USP paddle dissolution apparatus (Pharma Test) at 37°C and 50 rpm. Five grams

of liquid suppository were placed into a semi-permeable membrane tube (m.w. cutoff: 6,000-8,000; Spectrum Medical, Fort Mill, SC, USA). Both sides of the tube were tied up with a thread to prevent leakage. The semi-permeable membrane tube was then immersed in 300 mL Sorensen's phosphate buffer, pH 7.4. Sampling and analysis were performed as described above.

2.4.7. *Kinetic analysis of the release data*

In order to describe the release model, the *in vitro* release data from solid suppositories were analyzed according to a zero-order kinetic model (Q *vs*. t), a diffusion controlled model (Q *vs.* square-root of t), and a first-order model (log (Qo – Q) *vs*. t), where Q is the amount of drug released at time t and Qo is the initial amount of the drug. The model that consistently produced the highest correlation among the suppository preparations was used for the assessment of drug release rates (*11*).

To analyze the release mechanism of the drug from liquid suppositories, the release data obtained were fit to the Power Law (*12*):

$$M_t/M_\infty = K \cdot t^n$$

where, M_t/M_∞ is the fraction of drug released at time t and k denotes the constant of the suppository system and n is the diffusion exponent related to the mechanism of the drug release. The n value of 1 corresponds to zero-order dissolution kinetics, $0.5 < n < 1$ means a non-Fickian dissolution model and $n = 0.5$ indicates Fickian diffusion (Higuchi model) (*10*).

2.4.8. *Effect of fenoterol HBr suppository on histamine-induced bronchospasm in Guinea pigs*

Bronchospasm was induced in Guinea pigs by exposing them to a histamine aerosol. Guinea pigs weighing about 350 g were divided into three groups of six animals each. Group I served as control, Group II received selected fenoterol HBr suppositories (5 mg/kg), and Group III received an oral solution of fenoterol HBr (5 mg/kg). Groups 2 and 3 received medication half an hour before exposure to the histamine aerosol. The animals were exposed to 1% histamine aerosol under constant pressure in an aerosol chamber (24 × 14 × 24 cm) made of perplex glass. The end point, preconvulsive dyspnea (PCD), was determined from the time of aerosol exposure to the onset of dyspnea leading to the appearance of convulsions (*13*). As soon as PCD commenced, the animals were removed from the chamber and exposed to fresh air. The investigations were performed after approval of the local ethical committee at Faculty of Pharmacy, Cairo University.

3. Results and Discussion

3.1. *Weight variation, content uniformity, and hardness*

The weight variation study for all the suppositories were found to be within the acceptable range of < 5% (Table 1), which indicated perfect calibration of mold. Also, the drug content of five suppositories, from each formulation, did not deviate by more than 10% from the labeled amounts (Table 1). The mechanical strength of all tested suppositories was in the range of 1.50 to 4.00 kg showing optimum hardness for handling and transportation (Table 1).

3.2. *In vitro release of fenoterol HBr from hydrophilic bases*

Table 1. Codes, composition and characterization of tested solid suppository formulations

Code	Suppository composition	Weight variation (g)	Drug content (%)	Hardness (kg)	Extent release at 120 min (%)	Kinetic mechanism of release data
F1	PEG I (PEG 1500/PEG 4000, 3:1)	2.23 ± 0.08	95.43 ± 4.56	3.8 ± 0.11	69.8 ± 2.3	Diffusion
F2	PEG II (PEG 1500/PEG 4000, 9:1)	2.21 ± 0.09	92.27 ± 3.89	3.3 ± 0.06	50.8 ± 1.2	Diffusion
F3	PEG III (PEG 1500/PEG 6000, 2:1)	2.26 ± 0.04	90.09 ± 2.09	4.2 ± 0.04	100.0 ± 0.5	Diffusion
F4	P188/propylene glycol (10:0)	2.23 ± 0.05	96.70 ± 3.87	2.1 ± 0.09	22.7 ± 2.1	Zero
F5	P188/propylene glycol (9:1)	2.24 ± 0.04	94.25 ± 4.65	1.8 ± 0.02	38.9 ± 1.7	Diffusion
F6	P188/propylene glycol (8:2)	2.18 ± 0.06	93.87 ± 2.82	1.5 ± 0.06	57.1 ± 1.3	Diffusion
F7	Witepsol H15 (W_{15})	2.01 ± 0.03	97.58 ± 4.73	2.4 ± 0.04	24.0 ± 3.1	Diffusion
F8	Witepsol E75	2.05 ± 0.00	92.68 ± 1.33	2.6 ± 0.06	13.0 ± 1.9	Zero
F9	Suppocire AP	2.03 ± 0.05	94.37 ± 1.58	2.3 ± 0.04	3.2 ± 0.1	Diffusion
F10	Suppocire BM	1.98 ± 0.06	93.02 ± 2.99	2.6 ± 0.05	17.2 ± 2.5	Diffusion
F11	W_{15} + Tween 60 (2%)	2.06 ± 0.02	92.98 ± 4.45	2.4 ± 0.08	28.4 ± 1.1	Diffusion
F12	W_{15} + Tween 60 (5%)	2.09 ± 0.03	92.49 ± 1.56	2.1 ± 0.07	44.6 ± 1.6	Diffusion
F13	W_{15} + Tween 60 (10%)	2.07 ± 0.05	92.67 ± 3.84	1.8 ± 0.05	34.9 ± 0.7	Diffusion
F14	W_{15} + Span 20 (2%)	2.03 ± 0.06	92.67 ± 3.61	2.4 ± 0.05	73.9 ± 2.4	Diffusion
F15	W_{15} + Span 20 (5%)	2.07 ± 0.05	91.54 ± 2.75	2.1 ± 0.01	61.3 ± 1.6	Zero
F16	W_{15} + Span 20 (10%)	2.09 ± 0.06	90.43 ± 2.90	2.0 ± 0.03	29.1 ± 1.3	Zero
F17	W_{15} + Span 20 (2%) + drug solution	2.06 ± 0.01	93.67 ± 4.56	2.3 ± 0.01	100 in 15 min	--

The results are the mean of three determinations ($n = 3$).
Abbreviations: PEG, polyethylene glycol; P188, poloxamer 188; W_{15}, Witepsol H15; P407, poloxamer 407.

The dissolution profiles of fenoterol HBr from suppositories manufactured using different compositions of PEG, *viz.* F1, F2, and F1 are shown in Figure 1A. The release of fenoterol HBr from the suppositories was relatively high (ranging from 50.8 to 100%). The literature abounds with reports on improvement of dissolution of drugs from a PEG suppository base. This is due to the water absorbing properties of PEG (*7,14,15*), which result in the formation of a hydrophilic matrix with subsequent solubility enhancing effects. This result was in agreement with the kinetic analysis of the results, which revealed a diffusion mechanism for all tested PEG formulations (Table 1). It is known that, as the molecular weights of PEG increase, their water solubility and hygroscopicity decrease (*16*). It is also reported that PEG of different molecular weights can be combined to achieve a suppository base with a specific drug release rate profile (*17*). Ranking the tested combinations in descending order, according to % extent of drug release, was as follows: F3 > F1 > F2 (Table 1). Drug partitioning is a function of the nature of base and the affinity of the drug towards the base, *i.e.* when there is a low affinity between the drug and the base, the release rate of the substances having high solubility in aqueous media was expected to be high. Knowing that fenoterol HBr is freely soluble in water, it is to be noticed that the base with the lowest hydrophilicity, F3, is accompanied by the highest drug release profile (100% extent release). F2 has a high ratio of PEG 1500, which is most likely to have more hydrophilic character, and this was reflected in the slowest drug release (50.8% extent release). It is apparent that this is attributed to the privileged partition of the water soluble fenoterol HBr towards the base. In-between came F1 (69.8% extent release), which has an intermediate hydrophilic character.

Figure 1B shows the release profiles of fenoterol HBr from poloxamer/propylene glycol bases. It is to be noticed that, PEG-based suppositories had significantly higher dissolution rates of fenoterol HBr than any poloxamer-based suppositories (Figures 1A and 1B). These results suggested that PEG was soluble in the dissolution medium, while poloxamer was not soluble but rather gelled (*18*). The formed gel neither absorbed water nor was it hydrophilic. This may be due to the hindrance of the hydrophobic polyoxypropylene segment of the molecule by the hydrophilic polyoxyethylene moiety of the molecule (*19*). The mechanism of drug release was therefore found to be zero-order kinetics (Table 1).

The rank of poloxamer/propylene glycol bases in descending order, according to % extent of drug release, was as follows: F6 > F5 > F4 (Table 1). It is obvious that increasing the amount of propylene glycol was accompanied by increased dissolution rates of fenoterol HBr. This finding is probably due to the hygroscopic properties of propylene glycol (*20*), which caused increased water absorption and the formation of a hydrophilic polymer matrix. This effect changed the mechanism of release of fenoterol HBr from zero order in the case of poloxamer alone (F4) into diffusion through the hydrophilic matrix of poloxamer/propylene glycol bases (F5 and F6).

3.3. *Release of fenoterol HBr from fatty bases*

Release of fenoterol HBr from four different types of semi synthetic fatty bases was studied (Figure 2). Ranking them in descending order according to percent extent drug release was as follows: Witepsol H15 > Suppocire BM > Witepsol E75 > Suppocire AP (Table 1).

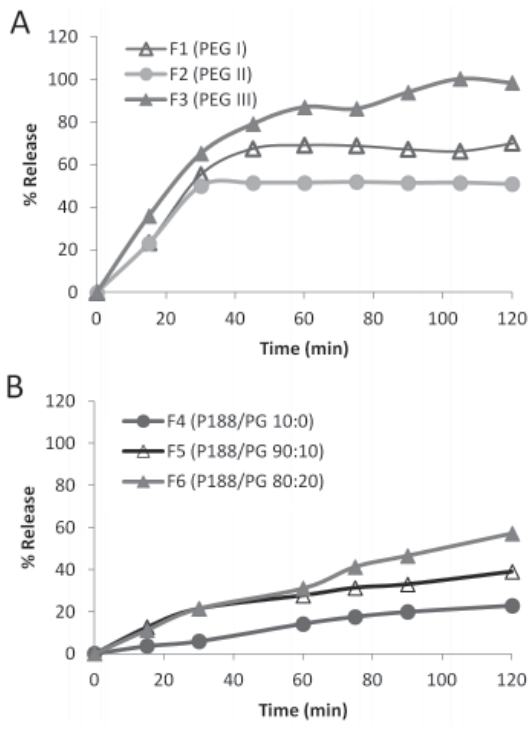

Figure 1. Release of fenoterol HBr from various formulations tested. (A) Hydrophilic PEG bases. **(B)** Hydrophilic poloxamer (P188) bases with different propylene glycol (PG) proportions. The results are the mean of three determinations ($n = 3$).

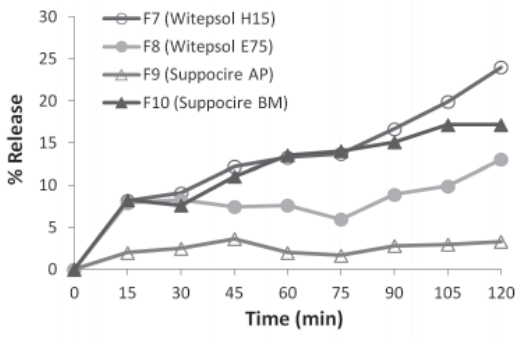

Figure 2. Release of fenoterol HBr from fatty bases. The results are the mean of three determinations ($n = 3$).

Semi-synthetic suppository bases are mixtures of fatty acids and esters with certain amounts of glycerides. The hydroxyl values reported for specific bases represent the proportions of free mono- and di-glycerides, *i.e.* free hydroxyl functional groups that are available for interaction. A high hydroxyl value is an indication of the potential for a base to adsorb water. The presence of a high hydroxyl value in fatty bases could thus favor the formation of a water-in-oil emulsion, which will generally result in a very slow transfer of drug molecules from the inner aqueous phase, *i.e.* retarded drug release (*3*). In this respect, drug release from formulations manufactured with Suppocire AP, which has a high hydroxyl value of 30-50, was slower than that from formulations manufactured using Suppocire BM (hydroxyl value < 6) or from either type of Witepsol (hydroxyl value 5-15) (*21*).

The extent of drug release from formulations manufactured with Witepsol H15 was higher than that from formulations manufactured using Witepsol E75 despite having similar hydroxyl values. This finding is related to the melting characteristic of the bases. Witepsol H15 has a melting point value of 33.5-35.5°C while Witepsol E75 has a melting point of 37-39°C (*20*). A complete melting of a suppository in the dissolution medium is certainly required for the drug to have the potential to be completely released. These results explain the kinetic analysis of the release data, which revealed a diffusion model for Witepsol H15 and a zero-order mechanism for Witepsol E75.

From the results it is evident that, PEG and poloxamer formulations released fenoterol HBr to a greater extent than those from fatty base suppositories. This is in accordance with previous reports (*22*,*23*), which stated that lipophilic bases for conventional suppositories led to much slower release than the hydrophilic bases. Nevertheless, the research conducted focused on improving the rate and extent of release of fenoterol HBr from fatty bases, since PEG bases have been reported to cause some irritation to mucosal tissues (*24*,*25*).

3.4. *Effect of surfactants on fenoterol HBr release*

In this study, the possibility of increasing the release of fenoterol HBr from fatty base suppositories was evaluated by the incorporation of non-ionic surfactants namely, Tween 60 as an example of a hydrophilic surfactant (hydrophilic-lipophilic balance (HLB) = 14.9) and Span 20 as an example of a lipophilic surfactant (HLB = 8.6) into a Witepsol H15 suppository base. Witepsol H15 was selected for further studies since it has an intermediate hydroxyl value, an optimal melting range and the greatest release profile among the tested fatty bases. The incorporation of non-ionic surfactants affected the rate of medicament release depending on nature and concentration of surfactant (*26*).

3.4.1. *Effect of Tween 60*

The release data for suppositories containing 2, 5, and 10% (w/w) Tween 60 are presented in Figure 3A. Percent drug release increased significantly upon incorporating up to 5% Tween 60. It is probably due to the fact that Tween 60 lowered the interfacial tension, and hence increased dispersibility of the suppository base with the dissolution fluid (*27*). However, further addition of Tween 60 up to a concentration of 10% increased the release rate of the drug to a smaller extent than that achieved with 5%. At higher concentrations, the surfactant might have exceeded its critical micellar concentration (CMC), and thus retarded drug release, as a result of micellar entrapment of the drug (*3*). The addition of Tween 60 to Witepsol H15 did not change the mechanism of drug release, which was found to be a diffusion model.

3.4.2. *Effect of Span 20*

The release data for suppositories containing 2, 5, and 10% (w/w) Span 20 are presented in Figure 3B. Two percent of Span 20 increased drug release significantly, which may be attributed to enhanced

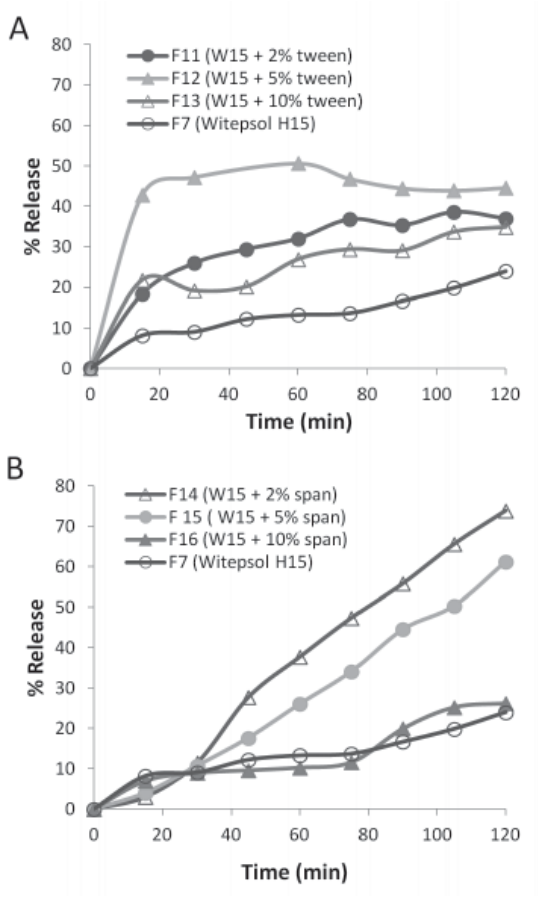

Figure 3. Release of fenoterol HBr from Witepsol H15 containing Tween 60 (A) or Span 20 (B). The results are the mean of three determinations (n = 3).

wetting of the matrix (*27*) and subsequently facilitated dissolution of the drug in the suppository and in the dissolution medium. Further addition of Span 20 up to a concentration of 10% led to a lesser improvement in rate and extent of fenoterol HBr release. Span 20 at higher concentrations may lead to a decrease in drug release due to formation of a water-in-oil emulsion (*28*). Other studies have reported that incorporation of surfactants may increase or decrease drug release from suppositories (*23*,*29*). It is worthy to note that for each surfactant there is an optimum concentration at which the drug exhibits maximum release rate.

The release profiles are consistent with the kinetic analysis of the data. The release rate followed diffusion only in the case of 2% Span 20 but changed to zero-order at higher concentrations of Span 20 which may be due to the formation of the water-in-oil emulsion.

From the previous results it is concluded that, low concentrations of surfactant are more effective in enhancing drug release than higher concentrations. This finding makes surfactant incorporation in suppository bases applicable as it has been demonstrated by Nakanishi *et al.* (*30*) that higher concentrations of nonionic surfactants are associated with adverse histological changes in the rectal tissue of rats. The enhancement of release rate produced by Span 20 was greater than that produced by Tween 60 (Table 1). This may be attributed to the much higher hydroxyl value of Span 20 (330-358) relative to Tween 60 (81-96) (*31*), which may increase the ability of the surfactant in wetting the matrices and producing a greater number of channels for the dissolution fluid to leach out the drug (*32*). However, this high hydroxyl value is probably the reason why at higher concentrations of Span 20 a water-in-oil emulsion is formed and drug release rate is decreased.

3.5. *Effect of incorporating fenoterol-HBr in a solution form in the suppository base on its release*

All previous trials to enhance drug release from Witepsol H15 succeeded only to a limited extent, because the highly hydrophilic drug fenoterol HBr is present in the base in a suspended form rather than a dissolved one. In this case, drug release is the result of a series of successive steps that involve melting of the base, migration of drug particles to the interface between the melted excipients and the dissolution medium, and finally the passage of the particles through this interface to be released in a molecular form (*3*).

The drug was thus incorporated in the base in the form of an aqueous solution instead of the powder form using Span 20 for emulsification. A 100% release was achieved in the first 15 min, because once the base was melted, the drug which was already dissolved was released all at once into the medium.

3.6. *Evaluation of liquid suppository*

Conventional suppositories are solid at room temperature and melt or soften at body temperature. Due to the characteristics of the solid, patients feel discomfort and, as a result, prefer oral administration of drugs, rather than *via* conventional suppositories.

In an attempt to solve this problem, suppositories that are liquid at room temperature and gel at body temperature have been developed and have been proposed as alternatives to conventional solid suppositories for the administration of drugs. Liquid suppositories are usually prepared from poloxamers since an aqueous solution of poloxamers, at high concentration, exhibits reversible thermal gelation (*33-36*). Hence, liquid suppositories are converted to solids in the rectum by thermal gelation following rectal administration. Since they can be administered as liquids, they would be expected to be more acceptable to patients and to cause less irritation to rectal mucosa compared to conventional suppositories. The problem associated with liquid suppositories is the migration of drugs that may undergo first-pass metabolism, up to the colon (*17*,*36*). This problem could be solved by the incorporation of a mucoadhesive polymer to the preparation. In the present study, sodium alginate was chosen as a mucoadhesive polymer because it causes no irritation to rectal mucosa and it exhibits a large mucoadhesive force (*37*).

3.6.1. *Gelation temperature*

The gelation temperature of liquid suppositories was dependent on the concentration of poloxamers (P188 and P407). F18 (15% (wt) each of P188 and P407) exhibited a gelation temperature of 39 ± 1°C (Table 2). Increasing the percentage of P407 to 20% (F19) and 25% (F20) was accompanied with a reduction in the gelation temperature to 37 ± 0.5°C and 36 ± 0.3°C, respectively (Table 2). F20 revealed therefore an optimal gelation temperature for the *in situ* gelling of the liquid suppository.

Table 2. Codes, composition and characterization of tested liquid suppositories

Code	Suppository composition	Extent release at 120 min (%)	Diffusion exponent (n)	Kinetic mechanism of release data	Gelation temperature (°C)
F18	Thermogel: 15% P188, 15% P407, 0.6% sodium alginate	22.9 ± 2.5	0.704	Non-Fickian	39 ± 1
F19	Thermogel: 15% P188, 20% P407, 0.6% sodium alginate	19.7 ± 1.9	0.748	Non-Fickian	37 ± 0.5
F20	Thermogel: 15% P188, 25% P407, 0.6% sodium alginate	21.6 ± 1.5	0.745	Non-Fickian	36 ± 0.3

The results are the mean of three determinations (n = 3).

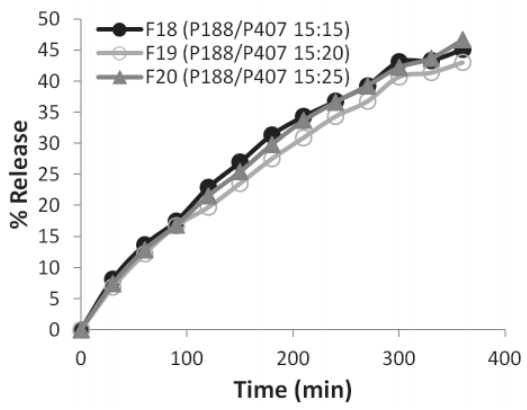

Figure 4. Release of fenoterol HBr from liquid suppositories composed of different proportions of P188 and P407. The results are the mean of three determinations ($n = 3$).

Table 3. Onset of dyspnea recorded for three groups of Guinea pigs with histamine-induced bronchospasm

Groups	Medication	Pre-convulsion dyspnea (min)
Gp I	Control	1.5 ± 1.2
Gp II	Oral solution of fenoterol HBr (5 mg/kg)	12.0 ± 2.3
Gp III	Fenoterol HBr suppository (F17) (5 mg/kg)	no reaction (animals were removed after 30 min from the chamber)

Values are mean ± S.D., $n = 6$ in each group. $p < 0.05$ as compared to control (unpaired Student's *t*-test).

3.6.2. *Release of fenoterol HBr from liquid suppositories*

The release profile of the drug from the tested formulations (F18, F19, and F20) was very similar, *i.e.* the change in proportions of P188 to P407 did not significantly influence drug release (Figure 4, Table 2). Drug release was generally retarded probably due to the mucoadhesive polymer. It was reported that, among many tested mucoadhesive polymers, sodium alginate exhibited the highest release retardation (*37*). Sodium alginate seems to affect the release rates by influencing the physicochemical properties of gel matrix. The polymer may have distorted or squeezed the diffusion channels, thereby delaying the release process. F20 with optimal gelation temperature thus presents a promising sustained release formulation for fenoterol HBr.

Kinetic analysis of release data revealed n values between 0.5 and 1.0 (Table 2), the mechanism of drug release is defined as anomalous (non-Fickian), where the release is controlled by a combination of diffusion and polymer relaxation (*38*).

3.7. *Histamine-induced bronchospasm in Guinea pigs*

All groups of Guinea pigs were subjected to histamine aerosols in order to induce bronchospasm, which was demonstrated by dyspnea followed by convulsions. The onset of dyspnea was recorded for all animals and the results are shown in Table 3.

The oral solution of fenoterol HBr succeeded in delaying the onset of dyspnea about 8 times (12 ± 2.3 min). For the study the suppository formula, in which the drug was incorporated in Witepsol H15 as aqueous solution (F17), was selected because of its very fast drug release. For the group of animals, that received the suppositories no dyspnea was recorded up to 30 min (after 30 min the animals were removed from the chamber to prevent their suffocation due to depletion of oxygen). This result implies that suppositories possess greater bioavailability than oral solutions probably due to avoidance of first pass effect.

4. Conclusion

In conclusion, hydrophilic bases were superior to lipophilic bases in terms of their ability to release fenoterol HBr from the suppository formulations. Incorporation of non-ionic surfactants to Witepsol H15 at lower concentrations improved drug release. A very fast release of the drug was achieved by incorporating the drug as an aqueous solution in Witepsol H15. This formula succeeded in preventing histamine-induced bronchospasm in Guinea pigs for up to 30 min. Liquid suppositories composed of various proportions of P188 and P407 in addition to the mucoadhesive polymer, sodium alginate, have shown remarkable retarded release of the drug. The formula that contained 15% P188 and 25% P407 exhibited optimal gelation temperature for the *in-situ* gelling of the liquid suppository. This formula could be regarded as a promising sustained release formulation suitable for further investigation.

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