Drug Discoveries & Therapeutics

ISSN 1881-7831 Online ISSN 1881-784X
Volume 2, Number 5, November 2008
www.ddtjournal.com

Japan-China Joint Medical Workshop on Drug Discoveries and Therapeutics 2008
September 29 – October 1, 2008, The University of Tokyo, Tokyo, Japan

Shandong University China-Japan Cooperation Center for Drug Discovery & Screen
International Advancement Center for Medicine & Health Research
Drug Discoveries & Therapeutics

Drug Discoveries & Therapeutics is a peer-reviewed international journal published bimonthly by Shandong University China-Japan Cooperation Center for Drug Discovery & Screen (SDU-DDSC) and International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.).

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

Subject Coverage: Basic and clinical pharmaceutical research including Pharmaceutical and therapeutical chemistry, Pharmacology, Pharmacy, Pharmacokinetics, Industrial pharmacy, Pharmaceutical manufacturing, Pharmaceutical technology, Drug delivery, Toxicology, and Traditional herb medicine.

Language: English
Issues/Year: 6
Published by: IACMHR and SDU-DDSC
ISSN: 1881-7831 (Online ISSN 1881-784X)

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Japan-China Joint Medical Workshop on Drug Discoveries and Therapeutics 2008: The need of Asian pharmaceutical researchers' cooperation

Munehiro Nakata¹, Wei Tang²

Keywords: Drug discovery, Therapeutic, Influenza

The Japan-China Joint Medical Workshop on Drug Discoveries and Therapeutics 2008 (JCMWDDT 2008) was held from September 29 to October 1, 2008 at The University of Tokyo, Tokyo, Japan. JCMWDDT is an international workshop that is mainly organized by Asian editorial members of Drug Discoveries & Therapeutics (http://www.ddtjournal.com/home) for the purpose of promoting research exchanges in the field of drug discovery and therapeutic. This year's JCMWDDT is the second workshop and focused particularly on novel development and technological innovation of anti-influenza agents. The workshop began with an announcement by the Japanese Co-chairperson, Dr. Sekimizu (Department of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Japan; Editor-in-Chief of Drug Discoveries & Therapeutics, DDT) followed by a speech by the Chinese Co-chairperson, Dr. Wenfang Xu (School of Pharmaceutical Sciences, Shandong University, Shandong, China; Editor in China Office of DDT), with additional speeches by Dr. Norio Matsuki (The University of Tokyo, Japan; Editor of DDT) and Dr. Guanhua Du (Chinese Academy of Medical Science, China; Editor of DDT).

Fifty-nine titles were presented in 6 specialized sessions (Research Advances in Drug Discoveries and Therapeutics, Drug Synthesis/Clinical Therapeutics, Medicinal Chemistry/Natural Products, Anti-influenza Drugs, Anti-infection/antiviral Drugs, Biochemistry/Molecular Biology/Pharmacology) and a poster session (Drug Discov Ther 2008; 2, Suppl; available at http://www.ddtjournal.com/Announce/index.htm). An annual outbreak of avian influenza in Asian countries including China and Japan has sparked fears that the virus will mutate and then cause an epidemic in humans. Therefore, Asian researchers need to work together to control this infection. This year's JCMWDDT helped provide an opportunity to reiterate the crucial role of medicinal chemistry in conquering influenza and created an environment for cooperative research in Asian countries. (reported on October 1st, with grateful thanks to all participants)

Keywords: Drug discovery, Therapeutic, Influenza
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- Structure-activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their in vitro anti-viral activities by Guanhua Du (Chinese Academy of Medical Sciences and Peking Union Medical College, China)
- Mechanisms and consequences of phagocytosis of influenza virus-infected cells by Yoshinobu Nakanishi (Kanazawa University, Japan)
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Session V. Anti-infection/Antiviral Drugs

- Emerging infectious diseases and anti-viral drugs: Urgent need to develop effective drugs which cause less resistant virus by Nobuyuki Kobayashi (Nagasaki University, Japan)
- Design, synthesis and antiviral evaluation of novel heterocyclic compounds as HIV-1 NNRTIs by Xinyong Liu (Shandong University, China)
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- Effect of andrographolide derivatives having α-glucosidase inhibition, on HBsAg, HBeAg secretion in HepG2 2.2.15 cells by Hideki Tani (Shandong University, China)
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- A novel conjugate of low-molecular-weight heparin and Cu,Zn-superoxide dismutase: Study on its mechanism in preventing brain reperfusion injury after ischemia in gerbils by Fengshan Wang (Shandong University, China)
- A novel gene fusion in SCCmec region regulates the colony spreading ability and virulence in Staphylococcus aureus by Chikara Kaito (The University of Tokyo, Japan)
- Water soluble fluorescent boronic acid sensors for tumor cell-surface saccharide by Hao Fang (Shandong University, China)
- Molecular characterization of the biosynthetic enzyme for the biotechnological production of tetrahydrocannabinol, the active constituent of marijuana by Tatsushi Taura (Kyushu University, Japan)
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Pulmonary drug delivery: Implication for new strategy for pharmacotherapy for neurodegenerative disorders

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ABSTRACT: Innovative drug delivery in the treatment of brain neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) has the potential to avoid many unwanted side effects over current medications. Advances in understanding of these diseases and their treatments have led to the search for novel modes of drug delivery. In this review, we have highlighted new strategies and future prospects for pulmonary delivery of drugs for the management of these important neurological disorders. The advancement of knowledge on pulmonary drug delivery will provide novel therapeutic formulations for better management of the PD and AD patients throughout the world.

Keywords: Pulmonary drug delivery, Dry powder inhaler, Metered dose inhaler, Nebulizer, Parkinson's disease, Alzheimer's disease

1. Introduction

Pulmonary delivery, a non-invasive route of drug delivery is becoming a route of choice for most drugs. Pressurized metered dose inhalers (pMDI) and nebulisers (liquid jet and ultrasonic) are the preliminary devices to deliver drugs into lung; however, currently, breath actuated dry powder inhalers (DPI) are designed to deliver medicaments as a powder form through the airways in the lung to achieve both systemic and local effects.

Direct delivery of drugs into the pulmonary regions of the lung enables lower doses with an equivalent therapeutic action compared to oral or parenteral routes because of the large surface area (~100 m²) of the lungs. Advantages of DPI formulations over other dosage forms (i.e., parenteral and other liquid dosage forms) are solid dosage form stability, ease of use, less expensive, painless and user friendly. The inhaled route allows the delivery of small doses of drug directly to the alveoli attaining a high concentration of drug in the local area and minimizes systemic side effects resulting in a high therapeutic ratio of drugs compared with that of systemic delivery administered either by oral or parenteral routes. Oral tablets and capsules need to be swallowed which is sometime difficult for some patients especially for children. Respiratory delivery also offers effective therapy with minimum adverse effects by using small doses of drugs through inhalation and allows substantially greater bioavailability of polypeptides (1).

Currently, delivery of drugs for the management of neurological disorders especially PD and AD are done by oral, parenteral and transdermal routes. Pulmonary delivery of drugs is well established in the management of asthma and COPD (chronic obstructive pulmonary disorder). However, no DPI drugs are approved yet for the management of other diseases like AD and PD. This mini-review discusses advantages of pulmonary delivery of drugs, pulmonary delivery technologies, and current situations and future trends in managing major AD and PD by delivering drugs into the deep lung via DPI or MDI devices.

2. Pulmonary delivery technology

Aerosol delivery of drugs, formulated as liquid solutions, suspensions, emulsions or micronized dry powders, are aerosolised via some commonly used different types of delivery devices (nebulizer, pMDI, and DPI). In this section both the formulations as well delivery devices are discussed.

2.1. Nebulisers

Nebulisers are probably one of the oldest forms of pulmonary drug delivery, deliver large volumes of
drug solutions or suspensions and are frequently used for those drugs which can not be formulated into pMDIs or DPIs. Currently, two categories of nebulisers are available on the market include air jet and ultrasonic nebulisers. Air jet nebulisers can generate both smaller particles (mass median aerodynamic diameter 2-5 μm) and coarse aerosols, and deliver medication quickly; however, it produces high oropharyngeal deposition of drugs. Most jet nebulisers operate by forcing pressurised gas (air or oxygen) through a nozzle or jet at high velocity so that the nebulizer solution is atomized. On actuation the gas expands resulting in the generation of a negative pressure which draws the liquid formulation into the gas stream. The aerosol mist impacts against a baffle, drains back into the reservoir incorporated with the nebuliser and recirculates. Ultrasonic nebulisers do not require compressed gas. The solution formulation is atomised by an energy source, piezoelectric crystal transducer, which vibrates at high frequency and these devices can generate slightly larger aerosols. However, the overall efficiency of the piezoelectric driven ultrasonic nebulisers is more or less similar to that of air-jet nebulisers. Patients who are seriously affected with obstructive lung conditions prefer to use nebuliser therapy. Nebulisers are suitable for drugs with high dose and little patient co-ordination or skill; however, treatment using nebuliser is time consuming and less efficient, resulting in the waste of active medicaments. They are not portable devices and have been limited to the treatment of hospitalised patients. A number of nebulisers include AeroDose® (Aerogen), AeroEclipse® (Trudel Medical International), Halolite® (Medic-Aid Limited), Respimat® (Boeringer Ingheim), etc are currently available on market to deliver various types of drugs.

2.2. Pressurised metered dose inhalers

Pressurised metered dose inhalers (pMDIs), also known as metered dose inhalers (MDIs), are the most commonly used delivery devices. In this device (Figure 1), drug is either dissolved or suspended in liquefied propellents (or a mixture of propellants) with other excipients and presented in a pressurised canister fitted with a metering valve. On actuation of the valve, a predetermined amount of drug is released as spray. Aerosol formulations are packed in tin-plated steel, plastic coated glass or aluminium containers. The propellents used in pMDI formulations are liquefied gases of chlorofluorocarbons (CFC), which are not environmentally friendly. This is the reason why currently hydrofluoroalkanes (HFAs), which have no remarkable effects on the ozone layers, are used in the formulation for MDIs. At room temperature and pressure these are gases but they are liquefied by applying high pressure or by lowering temperature. On spraying, drug formulation with propellents are expelled and aerosolised. Although pMDIs are widely used in respiratory drug delivery, some problems have been associated with these devices, including the need for coordination of inspiratory inhalation with valve actuation and the use of a propellant, which has possible adverse effects on the stratospheric ozone layer as mentioned before. Currently there are a good number of pMDIs available on the market such as Ventolin (albuterol, GlaxoSmithKline), Azmacort (triamcinolone acetate, Aventis Pharma), Symbicort

![Figure 1. Schematic diagram of aerosol delivery of drugs from pMDI. Modified from Dalby et al. (2).](https://www.ddtjournal.com)
(Budesonide and formoterol, AstraZeneca), Flovent (Fluticasone, GlaxoSmithKline), etc. for the treatment of asthma.

There are some breath actuated and microprocessor controlled MDIs available on the market (Autohaler®, Respimat®). These devices ensure the patient receives the drug at the correct point in the inspiration, and by slow inhalation with an indicator light to inform the patient whether the dose is inhaled or not. Anyway, as mentioned above, pMDIs have some disadvantages such as oropharyngeal deposition of drugs due to high velocity of propellants. The particles aerosolised from the MDIs have a high velocity, which exceeds the patients’ inspiratory force, therefore, a large number of particles deposit onto the oropharyngeal areas. Thus a small fraction of drug deposits into the patients lungs (3) due to a lack of co-ordination between actuation and inhalation. To overcome this difficulty several inhalation aids like spacers incorporated with MDIs have been developed (4,5).

2.3. Dry powder inhaler (DPI) system

Dry powder inhalers contain the drug in a powder formulation, where drug particles (< 5 μm) are blended with a suitable large carrier (e.g. lactose) to improve flow properties and dose uniformity (6,7) and drug powders are delivered into the deep lung via a device known as dry powder inhaler (DPI). Powder de-agglomeration and aerosolisation from these formulations are achieved by the patient’s inspiratory airflow, which needs to be sufficient to create an aerosol containing respirable drug particles for lung deposition. Good flow properties of the formulation are necessary to ensure accurate dose metering of the drug. Advantages of DPI over other inhaler systems (pMDIs) are independence of breathing co-ordination with dose actuation, the absence of propellants, low innate initial velocity of particles (reducing inertial impaction at the back of the throat) and solid state drug stability. There are two types of DPI formulations; one is loose agglomerates of micronized drug particles having controlled flow properties, and the second one is carrier-based interactive mixtures (Figure 2) which consist of micronized (< 5 μm) drug particles mixed with larger carrier particles (8). Drug dispersion form the interactive mixtures can be enhanced by the addition of fine excipients (lactose) in the formulation (9,10). Drug particle size and powder formulation, breathing patterns and complex physiology of respiratory tract are major factors affect delivery of drugs into the deep lung. The redispersion of drug particles depends upon the interparticulate forces within the powder formulation. DPIs are highly portable, breath activated and relatively less expensive. Since drugs are kept in solid state in DPIs, they exhibit high physicochemical stability of drugs particularly proteins and peptides In DPI formulation the device is an important factor in achieving adequate delivery of inhaled drug to lungs. The device must provide an environment where the drug can maintain its physicochemical stability and produce reproducible drug dosing.

There is a wide range of DPI devices, single, multi-unit or multiple dose devices, breath activated and

![Figure 2](image_url). Schematic diagram of the pulmonary delivery of drugs from DPI formulations. The formulation consists of micronized drugs adhered on the surface of large carrier particles. Drug particles detached from the surface of large carriers and deposits into the patients airways by inhalation. Modified from Telko and Hickey (11).
power driven, available on the market; however, no devices showed efficiency in maximal drug delivery. Currently, based on the design, DPI devices may be classified into three broad categories i.e., the first generation DPIs, the second generation DPIs and the third generation DPIs. The first generation DPIs were breath activated single unit dose (capsule) i.e., the Spinhaler® and Rotahaler®. The second generation of DPIs use better technology i.e., multi-dose DPIs (they measure the dose from a powder reservoir) or multi-unit dose (they disperse individual doses which are premetered into blisters, disks, dimples, tubes and strip by the manufacturers) and multi-unit dose devices are likely to ensure the reproducibility of the formulation compared to that of multi-dose reservoir. The third generation DPIs, also known as active devices, which employ compressed gas or motor driven impellers or use electronic vibration (12,13) to disperse drug from the formulation. The very first approved active device (Exubera®, Pfizer) with compressed air to aerosolise drug formulation for DPI insulin delivery was available on market; however, due to some unknown reasons, the production has been discontinued.

3. Mechanisms of drug deposition from aerosols

To achieve a desired therapeutic effect from aerosols, an adequate amount of drug must reach the alveolar sacs of the respiratory airways. The dynamic behavior of aerosol particles is governed by the laws of aerosol kinetics (14). The dominant mechanisms of depositing aerosol particles into the respiratory tract include inertial impaction, sedimentation (gravitational deposition), Brownian diffusion, interception and electrostatic precipitation (15). Inertial impaction and sedimentation are the most important for large particle deposition (1 μm < MMAD < 10 μm). A brief description of each mechanism of deposition is given below:

Inertial impaction: This is the main deposition mechanism at the tracheal bifurcation or successive branching points of airways. The airflow changes its direction at branching of the airways. The aerosol particles continue to move in their original direction and impact on any obstacle on the way. The deposition of aerosol particles by impaction increases with increasing air velocity, frequency of breathing and particle size (16). Large particles (> 5 μm) with high velocity are mainly deposited by impaction (17).

Sedimentation: Sedimentation occurs when the gravitational force exerted on a particle overcomes the force of the air resistance. Particles of smaller size (0.5-3.0 μm), which have tendency to escape from deposition by inertial impaction, may be deposited by sedimentation. Deposition of small particles by sedimentation mainly occurs in the smaller airways and alveolar regions and increased sedimentation is observed during breath-holding or slow steady breathing (17).

Diffusion: Deposition of aerosolized particles less than 0.5 μm occurs by diffusion due to Brownian movement. Deposition of aerosols by diffusion is independent of the density of particles but increases with decreasing size. Generally, the deposition of particles larger than 1.0 μm is dominated by inertial impaction and particles smaller than 0.1 μm are deposited by diffusion. Both sedimentation and diffusion are important for the particle size ranging between 0.1-1.0 μm (18).

Interception: Although particle deposition by interception is not common, the deposition of elongated particles (particles large in one dimension but with small aerodynamic diameters) is believed to occur by this mechanism. Deposition of particles in the respiratory airways by interception is important when the dimensions of the anatomic spaces of airways become comparable to the dimensions of the particles (17).

Electrostatic precipitation: Electrostatic charges may be generated in a DPI on particles of an aerosol. Particles are inhaled immediately after charge generation and before neutralisation of the charge can occur. A charged particle may induce an image charge of opposite polarity on the airway walls. This image charge attracts the particle which is subsequently deposited by electrostatic precipitation (17,19). Only fibrous particles are believed to be deposited by this mechanism, therefore, this mechanism may not be significant for DPI formulations.

4. Pulmonary delivery of various drugs

4.1. Current drugs

Currently, local delivery of medicaments to the alveoli of lungs from both DPIs and pMDIs are mainly used for the treatment of lung disorders including asthma and bronchitis and a limited number of therapeutic compounds such as β-adrenoceptor agonist, mascaicn agonist, corticosteroids and mast cell stabilizers are available. Recently certain combinations of drugs are also formulated due to a synergistic therapeutic benefit. Corticosteroids and long acting β-adrenoceptor agonists formulations are available as both pMDIs and DPIs (20). Zanamavir, an antiviral agent has been introduced in the market as an aerosol product for the treatment of influenza (21). Aerosol delivery of recombinant human deoxyribonuclease (rhDNase) and tobramycin are available as nebuliser for the treatment of cystic fibrosis (21,22). The very first approved aerosol delivery of insulin as DPI formulation (Exubera®, Pfizer) was introduced in the market; however, the production of this drug has been discontinued from market in early 2008 due to some unknown reasons.
4.2. Drugs delivered as aerosol

Aerosol delivery offers the greatest potential to delivery drugs into the lower airway of the lungs of a wide range molecule for systemic diseases. A list of various drugs administered via pulmonary route has been presented in the Table 1.

Aerosol delivery of macromolecules is a potential non-invasive way of administering drug, to avoid frequent injections. Lung delivery of insulin has already been established; however, insulin loaded chitosan nanoparticles (23); nanoparticles of calcitonin (24); and nanospheres of elcatonin coated with chitosan (25), have been demonstrated for successful deep lung delivery. Aerosol delivery of leoprolide has been investigated as both MDI and DPI formulation for the management of prostate cancer (26,27). Dry powders of other proteins like parathyroid hormone for osteoporosis (28,29), glucagon (30), growth hormone (hGH) for dwarfism (31), vasoactive intestinal peptide (VIP) for pulmonary diseases like asthma (32) have been successfully investigated.

Lung delivery of genes that directly target the regions of interest by avoiding problems associated with intravenous delivery has been developed. Recently, successful gene delivery into lungs for cystic fibrosis has been demonstrated (34,35). Lung delivery of genes complex with cationic lipids (lipoplex) and polymer-based (polyplex) are in progress (36,37) and a cationic lipid coupled with plasmid DNA (lipoplex), showed efficient lung delivery of gene (38). In another study, aerosol delivery of p53 and cytokine (IL-12) delivered via a nebuliser have been reported for therapeutic responses with reduced toxicity in animal lung tumor model (39-41). Based on the above mentioned researches it seems that there is a potential future of pulmonary gene therapy for various types of clinical applications.

Aerosol delivery of vaccines is an another area of interest and inhalation of measles vaccine was showed to be both safe and effective (42) and nebulised measles vaccine in human model found to produce better immunity with reduced side effects compared to that of subcutaneous injection (43). Dry powder inhaler formulation of measles vaccine (44), mucosal vaccination for influenza virus (45), malarial vaccine (46), and siRNA (47) have been investigated with significant success. Very recently, aerosol delivery of human immunodeficiency virus (HIV) treatments in infected patients found to be therapeutics with reduced toxicity and improved patient compliance (48). Therefore, it seems that pulmonary delivery of various genes is progressing and in future the world will see suitable vaccines against many pulmonary pathogens like Mycobacterium tuberculosis, respiratory syncytial virus (RSV), and severe acute respiratory syndrome (SARS).

Table 1. Drugs administered as aerosols against various diseases

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<td>Endometriosis, Pubertus praecox, Prostate carcinoma</td>
<td>Leuprolide</td>
<td>Powder/Liquid</td>
<td>26,27</td>
</tr>
<tr>
<td>Hormone replacement therapy</td>
<td>Testosterone</td>
<td>Liquid/Oral AREX</td>
<td>112</td>
</tr>
<tr>
<td>Immunosuppressor</td>
<td>Cyclosporin A</td>
<td>Microparticle/Liquid</td>
<td>113,114</td>
</tr>
<tr>
<td>Thrombosis and emphysema</td>
<td>Heparin</td>
<td>Microparticle</td>
<td>115,116</td>
</tr>
</tbody>
</table>
Inhaled rifampicin antibiotic, and rifampicin loaded poly(lactide-co-glycolide) microparticles (49), colistin sulphate (50,51), and mucoactive agent Nacystelyn (52) have been found to be promising against cystic fibrosis (CF). Moreover, DPI formulation of colistin (53), gentamicin (54), azithromycin (55), tobramycin (56), have been effective method of treating CF. Furthermore, deep lung delivery of amphoterin B desoxycholate, liposomal amphoterin B, amphoterin B lipid complex and amphoterin B colloidal dispersion via nebulizers has been shown to be valuable in the prophylactic treatment of pulmonary aspergilosis (57). A nebulised dispersion of amorphous itraconazole nanoparticles (300 nm) produced by ultra-rapid freezing technique, showed improved bioavailability in mice (58). This outcome offers the application of itraconazole nanoparticles for the efficient treatment of fungal infections.

Lung delivery of aerosolised chemotherapeutic agents for the direct local treatment of lung tumors has been explored and found advantageous over other methods of drug delivery systems. Pulmonary delivery of aerosolised 9-nitrocamptothecin (9-NC) and cisplatin in patients with lung cancer have shown safety and promising antitumor effect (59,60). In addition, lung delivery of doxorubicin (61), paclitaxel (62), celecoxib and docetaxel (63,64), gemcitabine (65), liposomal camptothecin (66), etc., has also been investigated and reduced toxicity was demonstrated. Recently, doxorubicin-loaded nanoparticles in dry powder aerosol form showed significant cytotoxicity in lung cancer (67). The researchers have indicated the potential of inhalation delivery of anticancer drugs in the treatment of lung cancer, however, further details investigation has been warranted.

Very recently, Dames and his co-workers developed targeted delivery of colloidal iron oxide nanoparticles (super magnetic iron oxide) (68), suspension of tocopherol nanoparticles coupled with biodegradable polymers for delayed release (69) and liposomal encapsulated cannabinoids for a prolonged psychoactive effect (70) have been demonstrated. In another study, aerosol delivery of apomorphine for sexual dysfunction (71), morphine and fentanyl for pain management (72), and ergotamine for migraine headaches (73,74). Furthermore, using nebuliser, aerosol delivery of radiopharmaceutical, ⁹⁹mTc with phosphate buffer for lung ventilation imaging purposes is widely used (75). Inhalation of radiolabeled sulfur colloid (SC) aerosol (⁹⁹mTc-SC, 0.2 μm) for studying particle uptake by airway surface macrophages has been demonstrated (76).

The aforementioned findings show the wider application of aerosol delivery of drug-aerosols; which offers the greatest potential to deliver drugs into the lower airway of lungs of a wide range of molecules (i.e., antibiotics, genes, peptides and proteins, antibodies and oligonucleotides) for systemic diseases and put forwards the most promising inhalable platform for efficient systemic administration.

5. Inhaled drugs for neurodegenerative disorders

Pulmonary delivery for drugs of the management of neurological diseases is not currently approved. As deep lung delivery of different drugs showed potential benefit, researchers are now focusing to expand research on delivering drugs into the deep lungs via DPIs/MDIs. Some drugs administered as aerosols for the treatment of neurological disorders are shown in Table 2.

5.1. Pulmonary delivery of drugs for Parkinson's disease (PD)

PD is a chronic and progressive movement disorder (77). Millions of people suffer from PD in the developed and developing world (77). It is estimated that approximately 15 percent of people with PD are diagnosed before the age of 50 with incidence increasing with age. Evidence suggests that PD occurs when trouble sprouts in the basal ganglia, a segment of brain areas known for their contribution to movement. In general, nerve cells in the brain substantia nigra inexplicably die or become impaired. Normally these cells communicate via the chemical dopamine (DA) with cells in another one of the areas, the striatum, which includes subareas called the putamen and the caudate nucleus. Without DA, the striatum can't send out the electrical signals needed for normal movement, and consequently PD develops.

Recently, a number of studies also indicate that the basal ganglia are involved with some of the cognitive problems that PD patients experience (78). Although there is presently no effective cure, there are many treatment options such as medication and surgery to manage the PD symptoms.

Levodopa is considered to be a temporary solution for minimising PD symptoms (79,80). Research suggests that the drug enters the brain and is transported into cells that can convert it into DA in the striatum. At first, symptoms diminish, but symptoms return in three to five years. Thus far, research suggests that the cells that convert levodopa die off. Higher doses of levodopa can make up for the decreasing number of cells but may cause jerking movements of the limbs, trunk and head as well as hallucinations. However, a number of attempts have been made to improve on the levodopa treatment by creating drugs that mimic DA by using DA agonists. Low doses of the DA agonists in combination with levodopa create less severe side effects and work for longer periods of time. Scientists are now taking this step further to determine if the drugs that mimic DA can replace levodopa altogether (81). At this point, researchers have developed drugs

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that each target a specific DA receptor site such as D1 or D2 (82). The drugs that are acting at D2 sites appear not to work effectively alone. But preliminary research on rodent models shows drugs that act on D1 sites work better than when administered without levodopa. Thus, these new drugs appear to work better than the use of levodopa alone because they continue to show benefits over time and cause lesser side effects. More importantly, several investigators have discovered other DA receptors sites such as, D3, D4, and D5. It is likely that in the future drugs could be developed that would act on these receptor sites (80-82).

Overall, levodopa (L-dopa) provides better therapeutic advantage for most early stage PD patients and current treatments of PD are primarily with oral formulation, such as levodopa/carbidopa, bromocriptine, selegiline, benztropine and trihexyphenidyl; however, the efficacy of orally administered formulations becomes problematic with the progression of disease condition and due to a lost ability to control L-dopa’s poor pharmacokinetics. Majority of the drugs approved and currently available on the market are oral (Tablet/capsules), some are parenteral (IV, IM, SC) and only one is a transdermal patch; however, no pulmonary delivery products been not developed.

Deep lung delivery of levodopa particles for treating a patient with PD has been reported (83); however, no further data is available for the readers. Using MDI, delivery of a DA agonist to the airways has been demonstrated and the authors indicated that the inhalation route provided effective delivery of the drug to the receptor (84). Pulmonary administration of a drug (ABT-431, a selective D1 receptor agonist) was found to be significantly greater than that of oral administration (85). For example with intratracheal instillation of the drug solution, bioavailability of the drug was 75% in dogs and tetrafluoroethane (HFC-134a) based MDI formulation showed 40% bioavailability. The lung bioavailability of the aerosolised drug was 34% compared to intravenous injection in the same dogs. The authors emphasised that a single rising dose in human study demonstrated that the absorption of ABT-431 following oral inhalation administration (bioavailability 25%) resulted in a dose-dependent increase in the AUC (area under the curve) versus time profile at dosages from 3.3 mg to 13.2 mg. sing a novel delivery device (AERx), Okumu and his associates (86) delivered ABT-431 (a selective dopamine D-1 receptor agonist) to healthy male volunteers and plasma samples were analysed following lung delivery and intravenous administration and 82-107% of pulmonary bioavailability was observed. This outcome demonstrated that the aerosol inhalation of this drug was a proficient means for systemic delivery.

Dugs for pulmonary delivery for the management of PD have not currently been approved; however,

Table 2. Drugs administered as aerosols against neurological disease

<table>
<thead>
<tr>
<th>Indication</th>
<th>Drug substances</th>
<th>Delivery method</th>
<th>Details of delivery method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain, dyspnea</td>
<td>Opioids/Morphine</td>
<td>Powder</td>
<td>Micronized powders and aqueous formulation aerosolized</td>
<td>117-119</td>
</tr>
<tr>
<td></td>
<td>Fentanyl/alfafentanyl and morphine</td>
<td>Liquid</td>
<td>Liposomal fentanyl delivered via a nebulizer and MDI</td>
<td>72,120</td>
</tr>
<tr>
<td>Smoking cessation</td>
<td>Nicotrol® Nicorette® (Nicotine)</td>
<td>Nicotine cartridge/ Liquid</td>
<td>Nicotine cartridges in an inhaler; pMDI</td>
<td>121,122</td>
</tr>
<tr>
<td>Migraine, Vascular cephalgia</td>
<td>Dihydroergotamine mesylate</td>
<td>Liquid</td>
<td>HFA 134a based suspension delivered by MDI</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Ergotamine tartrate</td>
<td>Liquid</td>
<td>Pulmonary delivery method is unavailable</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>Detorelix</td>
<td>Liquid</td>
<td>Intratracheal (i.t.) instillation of Liposomal drugs</td>
<td>124</td>
</tr>
<tr>
<td>Parkinson's disease</td>
<td>Dopamine D-1 agonist, ABT-431</td>
<td>Liquid</td>
<td>HFA based drug formulation was delivered by MDI and AERx</td>
<td>85,86</td>
</tr>
<tr>
<td>Parkinson's disease</td>
<td>L-Dopa/ L-Dopa + agonist</td>
<td>Powder/liquid</td>
<td>Alkermes AIR, delivery of small or large molecules to the deep lung; Drugs in propellent delivered by MDI</td>
<td>83,84,89</td>
</tr>
</tbody>
</table>

Note: pMDI is pressurised metered dose inhaler; MDI: metered dose inhaler; HFC is hydrofluoro alkane; AERx is a DPI device that deliver aerosolised drugs from a dosage form that consists of liquid drug formulation and a nozzle array.
only one formulation containing L-dopa has been investigated \cite{83,87}. The inhalation data for pulmonary delivery of L-dopa showed at least two fold less dose compared to that of oral dose \cite{88}. In another study, demonstrated aerosol delivery of L-dopa dry powder formulation in a rat model and pulmonary administered L-dopa showed rapid and higher plasma levels \(C_{\text{max}} = 4.8 \pm 1.10 \, \text{mg/mL} \) at 2 min compared to that of oral administration where the drug produced delayed and lower plasma level \(C_{\text{max}} 1.8 \pm 0.40 \, \text{mg/mL} \) at 30 min \cite{89}. The authors acknowledged that an inhalable formulation of L-dopa may provide PD patients with effective form of rescue therapy as well as replacement for first-line oral therapy. However, this formulation is in preclinical stage. Innovative drug delivery in treating PD has the potential to reduce many adverse effects of currently available drugs. Pulmonary delivery of current drugs, genes and liposomes will help encourage patient specific treatment for PD. Improved treatments with pulmonary delivery system may involve drugs that target one specific site or a combination of sites for better pharmacotherapeutics. Therefore, these advances, along with investigations into gene transfer, surgical and transplantation techniques, hold great promise for those with PD.

5.2. Pulmonary delivery of drugs and Alzheimer's disease (AD)

Dementia is a brain disease that significantly affects a person's ability to carry out daily routine activities. It is well established that the most common form of dementia among older people is AD, which initially involves the various parts of the brain that control thought, memory, and language \cite{90,91}. In recent years some progress has been made, however, the causes of AD remain unknown, and there is no effective cure. Like PD (see above), it is estimated that millions of people worldwide suffer from AD \cite{91}. Previous research has found that other brain changes in people with AD. For example, brain neurons die in areas of the brain that are vital to memory and other mental abilities, and connections between neurons are disrupted. Furthermore, AD may impair thinking and memory by disrupting neuronal transmissions and functions. AD is a slow disease, starting with mild memory problems and ending with severe brain damage. The course the disease takes and how fast changes occur vary from person to person. On average, AD patients live from 8 to 10 years after they are diagnosed, though some people may live with AD for as many as 20 years \cite{90,91}.

Given the complex disease process with AD, there is no better therapeutic strategy to reduce or minimize AD. However, some drugs such as, tacrine, donepezil, rivastigmine, or galantamine may help prevent some symptoms from becoming worse for a limited time in some people in the early and middle stages of the disease. Most drugs for the treatment of AD are oral (very slow absorption) or parenteral (expensive) and aerosol formulations have not been studied. Thus, it would be worthwhile for the researchers to focus on

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pulmonary delivery of drugs for the management of AD. Like PD, current treatments of AD are primarily with oral formulation as described above. Improved treatments with pulmonary delivery system may involve drugs that target one specific site or a combination of sites for better pharmacotherapeutics.

6. Future directions

Lung delivery of drugs offer the greatest potential to deliver drugs into the lower airway of the lungs and the delivery of a powder form of a wide range of molecules (i.e., antibiotics, peptides, proteins, antibodies and oligonucleotides) to the deep lung for systemic diseases put forwards the most promising inhalable stage for increasing systemic administration. Pulmonary delivery of large molecules for chronic diseases is advancing tremendously and may become successful in the near future. Therefore, pulmonary drug delivery needs to focus not only on lung diseases but also on conditions in which fast onset is desirable such as cancer pain, allergic reactions, brain disorders, cardiovascular disorders, and sexual dysfunction. It has been reported that more than 40 drug formulations for widely varying designs of DPIs are in the pipeline for drug delivery of a powder form of a wide range of molecules (i.e., antibiotics, peptides, proteins, antibodies and oligonucleotides) to the deep lung for systemic diseases.

7. Conclusions

Pulmonary delivery of various drugs by aerosolisation has been used for centuries to treat respiratory tract diseases and the pressurised metered-dose inhaler was the only delivery device of choice. Currently, aerosol therapy is expanding with the advancement of science and technology specially in developing dry powder inhaler formulations to target the systemic circulation for the delivery of proteins and peptides, gene therapy, and influenza and measles vaccines. Moreover, advances in all of these areas have led to pulmonary delivery of medicaments being a route of choice for many drugs, not only for respiratory diseases but also for systemic delivery of drugs for other disorders. Now a days, mental health disorders and cardiac diseases are increasing with the changing world; however, pulmonary delivery of neuroactive and cardio active drugs has not been explored. Most drugs for treating AD or PD are in oral dosages forms (tablets, capsules and solutions); forms where drug absorption is very slow and only 70-90% is bioavailable. The longer range future of DPIs does include non-invasive and efficient delivery of large molecules for systemic conditions with improved patient compliance. Therefore, pulmonary drug delivery of drugs would extend the new era of drug delivery research, which will eventually extend the life of drugs (solid state stability), increase patient compliance, and reduce the total cost not only for brain neurodegenerative disorders but also for other chronic human diseases.

Acknowledgement

We gratefully acknowledge the contribution made by Gail Neilson in proofreading this review article.

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(Received November 4, 2008; Accepted November 6, 2008)
Effect of drug-polymer binary mixtures on the in-vitro release of ibuprofen from transdermal drug-in-adhesive layers

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ABSTRACT: We report on the formation of eutectic mixtures of ibuprofen using two different polymers together with investigations on the in-vitro release of ibuprofen from drug-in-adhesive layers. Ibuprofen, literature melting point (m.p.) = 73.5-76.5°C, was tested together with Pluronic F127, literature m.p. = 54.4-60.5°C, and polyethylene glycol 1000 (PEG 1000), literature m.p. = 37-40.9°C, as second components in binary mixtures, incorporated into an acrylic adhesive, either as solid physical mixtures (PM) or molten mixtures (MM). Studies of how the type of mixture preparation (PM versus MM) and the ratio of components in binary mixtures affecting the in-vitro drug release of ibuprofen, compared with ibuprofen-adhesive layers without polymer addition were conducted. Ibuprofen release did not improve using the eutectic composition with Pluronic F127, possibly due to increased ibuprofen solubilisation in the adhesive and a subsequent decrease in the thermodynamic activity of the formulation. A significant increase in ibuprofen release (P < 0.05) was shown for compositions adjacent to the eutectic one, with ibuprofen: Pluronic F127 (40:60) and ibuprofen: PEG 1000 (20:80, 25:75, 30:70), from both PM- and MM-adhesive formulations, compared to the ibuprofen-adhesive formulations.

Keywords: Transdermal patches, Drug-in-adhesive, Ibuprofen, Eutectic mixture, Thermodynamic activity

1. Introduction

The advantages of transdermal drug delivery include avoidance of the gastrointestinal tract, sustained drug release and increased patient compliance.

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eutectic composition may confer stability to the drug against crystallisation.

In our study we used ibuprofen as the model drug and two hydrophilic polymers with low melting points, PEG 1000 and Pluronic F127 that would enable a considerable suppression of the melting point of the drug. Ibuprofen is a non steroidal anti-inflammatory drug (NSAID) with analgesic and anti-inflammatory properties. The main side effect of the oral administration of NSAIDs is irritation of the gastrointestinal wall lining, which can lead to the development of ulcers following long-term administration. For this reason ibuprofen and other NSAIDs have been studied extensively as candidates for systemic delivery via the transdermal route (7). PEGs are non-irritant nor toxic to healthy skin and do not readily penetrate it (8). Poloxamers have previously been used as a vehicle for the topical delivery of NSAIDs due to their low toxicity and irritation (9).

Binary eutectic mixtures of ibuprofen with Pluronic F127 and PEG 1000 were formulated into drug-in-adhesive layers containing binary drug-polymer mixtures at several ratios, including the eutectic ratio. Hot stage microscopy (HSM) was used to study the melting properties of the solid dispersions and identify the eutectic composition. The HSM technique has been shown to be more efficient than differential scanning calorimetry in detecting the presence of drug crystals in solid dispersions and differences in the melting behaviour among samples, especially when a polymer with a low melting point is used as a drug carrier (10,11). The binary mixtures were prepared and incorporated in the adhesive layer according to two different methods: either as physical mixtures (PM) or as molten mixtures (MM) of the two components that would solidify after incorporation into the adhesive layer. The aim of our work was to study how the method of mixture preparation, as a PM or MM, and the ratio of components in the binary mixtures influence the in-vitro drug release of ibuprofen, compared with ibuprofen-adhesive layers without additive.

2. Materials and Methods

2.1. Materials

Ibuprofen was obtained from Knoll Pharmaceuticals (Nottingham, UK). DURO-TAK® 87-4287 was a gift from National Adhesives-Henkel (Slough, UK). Polyethylene glycol with an average molecular weight of 1000 Da (PEG 1000) was supplied by Sigma (St. Louis, USA). Pluronic F127 was supplied as Lutrol F127 and Pluronic F127 and was a gift from BASF AG (Ludwigshafen, Germany). The Scotchpak 9742 release liner was a gift from 3M Corporation (St. Paul, USA).

2.2. Preparation of solid dispersions

Solid dispersions of ibuprofen with either Pluronic F127 or PEG 1000 were prepared in ratios ranging from 90%: 10% to 10%:90% (w/w) according to the fusion method. Appropriate amounts of ibuprofen and polymer to give a 2 g mixture were accurately weighed in test tubes and were placed in a water bath (Techne Inc., Princeton, USA) with a VMR 1122S temperature control. The initial temperature of the water bath was 30°C, gradually increased to 75°C, at a rate of 3°C/min, while the drug-polymer mixtures were gently stirred with a glass rod. The molten mixtures were then allowed to solidify at 20°C for a week.

2.3. Hot stage microscopy

The melting temperature of the solid dispersions, ibuprofen, Pluronic F127 and PEG 1000 were recorded using a Vickers microscope attached to a Mettler FP5 hot stage temperature control and recorder. The temperature range was set from 25 to 80°C with a heating rate of 2°C/min. Each solid dispersion was tested in triplicate (n = 3). Two temperatures were recorded per sample, the first being the initial temperature when melting began (lower limit) and the second being the temperature that melting was complete (upper limit). Phase diagrams were then plotted and the eutectic compositions were identified.

2.4. Preparation of the adhesive layers

The following binary mixtures of ibuprofen:polymer were prepared as physical mixtures and solid dispersions: 60:40, 40:60, 30:70, 25:75, 20:80 with PEG 1000. A binary mixture that would contain 0.05 g of ibuprofen was accurately weighed and added to the required amount of liquid acrylic adhesive to produce dried circular adhesive layers with an ibuprofen concentration of 10% (w/w). Ibuprofen-adhesive layers without polymer were also prepared by mixing 0.05 g of either solid or molten ibuprofen with the acrylic adhesive to produce layers of 10% (w/w) ibuprofen concentration. All layers had a mean surface area of 4.5 ± 0.35 cm², with one side attached to a release liner. The layers were stored for a week at 20°C before dissolution testing.

2.5. In-vitro drug release studies

In-vitro drug release studies were conducted according to the B.P. Dissolution method for transdermal patches. The release of ibuprofen from each set of layers (n = 3) was tested for 5 h using a paddle dissolution apparatus (Copley instruments Ltd, Nottingham, UK) with 900 mL of citrophosphate buffer (pH 5.6) as the dissolution medium under sink conditions. The temperature of the
and leaked out of the borders of the adhesive layers it did not solidify on cooling but remained as liquid incorporated into the adhesive as a molten mixture, composition of ibuprofen with PEG 1000 (15:85) was dispersed in the adhesive layer. When the eutectic with the PM method contained undissolved polymer with no drug crystals observed. The layers prepared with the MM method were transparent in appearance binary mixtures on drug release. All layers prepared crystallization and, thus, observe only the effect of order to avoid suppression of drug release by drug solubility of ibuprofen in the adhesive polymer, in w/w) was selected to be lower than the saturation found at the ibuprofen:Pluronic F127 ratio of 30:70, composition of ibuprofen with Pluronic F127 was found at the ibuprofen:PEG 1000 ratio of 15:85, with a melting point of 30.9°C (Figure 1). This temperature was found at ibuprofen:PEG 1000 solid dispersions. Lower limit points show the mean temperature (n = 3) reading where melting of the sample was complete. Error bars represent the standard deviation from each mean temperature reading.

2.6. Statistical analysis

The % cumulative amount of ibuprofen released (n = 3) at 5 h was plotted against time. Statistical differences were determined using a Student t-test (two independent samples) with significance at P < 0.05.

3. Results and Discussion

The eutectic composition of ibuprofen with PEG 1000 was found at ibuprofen:PEG 1000 ratio of 15:85, with a melting point of 30.9°C (Figure 1). This temperature is lower than normal skin temperature (32°C), implying that ibuprofen will be in liquid form when applied onto the skin. Theoretically this would favour drug permeation into the stratum corneum (4). The eutectic composition of ibuprofen with Pluronic F127 was found at the ibuprofen:Pluronic F127 ratio of 30:70, with a melting point of 46°C (Figure 2).

The drug concentration in the adhesive layers (10%, w/w) was selected to be lower than the saturation solubility of ibuprofen in the adhesive polymer, in order to avoid suppression of drug release by drug crystallization and, thus, observe only the effect of binary mixtures on drug release. All layers prepared with the MM method were transparent in appearance with no drug crystals observed. The layers prepared with the PM method contained undissolved polymer dispersed in the adhesive layer. When the eutectic composition of ibuprofen with PEG 1000 (15:85) was incorporated into the adhesive as a molten mixture, it did not solidify on cooling but remained as liquid and leaked out of the borders of the adhesive layers on storage. For this reason it was not possible to carry out dissolution studies with the ibuprofen-PEG 1000 eutectic composition.

% Cumulative ibuprofen release at 5 h was statistically higher (P < 0.05) for the ibuprofen:PEG 1000 ratios 30:70, 25:75 and 20:80 compared to the ibuprofen monolayer 100:0 and the 60:40 ratio, for both PM and MM (Figures 3 and 4). Similarly, the ibuprofen: Pluronic F127 composition with the significantly higher ibuprofen release (P < 0.05) for both MM and PM was the 40:60 ratio, which is adjacent to the eutectic composition (Figures 5 and 6).

The eutectic composition of ibuprofen with Pluronic F127 (30:70) showed lower drug release compared to the formulation containing ibuprofen alone (Figures 5 and 6). This could be attributed to the fact that the eutectic mixture increases the solubility of ibuprofen in the adhesive layer and so simultaneously decreases the thermodynamic activity of the formulation. This is in agreement with previous observations demonstrating that a decrease in the melting point of a compound via formation of a binary eutectic system can be used as an approach for increasing the drug solubility in the vehicle (5).

The agreement between MMs and PMs on the order of enhancing drug release using either PEG 1000 or Pluronic F127 is noteworthy indicating an interaction taking place. An interaction between the components of binary physical mixtures during mixing has been previously reported (12). In our case, the incorporation of the binary physical mixtures in the adhesive solution may have resulted in ibuprofen-polymer solid dispersion formation during drying of the monolayer.

In conclusion, our results showed that ibuprofen release was enhanced by binary mixtures adjacent to the eutectic composition that contain a higher proportion of ibuprofen than the eutectic. This enhanced ibuprofen release could be observed up to a certain ratio, after which any further increase in the amount of ibuprofen in the binary mixture showed no significant difference (P > 0.05) on drug release compared to the drug-adhesive alone. Our
Figure 3. % Ibuprofen released from the adhesive layers containing MM of ibuprofen with PEG 1000. Error bars represent the standard deviation from the mean ($n = 3$).

Figure 4. Ibuprofen released from the adhesive layers containing PM of ibuprofen with PEG 1000. Error bars represent the standard deviation from the mean ($n = 3$).

Figure 5. % Ibuprofen released from the adhesive layers containing MM of ibuprofen with Pluronic F127. Error bars represent the standard deviation from the mean ($n = 3$).

Figure 6. % Ibuprofen released from the adhesive layers containing PM of ibuprofen with Pluronic F127. Error bars represent the standard deviation from the mean ($n = 3$).
results also support the hypothesis that incorporation of an additional component as a eutectic mixture with the drug in the adhesive monolayer will increase the solubility of the drug in the adhesive, with a subsequent decrease in thermodynamic activity for the given ibuprofen concentration in the monolayer. This may indicate that using the eutectic composition, higher ibuprofen concentrations can be accommodated in the transdermal monolayer without compromising the stability of the formulation, considering the inherent stability of eutectic mixtures against crystallization.

Acknowledgements

The authors would like to thank National Adhesives-Henkel, 3M and BASF for their kind provision of materials.

References


(Received October 28, 2008; Accepted October 31, 2008)
Effect of benzyl-N-acetyl-α-galactosaminide on KL-6 mucin expression and invasive properties of a human pancreatic carcinoma cell line

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ABSTRACT: KL-6 mucin is a type of MUC1 mucin and its aberrant expression has been shown to be associated with aggressive metastasis and poor clinical outcome in tumors. The present study is to investigate the effects of benzyl-N-acetyl-α-galactosaminide (GalNAc-O-bn), an O-glycosylation inhibitor, on KL-6 mucin expression and invasive properties of a human pancreatic carcinoma cell line, Suit-2 cells. Expression profiles of KL-6 mucin in the cells pretreated with or without 5 mM GalNAc-O-bn for 48 h were examined by Western blotting and immunocytochemical staining and invasive properties were examined by transwell chamber assay. Western blotting and immunocytochemical staining showed that the expression profiles of KL-6 mucin changed significantly after GalNAc-O-bn treatment. Meanwhile, the invasive ability of Suit-2 cells decreased significantly after GalNAc-O-bn treatment (p < 0.05). These results suggest that glycosylation of KL-6 mucin may be closely related to aggressive behaviors of pancreatic cancer cells like metastasis and invasion.

Keywords: KL-6 mucin, O-glycosylation, Benzyl-N-acetyl-α-galactosaminide, Invasion, Pancreatic carcinoma

1. Introduction

Patients with pancreatic cancer still have a poor prognosis, a 5-year survival rate of ~3% and a median survival of < 6 months (1), although recent efforts have improved cancer prevention, screening, and therapy. The major problem in the management of postsurgical cases is failure to control cancer metastases, which results from a lack of early detection and effective treatment.

MUC1 is a polymorphic, highly glycosylated, type I transmembrane glycoprotein expressed by ductal epithelial cells of secretory organs, including the pancreas, breast, lung, and gastrointestinal tract, that is overexpressed and aberrantly glycosylated in most cases of adenocarcinoma (2). The deduced amino acid sequence of MUC1 mucin reveals four distinct domains: an NH2-terminal domain consisting of a highly O-glycosylated tandem-repeat domain, a transmembrane domain, and a cytoplasmic domain (3). A number of studies have suggested that overexpression of MUC1 plays an important role in pancreatic cancer metastasis and that MUC1 seems to be an attractive target for treatment of pancreatic cancer (4,5). Therefore, having MUC1 target monoclonal antibody (mAb) with high specificity and affinity may represent an effective strategy.

KL-6 mucin, a type of MUC1 categorized as cluster 9, is recognized by KL-6 mAb, and its epitope includes sialo-oligosaccharide moiety in MUC1 molecules (6,7). This mucin was first established in the serum of patients with intestinal pneumonia but has recently been detected in various cancer tissues (8,9). Previous immunohistochemical studies by the current authors have shown that overexpression of KL-6 mucin was associated with worse tumor behaviors such as invasion and metastasis in ampullary carcinoma, primary
colostral carcinoma, and metastatic liver cancer tissues (10-12). In addition, the relationship between expression of KL-6 mucin and metastatic potential has also been reported in colorectal carcinoma cell lines (13). However, the role of glycosylation in KL-6 mucin in tumor behaviors remains to be elucidated. Reports have indicated that extension of O-glycosylation in cultured cells can be blocked in the presence of benzyl-N-acetyl-α-galactosaminide (GalNAc-O-bn) (14-16). The present study aimed to evaluate the effect of O-glycosylation inhibitor GalNAc-O-bn on the expression profiles of KL-6 mucin and the invasive properties of the human pancreatic carcinoma Suit-2 cell line.

2. Materials and Methods

2.1. Reagents

GalNAc-O-bn, trypsin, cell lysis regents, trypsin blue, and culture media were purchased from Sigma-Aldrich Japan, Tokyo, Japan. KL-6 mAb was provided by Eisai Co., Ltd, Tokyo, Japan. The Histofine SAB-PO kit and BD BioCoat™ Tumor Invasion System were from Nichirei Corporation, Tokyo, Japan and BD Biosciences, San Jose, CA, USA, respectively.

2.2. Cells and culture conditions

Human pancreatic carcinoma Suit-2 cell line was obtained from JCRB Cell Bank, Tokyo, Japan. The cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere with 5% CO2 in air at 37°C. For maintenance and subculture, cells in the exponential phase were treated with 0.25% trypsin solution containing 0.02% EDTA. Cells reaching 80% of confluence were collected and subjected to the following experiment.

2.3. Cell proliferation assay

Cells were reseeded in 96-well plates (3 × 10^4 cells per well) incubated with 0, 1, 2, 4, 8, and 16 mM of GalNAc-O-bn for 48 h. Cell proliferation was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described elsewhere (17). Absorbance was detected at 550 nm with 750 nm as a reference wavelength.

2.4. Western blotting

Cells treated with or without 5 mM of GalNAc-O-bn for 48 h at 37°C were lyzed in lysis buffer (50 mM Tris-HCl, pH 7.4, containing 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM NaF, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin) with occasional sonication. After centrifuging at 12,000 × g for 30 min at 4°C, cell extracts (10 μg protein each) were subjected to SDS-PAGE (8% gel) and then electrotransferred onto polyvinylidene difluoride membranes. After blocking with 20 mM Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween-20 overnight at 4°C, the membranes were incubated with KL-6 mAb (1:750 dilution) for 1 h at room temperature. After they were washed three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Reactivity was visualized by enhanced chemiluminescence using the ECL Western Blotting Starter Kit (RPN2108; GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Data were obtained from three independent experiments.

2.5. Immunocytochemistry

Cells (1 × 10^6) treated with or without GalNAc-O-bn were seeded on MAS-coated slides (Matsunami, Inc., Osaka, Japan) and incubated for 4 h. Cells were then fixed with 3.7% paraformaldehyde for 30 min followed by treatment with 0.2% Triton X-100 for 10 min. Next, immunohistochemical staining by KL-6 mAb was done as described before (10). Briefly, cells were blocked with normal goat serum for 30 min at room temperature, and then incubated with or without KL-6 antibody (1:200 dilution) for 60 min at room temperature. After the incubation of biotin-labeled secondary antibody, detection of KL-6 mucin was achieved by the biotin-streptavidin-peroxidase complex method using Histofine SAB-PO kit. 3,3’-Diaminobenzidine was used as the chromogen, and haematoxylin was used as a counterstain.

2.6. Transwell chamber assay

Motility and the invasive abilities of cells were assessed in 24-well transwell plates (Corning, NY, USA). Cells were pre-incubated with or without 5 mM of GalNAc-O-bn for 48 h at 37°C in a CO2 incubator and then detached and resuspended in serum-free RPMI-1640. A suspension of cells (2 × 10^5 cells/mL) was placed on matrigel-coated filters and control filters, respectively. The lower chambers were filled with 0.75 mL of RPMI-1640 medium supplemented with 5% FBS. Cells were allowed to migrate for 22 h at 37°C. Cells that invaded the matrigel and reached the opposite surface of the filter were stained with a Diff-Quik kit (Dade Behring, Newark, DE, USA) in accordance with the manufacturer's instructions. The invaded cells were quantified by counting the number of cells in eight random microscopic fields per filter at a magnification of ×100. Each data point was calculated from two separate experiments performed.
in triplicate. A p value less than 0.05 was considered statistically significant.

3. Results and Discussion

Control of metastatic pancreatic cancer remains a major objective in pancreatic cancer treatment. The overexpression of MUC1 mucin plays an important role in the process of pancreatic cancer metastasis and invasion. MUC1 targeting mAb with high specificity and affinity might represent a useful targeted therapy. KL-6 mucin is a type of MUC1 recognized by KL-6 mAb, and its epitope includes sialo-oligosaccharide moiety in MUC1 molecules (6-9). Many studies have shown that overexpression of KL-6 mucin may be associated with worse tumor behaviors such as invasion and metastasis in many kinds of cancers (6,10-12).

Since MUC1 is a highly O-glycosylated cell surface glycoprotein, GalNAc-O-bn, an O-glycosylation inhibitor, was used to inhibit O-linked oligosaccharide of KL-6 mucin. GalNAc-O-bn is a synthetic analogue of N-acetylgalactosamine and inhibits elongation of O-glycans. The inhibition is competitive and instead of monosaccharide transfer in which GalNAc is bound to serine or threonine, the elongation of the O-glycan chain occurs in benzyl-N-acetyl-α-galactosaminide molecules (18,19).

First, the inhibitory effects of GalNAc-O-bn on Suit-2 cell proliferation were examined using MTT assay. Inhibition of Suit-2 cell proliferation was not observed in the presence of up to 16 mM GalNAc-O-bn (data not shown). Therefore, a concentration of 5 mM was used in the following experiments as reported previously (18). Cell viability, as judged by trypan blue exclusion, was also unaffected in the presence of 5 mM GalNAc-O-bn (data not shown).

Using the KL-6 antibody, KL-6 mucin expression was next detected in Suit-2 cells with or without GalNAc-O-bn treatment by means of Western blotting. As shown in Figure 1A, KL-6 mucin was detected with variations in molecular weight. Notably, the intensity of the major band around 209 kDa was significantly lower after treatment with 5 mM GalNAc-O-bn. In contrast, the intensity of other two bands with a molecular weight of around 110 and 178 kDa, respectively, seemed to be higher in GalNAc-O-bn treated cells than in the untreated cells. To examine the subcellular expression of KL-6 mucin, Suit-2 cells with or without GalNAc-O-bn treatment were subjected to immunocytochemical staining. Before GalNAc-O-bn treatment, strong KL-6 mucin stains were observed in Suit-2 cells in the entire cell, including its membrane and cytoplasm (Figure 1B). After GalNAc-O-bn treatment, KL-6 mucin stains decreased significantly especially in the cell membrane. Cell morphology was also found to have changed after GalNAc-O-bn treatment (Figure 1B).

One of the earliest steps in metastasis is the invasion of the basement membrane. Next evaluated was the effect of GalNAc-O-bn on the motility and invasive ability of Suit-2 cells migrating through a matrigel-coated polycarbonate membrane. As shown in Figure 2, the invasive potential of Suit-2 cells decreased significantly after 5 mM GalNAc-O-bn treatment. The percentage of cells penetrating the matrigel-coated polycarbonate filters without or with GalNAc-O-bn treatment was 93.5% and 10.3%, respectively (p < 0.05).

The present study suggests that GalNAc-O-bn, an O-glycosylation inhibitor, may significantly alter the expression profiles of KL-6 mucin, especially on the cell surface, and it may alter the invasive ability of Suit-2 cells. Since KL-6 mAb recognizes sialo-oligosaccharides in addition to part of the core polypeptide of KL-6 mucin (8), the decrease in KL-6 mucin expression as a result of treatment with GalNAc-O-bn may be due to the insufficient elongation of oligosaccharide chains. Although the present study did...
not examine the changes in levels of expression of the KL-6 mucin core polypeptide for GalNAc-O-bn-treated and untreated cells, the current findings suggest that an extracellular domain containing sialo-oligosaccharide chains of KL-6 mucin may play a role in the invasive ability of the cells.

GalNAc-O-bn has been reported to inhibit O-glycosylation in other cell surface glycoprotein such as brush border glycoprotein sucrose-isomaltase (20) and to cause morphological changes with an accumulation of GalNAc terminal glycoproteins that may be mucin precursors at the cell surface (21). Further study is needed to clarify the detailed mechanism by which GalNAc-O-bn acts on KL-6 mucin expression and the underlying role of KL-6 mucin in the metastatic progression of the pancreatic carcinoma. These experiments may provide in vitro evidence for the O-glycosylation of KL-6 mucin playing a role in the invasion of cancer cells and portend that therapeutic strategies targeting oligosaccharide moieties of KL-6 mucin should be useful in the treatment of aggressive pancreatic cancer.

Acknowledgments

This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, and JSPS and CAMS under the Japan-China Medical Exchange Program.

References


(Received November 9, 2008; Accepted November 21, 2008)
Brief Report

Antioxidant activity of wild plants collected in Beni-Sueif governorate, Upper Egypt

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ABSTRACT: Antioxidant activity of a selection of commonly occurring wild plants growing in Beni-Sueif governorate, Upper Egypt, has been tested. The plants selected are Tamarix nilotica, Ambrosia maritima, Zygophyllum coccenium, Conyza dioscoridis, Chenopodium ambrosioides, and Calotropis procera. The in vitro antioxidant assays used in this study were 1,1-diphenyl-2-pieryl hydrazyl (DPPH) radical scavenging activity, superoxide anion scavenging activity and iron chelating activity. Extracts prepared from the leaves and flowers of Tamarix nilotica have shown the highest antioxidant activity in the three kinds of assay.

Keywords: Screening, Wild plant, DPPH, Superoxide anion, Iron chelation, Tamarix nilotica

1. Introduction

Plants are a valuable source of natural products. These plant metabolites can be new sources of such economic materials as oils, gums or tannins, new therapeutic agents and precursors of synthesis of complex chemical substances. Of the several hundred thousand plant species present on earth, only small proportion has been studied both chemically and biologically. The combination of both chemical and biological screening will provide important information about plant natural products (1).

Beni-Sueif governorate occupies a land area of approximately 10,954 km² in north part of Upper Egypt, with a total inhabitancy of 1,369.41 km². It boasts a population of over 2,315,512. Three phytogeographical regions can be distinguished in Beni-Sueif; the desert on the western side of the Nile that is considered as extension of the African Sahara region, the desert of the eastern side of the Nile that extends with the official border of the governorate to Red sea and the fertile land on both sides of the Nile including canal banks distributed throughout the governorate. In our continued efforts for chemical and biological screening of wild plants growing in Beni-Sueif (2), antioxidant activity of extracts prepared from commonly occurring wild plants was tested.

2. Materials and Methods

2.1. Plant material

The plant materials used in this study consisted of mature leaves, flowers and latex of different plants. The plants have been collected from the wild in Gerza village area, Beni-Sueif, Egypt, during flowering stage of each plant (2007). Voucher specimens were deposited in the Herbarium of Faculty of Pharmacy, Beni-Sueif University. The plant materials were rinsed with tap water and air dried in shade. Aqueous methanol extract (80%) of different plants was prepared by extracting the plant material twice. The extracts were stored in refrigerator at 4°C till use.

2.2. DPPH radical scavenging activity

The free radical scavenging activity (hydrogen donation) of plant extracts and n-propyl gallate was measured using 1,1-diphenyl-2-pieryl-hydrazyl (DPPH) radical. A solution of DPPH in methanol (0.08 mM) was prepared. Then, 1 mL of this solution was added to 0.3 mL of extracts or n-propyl gallate solutions at concentration of 500 μg/mL. The mixture was then shaken vigorously and allowed to stand at room temperature for 30 min, with the absorbance measured at 517 nm in a spectrophotometer against blank samples.

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2.3. Superoxide anion scavenging activity

Superoxide radicals were generated in phenazine methosulphate (PMS)-nicotinamide adenine dinucleotide (NADH) systems by NADH oxidation and assayed by nitroblue tetrazolium (NBT) reduction. In this experiment, the superoxide radicals were generated in 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 0.5 mL of NBT (300 μM) solution, 0.5 mL NADH (936 μM) solution, and 0.5 mL of plant extracts or n-propyl gallate solution at concentration of 500 μg/mL. The reaction was started by adding 0.02 mL FeCl₂ (2 mM). The reaction mixture was shaken vigorously and left standing at room temperature for 10 min. After equilibrium had been reached, absorbance of the solution was measured spectrophotometrically at 560 nm.

3. Results and Discussion

Free radicals are involved in a number of pathological conditions such as inflammatory diseases, atherosclerosis, cerebral ischemia, AIDS, and cancer (3). The free radicals are induced in the human body due to environmental pollutants, chemicals, physical stress, radiations, etc. Catalase and hydroperoxidase enzymes are among the most important antioxidants produced by the immune system. Consumption of antioxidants or free radical scavengers is necessary to compensate depletion of antioxidants of the immune system.

There is an increasing interest in the use of medicinal plants as antioxidants. Silymarin and wheat germ oil are well-known plant extracts used as antioxidants in the pharmaceutical market. In the present study, plant extracts prepared from commonly occurring wild plants in Beni-Suef governorate, have been screened for their in vitro antioxidant activity. The reported medicinal properties of these plants are described in Table 1.

The antioxidant activity of aqueous methanol (80%) extracts tested is listed in Table 2. In the DPPH radical scavenging assay, only extracts of leaves and flowers of Tamarix nilotica showed significant activity. In superoxide anion scavenging activity the same extracts and that of Conyza dioscoridis have shown significant activity. In iron chelating activity extracts of leaves and flowers of T. nilotica and that of Calotropis procera leaves have shown the highest activity. Extracts prepared from T. nilotica flowers have shown the highest antioxidant activities in the three used assays. Extracts of T. nilotica have been used in traditional Egyptian medicine as antisepic agents. The polyphenolic and flavonoids of T. nilotica have been previously investigated (4-7). The methanol extract of

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**Table 1. Plants tested for their antioxidant activity in this study**

<table>
<thead>
<tr>
<th>Plant name (Plant part)</th>
<th>Family</th>
<th>Parts used</th>
<th>Reported medicinal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamarix nilotica</td>
<td>Tamaraceae</td>
<td>Leaves and flowers</td>
<td>Antiseptic in traditional Egyptian medicine (4)</td>
</tr>
<tr>
<td>Ambrosia maritima</td>
<td>Asteraceae</td>
<td>Leaves</td>
<td>Hepatoprotective (8), Molluscicidal (9)</td>
</tr>
<tr>
<td>Zygophyllum coccenium</td>
<td>Zygophyllaceae</td>
<td>Leaves</td>
<td>Anidiarrheal (10), Antidiabetic (11)</td>
</tr>
<tr>
<td>Conyza dioscoridis</td>
<td>Asteraceae</td>
<td>Leaves and flowers</td>
<td>Trypanocidal (12), Antileishmania (13)</td>
</tr>
<tr>
<td>Chenopodium ambrosioides</td>
<td>Chenopodiaceae</td>
<td>Leaves and flowers</td>
<td>Protection against acetaminophen induced liver damage (14)</td>
</tr>
<tr>
<td>Calotropis procera</td>
<td>Asclepiadaceae</td>
<td>Leaves</td>
<td>Contractions of gastrointestinal smooth muscle (15)</td>
</tr>
</tbody>
</table>

**Table 2. Antioxidant activity of aqueous methanol (80%) extracts of different plants**

<table>
<thead>
<tr>
<th>Plant name (Plant part)</th>
<th>Type of assay</th>
<th>DPPH radical</th>
<th>Superoxide anion</th>
<th>Fe³⁺ chelation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamarix nilotica (leaves)</td>
<td>DPPH radical</td>
<td>73.13 ± 1.15</td>
<td>96.34 ± 0.83</td>
<td>79.30 ± 4.49</td>
</tr>
<tr>
<td>Tamarix nilotica (flowers)</td>
<td>DPPH radical</td>
<td>89.34 ± 0.82</td>
<td>92.82 ± 3.88</td>
<td>79.56 ± 2.94</td>
</tr>
<tr>
<td>Ambrosia maritima (leaves)</td>
<td>DPPH radical</td>
<td>21.87 ± 1.52</td>
<td>50.99 ± 3.17</td>
<td>60.24 ± 1.81</td>
</tr>
<tr>
<td>Zygophyllum coccenium (leaves)</td>
<td>DPPH radical</td>
<td>33.79 ± 2.19</td>
<td>22.43 ± 3.33</td>
<td>60.24 ± 1.81</td>
</tr>
<tr>
<td>Conyza dioscoridis (leaves and flowers)</td>
<td>DPPH radical</td>
<td>28.01 ± 0.69</td>
<td>93.98 ± 3.17</td>
<td>40.99 ± 3.19</td>
</tr>
<tr>
<td>Chenopodium ambrosioides (leaves and flowers)</td>
<td>DPPH radical</td>
<td>55.78 ± 1.10</td>
<td>73.73 ± 1.57</td>
<td>40.99 ± 3.19</td>
</tr>
<tr>
<td>Calotropis procera (leaves)</td>
<td>DPPH radical</td>
<td>23.07 ± 7.59</td>
<td>84.13 ± 1.05</td>
<td>40.99 ± 3.19</td>
</tr>
<tr>
<td>Calotropis procera (latex)</td>
<td>DPPH radical</td>
<td>93.1 ± 0.24</td>
<td>92.82 ± 3.88</td>
<td>40.99 ± 3.19</td>
</tr>
<tr>
<td>n-propyl gallate</td>
<td>DPPH radical</td>
<td>93.1 ± 0.24</td>
<td>92.82 ± 3.88</td>
<td>40.99 ± 3.19</td>
</tr>
</tbody>
</table>

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*a* Extract concentration used was 500 μg/mL. *b* Percent of radical scavenging activity. *c* Percent inhibition. *d* Insignificant results (< 20%).
**T. nilotica** have shown higher DPPH radical scavenging activity (51.5 ± 8.14%) than that of silymarin (40.4 ± 2.05%). Further work is underway to characterize the active principles acting as antioxidants in these promising plant extracts.

**Acknowledgments**

Sameh AbouZid gratefully acknowledges receiving a postdoctoral fellowship from the International Center for Chemical and Biological Sciences (ICCBS) and the academy of sciences for the developing world (TWAS). Thanks are due to Dr. Emad Elshereef, Faculty of Science, Beni-Sueif University, Egypt for his kind assistance and advice in collection and identification of the plants.

**References**


(Received November 15, 2008; Accepted November 23, 2008)
The neuroprotective effect of antidepressant drug via inhibition of TIEG2-MAO B mediated cell death

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ABSTRACT: Alcohol use disorders are common in the world. However, the development of novel drugs to prevent alcohol-induced brain damage is based upon an improved neurobiological understanding on the cellular changes that take place in the brain. We previously reported that ethanol exposure lowered cell proliferation and increased cell apoptosis in all cell types, but affects brain cell lines the most, while ethanol and the anti-depressant drug deprenyl, an monoamine oxidase B (MAO B) inhibitor, exposure in unison increases cell viability. Here we investigated the molecular mechanism of the neuroprotective effect of deprenyl (0.25 nM) on ethanol (75 mM)-induced harmful effect. Transforming growth factor-beta-inducible early gene 2 (TIEG2) is an activator for MAO B. MAO B levels increase has been shown to contribute to neuronal cell death. This study uses the neuronal cell line to address whether ethanol induced cell death is through the activation of TIEG2-MAO B apoptotic pathway, and whether deprenyl protects cells from the effects of alcohol through the inhibition of this pathway. We have found that ethanol exposure increases the levels of mRNA and protein/catalytic activity for both TIEG2 and MAO B, while ethanol and deprenyl exposure in unison reduce the expression of both TIEG2 and MAO B, however it increases cell viability. Additionally, TIEG2-overexpressed cells display more cellular death-induced by ethanol than control cells. In summary, this study demonstrates the role of TIEG2 in ethanol induced cell death. The inhibition of the TIEG2-MAO B pathway may be one of the mechanisms for the neuroprotective effect of deprenyl.

Keywords: Alcohol, Neuroprotection, Transforming growth factor-beta-inducible early gene 2, Monoamine oxidase B, Cell viability

1. Introduction

Alcohol use disorders are common around the world and also have a high correlation between alcohol use disorders and other psychiatric problems, such as major depression (1). Although short-term alcohol drinking causes euphoric and stress-relieving effects, numerous clinical and experimental studies have shown that alcohol use is a major risk factor for neurobehavioural diseases, inflammation disorders and enhanced susceptibility to bacterial infection (2-4). In particular, it affects the brain and alters its normal function (5). This includes altering the effects of neurotransmitters, suppressing nerve signals and causing cell death (6). In rodents, ethanol exposure during development significantly reduces the size of the brain as well as brain/body weight ratios (7). There are many adverse physical effects from long-term exposure to alcohol including the increased activity in the liver that causes cell death and chronic hepatic diseases (alcoholic fatty liver, alcoholic hepatitis and cirrhosis, etc) (7,8) and an increase in the number of apoptotic cells in various brain areas (9).

Monoamine oxidase (MAO) metabolizes biogenic and dietary amines in the central nervous system and peripheral tissues, including monoamine neurotransmitters serotonin, norepinephrine, dopamine, and phenylethylamine. MAO plays important roles in several psychiatric and neurological disorders (10). MAO exists in two forms, MAO A and MAO B. Their catalytic activity generates H2O2 and nitrogen species, which are toxic products and may cause oxidative damage to mtDNA and have potential implications for apoptosis, aging, and neurodegenerative processes.

Deprenyl (selegiline), an irreversible inhibitor of monoamine oxidase B (MAO B), was synthesized as an antidepressant and used to treat Parkinson's disease (11). Because MAO degrades serotonin and produces reactive oxygen that may cause cell death, an MAO inhibitor prevents cell apoptosis (12,13). Deprenyl or related compounds may be neuroprotective in general through the inhibition of "death" signal transduction-
mediated by MAO, induced by endogenous and environmental factors (11). Deprenyl in low concentrations that induce MAO B inhibition potently inhibits serum withdrawal induced apoptosis in tissue cultures of neuro-ectodermal origin (14). This report is consistent with our previous studies (15) that ethanol can induce apoptosis in neuronal cells, and deprenyl in a low concentration can protect cells from the harmful effects of ethanol.

Transforming growth factor-beta-inducible early gene 2 (TIEG2, also called KLF11--Kruppel-Like Factor 11) is a human transforming growth factor-beta-(TGF-β) inducible early gene. It is a recently identified human TGF-β-inducible zinc finger protein belonging to Sp1-like family of transcription factors. TIEG2 protein is a negative regulator of cell growth and induces apoptosis (16-18) by binding to GC-rich sequences (19) located in the promoter region of several genes including MAO B promoter. Ethanol has been shown to potentiate TGF-β1-mediated growth inhibition in the rat neuroblasticoma cells (20), and ethanol exposure increases TGF-β1 signal (21) that may increase TIEG2 protein level and lead to apoptotic death of cells (22). Our previous data have shown that TIEG2 activates MAO B gene expression (23). This study investigates the neuroprotective effect of antidepressant drug (deprenyl) on ethanol-induced apoptosis possibly mediated by TIEG2 and MAO B.

2. Materials and Methods

2.1. Cell lines, DNA plasmids and reagents

SH-SY5Y, a human neuroblastoma cell line, was purchased from The American Type Culture Collection (ATCC). SH-SY5Y was cultured in RPMI1640 supplemented with 10% fetal bovine serum and antibiotics. TIEG2-expression vector was a gift from Dr. Raul Urrutia, Mayo Clinic. TIEG2 coding sequence was cloned into pcDNA3.1 His A expression vector. MAO B inhibitor, selegiline (deprenyl), was purchased from Sigma-Aldrich USA. The antibodies used in this study were purchased from Santa Cruz Biotechnology, except that anti-TIEG2 antibody was from BD Transduction Laboratory.

2.2. TIEG2-stably transfected cell line

In generating the TIEG2-stable cell line, SH-SY5Y were plated at a density of 5 × 10^6 cells in a 10-cm dish. The next day the TIEG2 expression vector or pcDNA 3.1 was transfected into cells with a Superfect transfection reagent (Qiagen Inc). After 24 h, cells were treated with Geneticin (G418; 600 μg/mL). Resistant clones isolated into separate dishes after 6 days and cultured under continuous G418 selection (13).

2.3. Cell culture and treatments with ethanol and deprenyl

Before treatments, SH-SY5Y cells were seeded on 10-cm dishes or 6-well plates. After overnight culture in medium, the medium was replaced with new medium containing 75 mM of ethanol with or without 0.25 nM of deprenyl for three days. As ethanol is volatile, a closed chamber system was utilized to stabilize the ethanol concentration in the culture medium (24,25). With this system, ethanol concentrations are maintained at steady ethanol levels (more than ~90% of the original concentration) for 3 days in medium. Briefly, cell culture dishes or 6-well plate containing SH-SY5Y cells were placed on a rack inside a plastic container that could be tightly sealed. A separate sealed container was used for each ethanol concentration. The bottom of each container was a reservoir that was filled with 200 mL of an aqueous solution with the same ethanol concentration that was present in the culture medium. A nonethanol control had a bath of water only. The underlying principle of this method is that the alcohol in the bath evaporates into the air inside the sealed container establishing a stable vapor pressure so that there is no net loss of ethanol from the culture medium. Before sealing the containers, a small amount (60 cc) of CO₂ was injected into each container. The concentration of CO₂ in the chamber was routinely tested and was determined to be stable at 5%. The containers were sealed and maintained in an incubator at 37°C for up to 3 days as needed (25).

The ethanol concentration we used (75 mM for examining the effect of deprenyl) was within the standard range of in vitro study (26). When a heavy drinker's ethanol concentration in blood reaches ~50-100 mM, he probably shows slurred speech and unsteadiness (27). Therefore the ethanol concentration for this study is around the physiological effect of ethanol in alcoholics.

2.4. Real-time PCR (RT-PCR)

Total RNAs were extracted with Trizol from cultured cells. Reverse transcription was carried out with SuperScript first strand synthesis system for RT-PCR (Invitrogen Inc) following the manufacture's instruction. Specific primers for the human MAO B and TIEG2 were designed as follows:

MAO B Sense,
5'-GACCATGTGGGAGGCAGACTTAC-3'
Antisense,
5'-CGCCCACAAATTTCCTCTG-3'

TIEG2 Sense,
5'-CCTGTGGCGAGTACCCCTCTAC-3'
Antisense,
5'-AAAGCCCGCAATCTGGAGTCTGGA-3'

The mRNA quantitative analyses for each group were
performed by Real-Time PCR using a Bio-Rad iCycler system. The real-time PCR was performed with a SYBR supermix kit (Bio-Rad). The data were analyzed by the software from Bio-Rad as described previously (23).

2.5. MAO B catalytic activity assay

SH-SY5Y was grown to confluence, harvested, and washed with phosphate-buffered saline. One hundred micrograms of total proteins were incubated with 10 μM 14C-labeled PEA (Amersham Biosciences) in the assay buffer (50 mM sodium phosphate buffer, pH 7.4) at 37°C for 20 min and terminated by the addition of 100 μL of 6 N HCl. The reaction products were then extracted with ethyl acetate/toluene (1:1) and centrifuged for 7 min. The organic phase containing the reaction product was extracted, and its radioactivity was obtained by liquid scintillation spectroscopy (28).

2.6. Western blot

Cells were cultured in medium with ethanol (75 mM) for 3 days, washed by PBS (pH 7.4), and sonicated in 500 μL of RIPA lysis buffer (10 mM Tris-HCl, pH 7.4/160 mM NaCl/1% Triton/1% Na dextranolate/0.1% SDS/1 mM EDTA/1 mM EGTA) supplemented with protease inhibitors (Sigma). The samples were then freeze thawed and centrifuged for 2 min at 12,500 rpm. The supernatant was then kept and transferred to a new tube. Thirty micrograms (for TIEG2) of total proteins were separated by 10.5% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. After the transfer, membranes were blocked at room temperature for 2 h with 5% nonfat dry milk in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl). The membranes were then incubated with mouse anti-actin antibody (1:1,000) overnight at 4°C. After incubation with respective secondary antibody at room temperature for 2 h, the bands were visualized by horseradish peroxidase (HRP) reaction using SuperSignal West Pico Chemiluminescent Substrate (PIERCE).

2.7. MTT assay for proliferation rate/cell viability evaluation

Cell viability and proliferation was measured by tetrazolium salt (MTT) (13,15). The medium in excess of 2 mL (6-well plates) was removed and 40 μL of MTT dye (5 mg/mL) in sterile PBS was added to 360 μL of medium or PBS depending on cell confluence. Plates were incubated for 4 to 5 hours, during which time the mitochondria in living cells converted the soluble yellow dye (MTT) into an insoluble purple formazan crystal. Cells and dye were then solubilized by the addition of 800-1,000 μL of DMSO to the 6-well plates. Optical density of each well at 572 nm was determined using the NanoDrop Spectrophotometer.

2.8. Statistical analysis

All values are presented as means ± SD. A one-way ANOVA followed by a post hoc Bonferroni’s t-test was employed when three or more groups were to be compared. A paired t-test was performed for the statistical analysis of two groups. A P value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Ethanol increases the MAO B mRNA level and enzymatic activity, but deprenyl reverses the effect of ethanol

SH-SY5Y cells were treated with 75 mM ethanol for three days. Then the cellular mRNA and MAO B activity were determined by real time-PCR and traditional 14C method assay. The ethanol concentration we used (75 mM) was clinically relevant, because the ethanol at 50-100 mM reflects blood ethanol levels in chronic alcoholics (29,30). Therefore, the ethanol concentration in our study was within the levels that results in physiological effects observed in alcoholics.

The results showed that ethanol induced cellular MAO B mRNA level increased about 3.5 times more than the control; this increase could be reduced by 0.25 nM deprenyl treatment (Figure 1A, lanes 2 vs. 1). At the same time, we found MAO B activity in ethanol treated cells increased 1.8 fold more than control cells (Figure 1B, lanes 2 vs. 1). However, deprenyl significantly decreased the MAO B catalytic activity (Figure 1B, lanes 3 vs. 2).

3.2. Ethanol increases the MAO B mRNA and protein levels, but deprenyl reverses the effect of ethanol

To test the possibility that TIEG2 may take part in the ethanol-induced cell death, TIEG2 mRNA levels and protein levels were detected with RT-PCR and western blot. The results show that ethanol could induce TIEG2 mRNA expression 4-fold more than that of the control cells (Figure 2A, lanes 2 vs. 1). deprenyl plus ethanol could just increase 2.8-fold (Figure 2A, lanes 3 vs. 1). Similarly, TIEG2 protein expression level was increased around 3.5 times, however deprenyl could inhibit TIEG2 expression (Figure 2B, a and b, lanes 2 and 3 vs. 1).

3.3. TIEG2 enhances, but MAO B inhibitor (deprenyl) protects, cell death induced by ethanol

Ethanol has been found to increase the MAO B gene expression and catalytic activity in the human glioma 1242-MG cells (16). With our previous experiment, 75 mM ethanol treatment in conjunction with 0.25 nM deprenyl provided the most protection against apoptotic activity for brain cells SH-SY5Y and U-118 MG (31).
Here, we use pcDNA and TIEG2 stably transfected SH-SY5Y cell lines (Figure 3Aa) to investigate the role of TIEG2 in ethanol-induced cell death and the neuroprotective effect of deprenyl. We have previously shown that TIEG2 is a transcriptional activator for MAO B (23). As shown in Figure 3Ab, the MAO B catalytic activity is increased by ~2-fold in TIEG2-overexpressed cells as compared to that in pcDNA3.1-transfected cells (Figure 3Ab, lanes 2 vs. 1).

Next, cells were exposed to 75 mM ethanol in conjunction with 0.25 nM deprenyl for three days, and the cell viability (in survival rate) was observed in

Figure 1. Effects of ethanol on MAO B mRNA level and catalytic activity. SHSY5Y cells were treated with or without 75 mM ethanol or with ethanol plus 0.25 nM deprenyl for three days. (A) Cellular mRNA was extracted and quantitative real-time RT-PCR was performed. (B) MAO B enzyme activity was determined by enzymatic activity assay. Data represent the mean ± S.D. of three independent experiments. Controls were untreated cells (0 mM of ethanol) which were taken as 1. *P < 0.01 versus control cells and # < 0.05 versus cells treated with ethanol alone (one-way ANOVA followed by a post hoc Bonferroni’s t-test).

Figure 2. Effects of ethanol on TIEG2 mRNA and protein levels. SHSY5Y cells were treated with or without 75 mM ethanol or with ethanol plus 0.25 nM deprenyl for three days. (A) Cellular TIEG2 mRNA level was detected with quantitative real-time RT-PCR, and (B) TIEG2 protein was examined by western blot. (a) TIEG2 protein levels in cells treated with or without ethanol or with ethanol plus deprenyl were shown by western blot, and actin was used as the internal control. (b) The optical density analysis with western blot shows TIEG2 has about 3.5 fold expression in ethanol treated cells, whereas, deprenyl inhibited the TIEG2 expression. Data represent the mean ± S.D. of three independent experiments. *P < 0.01 versus control cells without ethanol treatment and # < 0.05 versus cells treated with ethanol (one-way ANOVA followed by a post hoc Bonferroni’s t-test).
SH-SY5Y cells compared to that in cells treated with 75 mM ethanol alone. The results show that TIEG2-overexpression could induce more cell death with the presence of 75 mM ethanol than that in the control group which was stably transfected with empty pcDNA3.1 vector (Figure 3B, lanes 7 vs. 5). However, deprenyl could protect cells from ethanol’s harmful effect (Figure 3B, lanes 6 vs. 5 and 8 vs. 7).

Previously, we have shown that 0.25 nM of deprenyl produced the best neuroprotective effect on SH-SY5Y cells. In order to examine whether 0.25 nM deprenyl is also the most appropriate dosage in this study using TIEG2-overexpressed cells, the different concentrations (0, 0.125, 0.25, 0.5, and 1 nM) of deprenyl were used to test the inhibitory effects on MAO B catalytic activity and cell death. As shown in Figure 3Ca, the ethanol treatment in conjunction with deprenyl for three days exhibited the inhibition on MAO B catalytic activity in a concentration dependent manner. Furthermore, MTT assay was performed (Figure 3Cb) to determine the effects of different concentrations (0, 0.125, 0.25, 0.5, and 1 nM) of deprenyl on cell viability in TIEG2-overexpressed cells. The result showed that the ethanol treatment in conjunction with 0.25 nM deprenyl for three days increased the cell survival rate by 175% as compared to that of control cells (Figure 3Cb, lanes 3 vs. 1), suggesting that 0.25 nM of deprenyl has the most protection against apoptotic activity.

4. Discussion

An understanding of the molecular mechanisms of cellular apoptosis toward excessive alcohol consumption is crucial for the development of new

Figure 3. The effect of TIEG2 on ethanol-induced cell death. (A) Compare the expression of TIEG2 and MAO B catalytic activity in TIEG2/pcDNA stably transfected SH-SY5Y cell lines. (a) Western Blot analysis of the expression of TIEG2 in TIEG2/pcDNA overexpressed cell lines. (b) MAO B catalytic activity is about 2 times higher in TIEG2 stably transfected cells than pcDNA stably transfected cells. Values are expressed as means ± S.D. of at least three independent experiments. * P < 0.02 (paired t-test). (B) Effects of ethanol, ethanol plus deprenyl and TIEG2 on cell survival rates. pcDNA3.1 stably transfected cells or TIEG2 stably transfected cells were treated with 75 mM ethanol with/without 0.25 nM deprenyl for three days. Then the cell viability was determined by MTT assay. (C) Effects of different dosage of deprenyl on MAO B catalytic activity and the protection of ethanol induced cell death in TIEG2-stably transfected SH-SY5Y cell line. Cells were treated with or without 75 mM ethanol in the conjunction with 0.125, 0.25, 0.5 or 1 nM of deprenyl as indicated in the figure for three days. Then (a) the MAO B catalytic activity or (b) cell viability (MTT assay) was determined. All data are presented as the mean ± S.D. of at least three independent experiments. Controls were untreated cells (0 mM of ethanol in B and 0 nM of deprenyl in C) which were taken as 100%. * P < 0.05 versus control cells and # < 0.05 versus cells-stably expressing TIEG (one-way ANOVA followed by a post hoc Bonferroni’s t-test).
treatments for alcoholism. In this study, we examine the TIEG2-MAO B role in the ethanol induced apoptosis, and the neuroprotective effect of deprenyl via inhibition of TIEG2-MAO B mediated cell death with SH-SY5Y cell line.

The MAO B gene is located on the Xp11.2-11.4 chromosome and consists of 15 exons with identical exon- intron organization (32), and its activity increases progressively in the brain throughout adult life (33,34). An aberrant increase of MAO B activity has been implicated in several psychiatric and neurodegenerative disorders (35,36). Thus one predicted mechanism for cell death is an abnormal increase in monoamine oxidase (37). Previously, the physiologically relevant concentration of ethanol has been found to increase the MAO gene expression and catalytic activity in the human glioma 1242-MG cells (31). The increased activity of MAO may thereby increase production of hydrogen peroxide (H2O2, a major source for oxidative stress) and cause apoptosis (38). The SH-SY5Y, a human neuroblastoma cell line, treated with 75 mM ethanol, could increase the expression of mRNA and protein, in particular, MAO B catalytic activity also increased. Previously, we showed that the level of Caspase 3, an apoptotic marker protein, was increased significantly by ethanol treatment, suggesting that ethanol-induced cell death is mediated at least partially by apoptotic pathway (15).

Transforming growth factor-beta-inducible early gene 2 (TIEG2) is an activator for MAO B through Sp1 overlapping sites (GC-rich sequence) located at the promoter region of MAO B. Sp1-like protein plays key roles in the regulation of MAO B gene expression (23). It has been reported that TIEG2 induces apoptosis in murine OLI-neu cells (39). With the ethanol treated SH-SY5Y system, we were able to show that the mRNA and protein levels for TIEG2 were increased significantly along with the increase in MAO B activity. Using TIEG2-overexpressed stable cell line, we further demonstrated that TIEG2 could increase the MAO B catalytic activity, and also enhance the cellular apoptosis triggered by ethanol, whereas, deprenyl, an MAO B inhibitor, could protect cell death induced by ethanol, because ethanol and deprenyl exposure in unison reduced the expression of both TIEG2 and MAO B.

Deprenyl is an irreversible inhibitor of MAO B which is an antidepressant drug, and is now also used in the treatment of Parkinson’s disease. Deprenyl in much lower concentrations needed to induce MAO B inhibition (less than ~1 nM) potently inhibits serum withdrawal (40) and nitric oxide (41) induced apoptosis. However, in high concentration, deprenyl induces apoptosis in cell cultures (42). Our findings suggest that 0.25 nM deprenyl and ethanol exposure in unison for three days is able to inhibit MAO B catalytic activity and produce the best neuroprotective effect comparing to other concentrations (0.125, 0.5, and 1 nM) of deprenyl. This may be due to the higher concentration (more than 0.25 nM) of deprenyl used to start to induce apoptosis in cell culture (14).

In summary, TIEG2-MAO B-mediated apoptotic pathway may contribute to ethanol induced neurotoxicity. The inhibition of this apoptotic signaling pathway may be one of the mechanisms for the neuroprotective effect of deprenyl.

Acknowledgements

This study was supported by Public Health Service Grants P20 RR17701 and a NARSAD Young Investigator Award. The contribution by Dr. Raul Urrutia for providing TIEG2-pcDNA3.1 expression vector is also acknowledged. Shakevia Johnson and Shawna Tazik were supported by the Neuroscience Summer Scholars Program, Department of Psychiatry, University of Mississippi Medical Center.

References


(Received August 29, 2008; Revised October 15, 2008; Accepted October 19, 2008)
Regulation of the nitric oxide synthesis pathway and cytokine balance contributes to the healing action of *Myristica malabarica* against indomethacin-induced gastric ulceration in mice

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ABSTRACT: The role of the arginine-metabolism in the healing action of the methanol extract of *Myristica malabarica* (rampatri) (RM) and omeprazole (Omez) against indomethacin-induced stomach ulceration in mouse was investigated. Indomethacin (18 mg/kg) was found to induce maximum stomach ulceration in Swiss albino mice on the 3rd day of its administration, which was associated with reduced arginase activity (38.5%, *p* < 0.05), eNOS expression, along with increased iNOS expression, total NOS activity (5.37 fold, *p* < 0.001), NO generation (55.1%, *p* < 0.01), and ratio of pro-/anti-inflammatory cytokines. Besides providing comparable healing as Omez (3 mg/kg × 3 d), RM (40 mg/kg × 3 d, p.o.) shifted the iNOS/NO axis to the arginase/polyamine axis as revealed from the increased arginase activity (59.5%, *p* < 0.01), eNOS expression, and reduced iNOS expression, total NOS activity (73%, *p* < 0.001), and NO level (49.8%, *p* < 0.01). These could be attributed to a favourable anti/pro inflammatory cytokines ratio, generated by RM. The healing by Omez was however, not significantly associated with those parameters.

Keywords: Arginase, Cytokine balance, Gastric ulcer healing, Indomethacin, NOS

1. Introduction

Gastric ulcer is a complex pluricausal disease and is known to develop due to loss of balance between aggressive and protective factors (1). The non-steroidal anti-inflammatory drugs (NSAIDs) are known to cause gastric ulceration and delay ulcer healing, which is generally explained in terms of cyclooxygenase (COX) inhibition, reduced prostaglandin (PG) synthesis, and the impaired PG-mediated angiogenesis. However, the complete mechanism underlying this effect is not completely understood. Various other factors, especially the nitrogen-metabolizing enzymes are also key contributors in host immune defence mechanisms and wound healing (2,3). In acute inflammatory responses, such as wound healing, heat stroke and glomerulonephritis, arginase has been implicated as an important regulator of diverse pathways including generation of polyamines and the cytostatic free radical molecule, nitric oxide (NO) (4). Arginine pathway plays a vital role in wound healing since L-arginine becomes an essential amino acid after wounding with almost undetectable levels in the wound milieu (5). Studies have shown that arginine itself has advantageous effects on cutaneous healing by enhancing cell proliferation and collagen synthesis as well as breaking strength (6). Further, nitric oxide (NO), produced from arginine also plays an important role in inflammatory processes, being a mediator of macrophage function (4,6,7). The temporal switch of arginine as a substrate for the inducible nitric oxide synthase (iNOS)/NO axis to arginase/polyamine axis is subject to regulation by inflammatory cytokines. However, little is known on the interplay of cytokines and the NO synthesis pathway during indomethacin-induced gastric ulceration. After trauma, the Th1/Th2 imbalance with Th2 predominance is reflected by a predominance of the arginase inducing cytokines such as IL-4, IL-10, and TGFβ (8).

Very recently, we have documented impressive healing activity of the fruit rinds of the plant, *Myristica malabarica* (Myristicaceae), popularly known as rampatri (9). It was found that oral administration of the methanol extract of rampatri (designated as RM) at a dose of 40 mg/kg for three days could effectively heal the indomethacin (18 mg/kg, p.o., single dose)-induced stomach ulceration in mice, reducing the
ulcer index from 3.0 to 0.8 (~74%). The healing activity of RM could be partly attributed to its ability to prevent oxidative damages to lipids, thiols and antioxidant enzymes, as well as augmenting mucin status. Subsequently, malabaricones B and C (the chemical structures shown in Figure 1) were found as its active constituents (10). However, the extract was more potent than the individual malabaricones at their respective concentrations in the extract (9). Hence for the present study, we have used RM to understand the mechanisms of its healing action in terms of its capacity to regulate the arginine metabolism by modulating the balance of cytokines in the process. To this end, we have investigated the effect of RM in elevating arginase activity, and regulating NO production through modulation of NOS expression. In addition, the important role of the pro- and anti-inflammatory, as well as regulatory cytokines vis-a-vis their putative role during wound healing was also investigated.

2. Materials and Methods

2.1. Chemicals and reagents

The dry fruit rinds of *M. malabarica* were purchased from the local market. L-arginine, indomethacin, isonitrosopropiophenone, Bradford reagent, Triton X-100, leupeptin, phenylmethylsulfonyl fluoride (PMSF), glycine, sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, Tween 20, ethylene diamine tetraacetic acid (EDTA), 3,3’,5,5’-tetramethyl benzidine (TMB), MnCl2, urea, omeprazole (Omez), Trizma base, cetyl trimethylammonium bromide (CTAB), and nitrocellulose membrane were procured from Sigma Chemicals (St. Louis, MO, USA). Other reagents used were disodium hydrogen phosphate and sodium dihydrogen phosphate (BDH, UK), sulphuric acid, hydrochloric acid, phosphoric acid, sodium chloride (Thomas Becker, Mumbai, India), horseradish peroxidase (HRPO, Sisco Research Laboratory, Mumbai, India), rabbit polyclonal iNOS and eNOS antibodies (Santacruz Biotechnology, Delaware, USA), peroxidase conjugated anti-rabbit IgG antibody, enhanced chemiluminescence detection kit (Roche, Mannheim, Germany), NOS and NO assay kits (Calbiochem, CA, USA), TGF-β1 set (Promega Corporation, Madison, USA) and cytokine ELISA kits (Pierce Biotechnology, Rockford, IL, USA).

2.2. Instrumentation

The absorbance spectrophotometry was carried out at 25°C using an ELISA reader (Biotech Instruments, USA). The bands obtained from western blots were quantified using the Gelquant software (DNR Bioimaging System, version 2.7.0, Israel).

2.3. Preparation of plant extract

The dry fruit rinds (20 g) of *M. malabarica* were powdered with a grinder and extracted successively with ether, methanol, and water (60 mL × 4 d with each solvent) at room temperature. The supernatants in each case were decanted. The entire process was repeated three times, each of the combined supernatants was filtered through a nylon mesh and evaporated at < 40°C in vacuo to obtain the respective extracts, which were stored in a vacuum desiccator. The methanol extract (designated as RM, 28.9%) was used for the present work.

2.4. Preparation of the drugs

The drugs were prepared from RM and Omez as aqueous suspensions in 2% gum acacia as the vehicle, and administered to the mice orally.

2.5. Protocol for ulceration and biochemical studies

The mice, bred at the BARC Laboratory Animal House Facility, Mumbai, India were procured after obtaining clearance from the BARC Animal Ethics Committee, and were handled following International Animal Ethics Committee Guidelines. Male Swiss albino mice (25-30 g) were reared on a balanced laboratory diet as per NIN, Hyderabad, India and given tap water ad libitum. They were kept at 20 ± 2°C, 65-70% humidity, and day/night cycle (12 h/12 h). To perform all the experiments in a blinded fashion, the animals were identified by typical notches in the ear and limbs, and randomized, before the experiments. The mice were divided into four groups (each containing five mice), and each experiment was repeated three times. Ulceration was induced in the groups II-IV mice by administering indomethacin (18 mg/kg, p.o.) dissolved in distilled water and suspended in the vehicle, gum acacia (2%) as a single dose. The animals were deprived of food but had free access to tap water, 24 h before ulcer induction. The mice of groups I and II, serving as normal and ulcerated controls respectively were given the daily oral dose of vehicle (gum acacia in distilled water, 0.2 mL) only. The groups III and IV mice were given RM (40 mg/kg × 3 d, p.o.) and Omez (3 mg/kg.

![Figure 1](https://www.ddtjournal.com)
× 3 d, p.o.) respectively, starting the first dose 6 h post indomethacin administration. After 6 h of the last dose of the drugs, the mice were sacrificed after an overdose of thiopental, the stomach was opened along the greater curvature, and the wet weights of the tissues were recorded. The glandular portion from five animals were pooled, rinsed with appropriate buffer, homogenized in the same buffer under cold condition and used for assessing the expression of different NOSs and assaying arginase and MPO activities. Other biochemical (total NOS and NO) and immunological parameters were assayed using the serum samples.

2.6. Assessment of ulcer healing

The ulcerated portions of the stomach were fixed in 10% formol saline solution for 24 h, embedded in a paraffin block, and cut into 5 μm sections, which were placed onto glass slides, and stained with haematoxyline and eosin for histological examination under a light microscope. One centimetre lengths of each histological section was divided into three fields. The histological damage score (DS) was assessed as described previously (9).

2.7. Determination of myeloperoxidase (MPO) activity

The MPO activity was determined following a reported method (11) with slight modifications. Gastric ulcer tissues were homogenized for 30 sec in a 50 mM phosphate buffer (pH 6.0) containing 0.5% CTAB and 10 mM EDTA, followed by freeze thawing three times. The homogenate was centrifuged at 12,000 × g for 20 min at 4°C. The supernatant was collected, and the protein content determined. The supernatant (50 μL) was added to 80 mM phosphate buffer, pH 5.4 (250 μL), 0.03 M TMB (150 μL) and 0.3 M H2O2 (50 μL). After incubating the mixture at 25°C for 25 min, the reaction was terminated by adding 0.5 M H2SO4 (2.5 mL). The absorbance of the mixture at 450 nm was recorded using HRPO as the standard. The MPO activity was expressed as μU/mg protein.

2.8. Arginase assay

Following a known method (12) with minor modifications, the assay was carried out using the tissue homogenate prepared in ice-cold 25 mM Tris-HCl buffer (pH 7.5) followed by centrifugation at 12,000 × g for 30 min at 4°C. The reaction mixture (200 μL) containing 0.5 M L-arginine (pH 9.7), 1 mM MnCl2, and the tissue extract (100 μL) was incubated for 20 min at 37.4°C. The reaction was stopped by adding an acid mixture (800 μL, H2SO4:H3PO4:H2O, 1:3:7) and 3% isonitrosopropiophenone, followed by heating at 100°C for 45 min, and the absorbance at 540 nm was read. The data were quantified from a calibration curve prepared using urea (1.5-120 μg), and normalized for tissue protein. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of urea/min.

2.9. Total NOS assay

The serum NOS activity was measured using the commercially available colorimetric kit following manufacturer's protocol.

2.10. Western blot analyses of tissue iNOS and eNOS expressions

The glandular part of the gastric mucosa after being washed with PBS containing protease inhibitors was minced and homogenized in a lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100) containing leupeptin (0.005 μg/mL) and PMSF (0.4 μM/mL). Following centrifugation at 15,000 × g for 30 min at 4°C, the supernatant was collected, and the protein concentration measured. The proteins (40 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membrane was blocked for 2 h in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% Tween 20) containing 99% fat-free milk powder and incubated overnight at 4°C with rabbit polyclonal iNOS or eNOS antibody. The membrane was washed over a period of 2 h with TBST and incubated with peroxidase conjugated anti-rabbit IgG (1:2,500 dilution). The bands were detected using an enhanced chemiluminescence detection kit and quantified using the Gelquant software.

2.11. Assay of NO

Following manufacturer's instruction, the serum NO level was measured using a commercially available colorimetric kit that measures the total nitrite concentration of the sample.

2.12. Assay of cytokines

The serum IL-4, IL-6 and TNF-α levels were estimated using commercially available ELISA kits following manufacturer's protocols. The method of TGF-β1 estimation (13) in sera was adopted after acidification to include the active and latent forms of the cytokine. Briefly, 96-well high binding ELISA plates were coated with anti-mouse TGF-β1 monoclonal antibody and incubated overnight at 4°C. After blocking for 30 min at 37°C, the wells were washed once with TBST buffer, the samples were activated by acid treatment followed by neutralization. The samples along with the standards were seeded to each well at an appropriate dilution, and incubated at room temperature for 90 min. The wells were washed (5 times), diluted polyclonal antibody
(100 μL) added, and the mixture incubated further for 2 h at room temperature. The wells were washed, and incubated for 2 h after addition of TGF-β HRPO conjugate (100 μL). After the final wash, TMB (100 μL) was added to each well, the mixture incubated for 15 min, the reaction was stopped by 1 N HCl, and the absorbance at 450 nm was read.

2.13. Statistical analysis

The values are expressed as the mean ± S.E.M. The data were analyzed by a paired Student’s t test for the paired data, or one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparisons post hoc test.

3. Results

Earlier we have observed peak ulceration in mice on the 3rd day after indomethacin (18 mg/kg, single dose) administration, and a three-day treatment with RM (40 mg/kg/day) and Omez (3 mg/kg/day) provided optimal ulcer healing (9). The extent of stomach ulceration and healing by the drugs were quantified from the same histological slide. The optimized healing data, assessed in terms of histological damage scores are provided in Table 1. Hence, the present experiments were also carried out under the above conditions. We preferred using the crude extract (RM) since it was more potent than the individual active components on equivalent basis. This would also be economically beneficial.

3.1. Regulation of the mucosal MPO activity

Compared to the normal mice, the mucosal MPO status in the gastric tissues of ulcerated untreated mice increased by 34.3% (p < 0.05) (Figure 2). This was brought down significantly by both RM (22.4%, p < 0.05) and Omez (20.3%, p < 0.05). The effect of Omez was not significantly different from that of RM.

3.2. Regulation of the mucosal arginase activity

The indomethacin-mediated arginase activity

Table 1. The optimized healing capacities of RM and Omez against indomethacin-induced stomach ulceration in mice as revealed from the histological damage scores

<table>
<thead>
<tr>
<th>Group</th>
<th>Damage score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>3.0 ± 0.07</td>
</tr>
<tr>
<td>Extract-treated</td>
<td>0.8 ± 0.03 (73.3)</td>
</tr>
<tr>
<td>Omez-treated</td>
<td>0.4 ± 0.02 (86.7)</td>
</tr>
</tbody>
</table>

* Stomach ulceration in mice was induced by indomethacin (18 mg/kg, p.o.). RM (40 mg/kg × 3 d) and Omez (3.0 mg/kg × 3 d) were used for the experiments. The histologic damage (DS) were measured on the 3rd day after indomethacin administration by analyzing the data from the review of a minimum of three sections per animal. The values are mean ± S.E.M. (n = 15). Figures in parenthesis show % reduction from the experimental control.

3.3. Regulation of the NOS activity

Compared to the normal mice, a significant increase (5.37 fold, p < 0.001) in the total NOS activity was noticed in the ulcerated mice. Both RM and and Omez reduced it by ~73% (p < 0.001) (Figure 4).

3.4. Modulation of the mucosal eNOS and iNOS expressions

The Western blots of the eNOS and iNOS expressions

Figure 2. Effects of RM and Omez in modulating the mucosal MPO level in the indomethacin-induced ulcerated mice. The supernatant of the gastric tissue homogenate was incubated with TMB in a suitable buffer and the MPO activity (mean ± S.E.M., n = 15) was assayed from the absorbance at 450 nm. * p < 0.05 compared to normal mice; ** p < 0.05 compared to untreated mice.

Figure 3. Effects of RM and Omez in modulating the mucosal arginase activity in the indomethacin-induced ulcerated mice. The supernatant of the gastric tissue homogenate was incubated with L-arginine and MnCl2 in a suitable buffer and the arginase activity (mean ± S.E.M., n = 15) was assayed from the absorbance at 540 nm. * p < 0.05 compared to normal mice; ** p < 0.01 compared to untreated mice; *** p < 0.01 compared to Omez-treated mice.
in the gastric mucosa of the normal, ulcerated and drug (RM or Omez)-treated mice are shown in Figure 5. The eNOS expression was detected in both normal and ulcerated gastric tissues. In contrast, the iNOS expression was very high in the ulcerated tissues, but insignificant in normal gastric tissues. Quantification of the bands revealed that stomach ulceration increased the expressions of iNOS (62%, \( p < 0.001 \)) and eNOS (44%, \( p < 0.01 \)), compared to normal mice. Treatment with RM reduced the iNOS expression (21.6%, \( p < 0.05 \)), along with dramatic increase in the eNOS expression (56.3%, \( p < 0.001 \)), compared to the untreated mice. In contrast, Omez reduced the eNOS expression (23.6%, \( p < 0.05 \)) significantly, but showed insignificant effect on the iNOS expression.

3.5. Regulation of the serum NO level

At peak ulceration, there was a significant increase (55.1%, \( p < 0.01 \)) in the serum nitrite level compared to the normal mice. Treatment with RM and Omez reduced it by 49.8% (\( p < 0.01 \)) and 29.3% (\( p < 0.01 \)) respectively, the effect of RM being significantly better (\( p < 0.05 \)) than that of Omez (Figure 6).

3.6. Modulation of the serum TGF-\( \beta1 \) level

Compared to the normal value, ulceration reduced the level of serum TGF-\( \beta1 \) (Figure 7) by 48% (\( p < 0.001 \)). Treatment with RM and Omez, however, increased it by 79.6% (\( p < 0.001 \)) and 26.3% (\( p < 0.05 \))
respectively, compared to the untreated mice. RM was significantly \((p < 0.05)\) more potent than Omez.

3.7. Regulation of the serum Th1 (TNF-\(\alpha\) and IL-6) and Th2 (IL-4) cytokines

Compared to the normal value, ulceration drastically increased the serum TNF-\(\alpha\) and IL-6 ~4.4 and ~3.5 folds, respectively \((p < 0.001)\). RM suppressed the levels of both TNF-\(\alpha\) (66.9\%, \(p < 0.001\)) and IL-6 56\% \((p < 0.001)\) significantly, compared to the untreated mice. Omez, however, reduced both the cytokines by ~13.3\% \((p < 0.05)\), which were much less than that by RM.

In contrast, the serum IL-4 level in the ulcerated mice was reduced by 28.3\% \((p < 0.05)\), compared to the normal mice. Treatment with RM improved it appreciably (95.4\%, \(p < 0.001\)), while the effect of Omez (14.3\% increase) was significantly less \((p < 0.001)\) than that of RM. The results on the cytokine modulation are summarized in Figure 8.

4. Discussion

The non-steroidal anti-inflammatory drugs (NSAIDs) including indomethacin are most widely prescribed for the treatment of pain and inflammation. However, they are also known to cause gastrointestinal (GI) damage, characterized by hyperemia, and increased vascular permeability, as well as delayed ulcer healing \((14)\). Ulcer-healing is a complex process involving a combination of wound retraction and re-epithelization wherein several factors such as enzymes, cytokines, and soluble mediators, liberated during the inflammatory response play crucial roles. The impressive healing capacity of RM \((IC_{50} = 23.30 \pm 3.50 \text{ mg/kg})\) against the indomethacin-induced gastric ulceration in mice \((9)\) encouraged us to investigate its probable modulatory effect on arginase and NOS as well as the Th1/Th2 cytokines profiles since these are some of the established mediators of wound healing.

Earlier, we have established the healing action of mal B by histology \((11)\). Quantification of the histological slides in terms of damage score (DS) provided a better assessment of the quality of healing. This was also substantiated from our results with the MPO assay. The MPO activity, a marker of neutrophil aggregation at the site of inflammation is frequently increased in ulcerated conditions, and reduced during wound healing \((15)\). Our studies depicted that while indomethacin administration enhanced the gastric mucosal MPO activity, treatment with RM \((40 \text{ mg/kg } \times 3 \text{ d})\) and Omez \((3 \text{ mg/kg } \times 3 \text{ d})\) reduced it almost equally. These results are consistent with our previous

![Figure 8](https://www.ddtjournal.com)
histological results, where both RM and Omez were found to produce similar ulcer healing at the designated doses (9). It is worth mentioning that extending the period of treatment led to slightly better healing, but a large part of this was due to natural healing. Since that would not provide much information on the drug action, this was not pursued. Likewise, the effect of increasing daily dose of RM was only marginal. The phytochemical analysis of RM revealed the presence of four diarylnonanoids, malabaricinones A-D as its major constituents. All these compounds contain a resorcinol moiety, which is known to induce irreversible, hydrogen peroxide-dependent loss of activities of the heme-containing peroxidases such as MPO (16).

Metabolism of arginine that can be catalyzed by arginase, and NOS, plays a vital role in gastric ulceration and its healing. Upregulation of arginase increases the level of polyamines, which play a significant role in wound healing. The regulatory role of arginase in acute intestinal inflammation and tissue repair has been demonstrated (17,18). On the other hand, NOS catabolizes L-arginine by a different pathway to produce L-citrulline and NO. The signaling molecule, NO has long been the subject of extensive research with respect to its role in GI mucosal defense. The enhanced generation of NO in the inflamed gastric mucosa seems to act as a killer molecule, and is involved in inflammation. As NO concentrations of NO that dilates the blood vessels and increases blood flow in the gastric mucosa (19) and also contributes to angiogenesis (20,21). The eNOS expression plays a major role in wound healing that gets inhibited if the eNOS expression is depleted (20,22). On the other hand, iNOS that can be largely induced under certain pathological conditions (23) acts as a killer molecule, and is involved in inflammation. The enhanced generation of NO in the inflamed gastric mucosa may be toxic in the digestive systems, and contribute to the pathogenesis of peptic ulcer disease (24). An increase in iNOS activity, and a decrease in eNOS activity in the gastric mucosa are closely related to the development of gastric mucosal lesions. Because the potential high-output source of NO in mammalian cells is iNOS, factors involved in the induction and expression of iNOS activity are key determinants of the NO-mediated toxicity. Sustained overproduction of NO by iNOS is detrimental and contributes to inflammation in various gastroduodenal disorders (15).

The intense reciprocal regulation of NOS and arginase activities in vivo, and the temporal switch between them decides ulceration and healing (8,25). Our results showed considerable down regulation of the mucosal arginase level along with an increased expression of the iNOS due to ulceration. This suggested a shift of the arginine metabolism towards the NO/iNOS pathway during ulceration. The elevated expressions of both iNOS and eNOS accounted for the increased total NOS activity as well as serum nitrite level due to ulceration. The iNOS is probably derived from inflammatory neutrophils and macrophages that would contribute to stimulate inflammatory situation, explaining the ulcerogenic property of indomethacin. Simultaneous generation of superoxide and NO by the macrophages would produce peroxynitrite creating oxidative stress. Its stimulated generation, as observed in this study, may also delay proximal duodenal contractions, by exposing mucus to gastric acidity.

Treatment with RM and Omez, especially the former restored the arginase activity almost to normalcy. RM also raised the eNOS/iNOS ratio to a level favorable for efficient ulcer-healing. This would amount to generation of more polyamine at the expense of the iNOS-derived NO that may be a key contributing factor in the anti-ulcer effect of RM. The reduction of the total NOS activity and NO level by RM was primarily due to suppression of the iNOS expression. Even a moderate suppression of iNOS expression would reduce NO release substantially, since the enzyme is capable of generating NO many folds.

In contrast, despite showing less effect on modulating eNOS/iNOS expressions and NO production, Omez provided excellent healing. This may be due to other operative mechanism in its healing action as observed by us and others (10,26). The total NOS reduction by Omez was due to less eNOS expression. This was also evident from the fact that its effect on NO reduction was much less.

Although iNOS is very strongly induced in ulcerated tissue in the stomach, the NO-derived from it does not appear to play a role in modulating healing. Most of the previous studies of NO and inflammation focused on the role of iNOS, whereas the role of eNOS that can also be markedly enhanced by various stimuli or tissue injury has been underestimated. Using eNOS deficient mice, the importance of eNOS- derived NO in regulating microvascular structure during acute inflammation has been demonstrated (27). Our results suggested that the eNOS-derived NO contributed maximum to the ulcer healing property of RM, although a role for neuronal NOS-derived NO cannot be excluded.

Stimulation of inflammatory cytokines is extremely important in mucosal defense. One of the most prominent modes of mediation of indomethacin-induced gastropathy is the increased expression of the pro-inflammatory cytokines (28,29), which also correlates with the extent of ulceration. Even the cross-talk amongst NOS/NO and arginase/polyamine is guided by the cytokine profile of the host (2,3). In view of this, the immune response due to ulceration, and its modulation by RM and Omez was monitored. This enabled us to associate the inflammatory response with a better prognosis.

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Indomethacin administration raised the levels of pro-inflammatory cytokines (TNF-α and IL-6) while reducing the anti-inflammatory cytokines (IL-4 and TGF-β), thereby creating a cytokine imbalance. Increased TNF-α is known to increase iNOS activity by promoting binding of NF-kB to the iNOS promoter (25, 30). The increased levels of Th1 cytokines due to ulceration would augment the iNOS/NO pathway to produce excess NO, which is likely to promote oxidative stress and result in ulceration (31, 32). Our result on the decreased IL-4 level due to ulceration was in tune with a previous report (33).

Treatment with RM, however, restored the imbalance by increasing the levels of IL-4 and TGF-β beyond the normal values. The upregulation of the anti-inflammatory cytokines by RM is likely to inhibit the stimulatory effect of indomethacin on the level of pro-inflammatory cytokine release in blood and gastric mucosa. The immunosuppressive Th2 cytokine, TGF-β has a direct role in stimulating epithelial restitution (34). Besides suppressing the IFN-γ-induced iNOS gene expression and thereby generation of excess NO, it also increases arginase activity during inflammatory processes (8, 25, 35). The altered arginase activity and iNOS expressions observed by us during ulceration, and RM treatment are consistent with their respective effects in modulating the mucosal TGF-β status. The enhanced IL-4 level by RM would trigger the TGF-β-SMAD-signaling pathway to stimulate the extracellular remodeling and subsequent tissue repair. In contrast, except for the TGF-β, the other cytokines were not affected significantly by Omez, as reported earlier (33). This was also reflected in its marginal effect in regulating the enzymes, arginase and NOS.

Overall, RM modulated the cytokine profile to shift the balance in favour of arginase/polyamine via-vis iNOS/NO pathway, besides improving the eNOS expression. A combination of all these events might tilt the balance in favour of the repair mechanisms, explaining its ulcer-healing action. The bimodal nature of general immune responses is explained by the Th1/Th2 paradigm (36). The regulatory T cells and Th2 cytokines often collaborate to suppress the Th1 response. Perhaps even more importantly, they strongly promote the mechanism of wound healing. However, the role of cytokine imbalance in gastropathy has not been adequately emphasized. Our results highlighted that the balance of the pro- and anti-inflammatory, as well as regulatory cytokines could play a significant role in the NSAID-induced gastric mucosal injury.

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(Received November 14, 2008; Accepted November 22, 2008)
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Revised February 2008
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