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Biomarkers : An Important Strategy for Human Health Risk Assessment

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INTRODUCTION

Cancer epidemiologists estimates that as much as 90% of all cancer in humans is largely associated with our life style, i. e., diet and social habits [1,2]. It is believed that 80 to 90% of human cancer is preventable. Tobacco smoking accounts for approximately 30% of cancer deaths. The remaining 70% of cancer deaths is mainly due to the carcinogenic chamicals present in diet, drugs, cosmetics , and in the environment [3,4]. Therefore, it is important to determine the carcinogenic components that we expose to, so that we can avoid or reduce their intake.

Because most of the carcinogenic chemicals require metabolic activation, risk evaluation must be based on the quantitative estimate of the dose of the reactive metabolites delivered to the target tissues, and based on the carcinogenic potency of the metabolites. Thus, to perform risk assessment is highly challenging. Isolation, identification and quantification of toxic chemicals that we expose to are not easy. Accurate assessment of carcinogenic potency of a chemical is not easy either. Furthermore, production of tumors in an animal species does not prove that the chemical is a human carcinogen. Also, failure to produce tumors in experimental animals does not eliminate the possibility that the chemical would be carcinogenic in man. For animal tumorigenicity assay, tumorigenic potency of a chemical is species dependent, and strain dependent. Tumorgenic potency is also dependent on the route of administration, and other factors. In a word, there are a large number of uncertainties concerning the extrapolation of experimental animal data for interpretation of human health risk posed by carcinogenic chemicals. Uncertainties are introduced in all the four steps for performing risk assessment. They are:(i) hazard identification; (ii) exposure assessment; (iii) dose-response assessment; and (iv) risk characterization.

Regulatory agencies, such as FDA and EPA, must assess human health risks posed by exposure to carcinogenic chemicals in order to make regulatory decisions that protect public health. However, because of a lack of understanding of the underlying biological, chemical, and physical processes which determine exposures and effects, the regulatory agencies are limited in the ability to assess health risks quantitatively. Without sufficient knowledge, uncertainties (assumptions) are introduced into the risk assessment process that allows wide interpretation of the limited experimental data that are available. As a consequence, it is important to pursue critical data on the relationship between exposure, dose to target tissue (delivered dose), and associated health effects. Emphasis is on the laboratory and field researh to improve understanding of basic biological mechanisms, especially as they relate to our ability to extrapolate from one set of circumstances to another and will allow us to quantify the human health risks associated with human exposures.

In order to efficiently assess the human health risk posed by carcinogenic chemicals, a research strategy, biomarkers, has been recently pursued. This new approach can provide highly sensitive bioassays, and the

biological responses occurring in experimental animals can be potentially applied to humans.

TYPES OF BIOMARKERS

The research on biomarkers is to provide new and potentially useful human biological markers as practical bioassays for risk assessment. Biomarkers are indicators of variation in cellular or physiological components or processes, structures , or functions that are measurable in a biological system or sample. A variety of parameters can be utilized as biomarkers. These at least include: metabolizing enzymes, metabolism patterns, parent carcinogenic substrate in target tissue, mutagenticity in urine, metabolite(s), protein adducts, and DNA adducts. There are mainly three types of biomarkers. They are: (i) biomarkers of exposure; (ii) biomarkers of susceptibility; and (iii) biomarkers of effect.

Biomarkers of Exposure

Biomarkers of exposure are the surrogates to determine the absorbed dose and the delivered (or target) dose of a chemical in the animal and human body. Biomarkers of exposure will be used as essential tools in monitoring and controlling exposure to a broad range of contaminants. For example, genotoxic solvents represent some of the most extensively used chemicals in industry, in research institutes, and even sometimes at home. Exposure to hazardous solvents can cause long-term genetic damage. Benzene, a leukemogen [5], is a widespread genotoxic environmental contaminant and in some cases is also present in the cosmetics and drinking water. Benzene is regulated under the Clean Air Act posed by U. S. EPA. The dose-response relationship between human exposure to benzene and the development of toxic responses has not been fully established. Metabolism of benzene by humans and rodents results in the reactive intermediate, benzene oxide, that binds to in albumin to form the cysteine adduct s-phenylcysteine. This s-phenylcysteine adduct in albumin has been considered as an effective biological marker for exposure to benzene. Another example is aflatoxin B_1 (AFB_1), which is a major food contaminant and is the agent suspected to cause hepatocarcinoma in humans. Metabolism of AFB_1 yields AFB_1 8, 9-oxide and AFB_1 8, 9-dihydrodiol, Covalent binding of AFB_1 8, 9-oxide results in the formation of the corresponding DNA adduct. On the other hand, binding of AFB_1 8, 9-dihydrodiol with human serum albumin to yield protein adducts has been reported [6, 7]. Both these DNA and protein adducts of AFB_1 have been utilized as biomarkers of AFB_1 exposure.

Biomarkers of Susceptibility

Carcinogenic compounds require metabolic activation in order to exert their biological activities, including carcinogenicity. It is known that the relative quantity of the metabolizing enzymes, such as the cytochrome p-450 isozymes, in humans and in experimental animals can be different. Thus, the metabolism rate of metabolic activation as well as detoxification of a chemical carcinogen can be considerably different from person to person, resulting in the differences in the susceptibility of carcinogen exposure. As a consequence, Differences in metabolizing enzymes can be useful biomarkers of susceptibility. For example, it is known that there are two distinct types of human acetyltransferases, the fast and the slow acetylator phenotypes[9-11]. A positive correlation has been found that, when exposed to aromatic amines, persons who have a slow acetylator phenotype encounter with a higher bladder cancer incidence than those with a fast acetylator phenotype [8]. On the other hand, persons with a fast acetylator phenotype have a higher tumor incidence than those with a slow acetylator phenotype [9, 10]. Evidently, acetyltransferase is a biomarker of susceptibility for assessment of human health risk.

Biomarkers of Effect

It has been well supported by the experimental results that tumors induced by chemical carcinogens involve cytogenetic changes. Thus, if a carcinogen exerts its carcinogenic effect via a genetic mechanism, its DNA adduct can be a potential biomarker of effect. For example, AFB_1 DNA adduct has also been employed as a biomarker of effect of AFB , However, not all the

carcinogen-modified DNA adducts can be used as biomarkers of effect. A representative example is 2-acetylaminofluorene (2-AAF). Upon metabolic activation of 2-AFF, reaction of cellular DNA with the resulted reactive metabolite, N-hydroxy-2-acetylaminofluorene generates three DNA adducts. They are: N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF), N-(deoxyguanosin-8-yl)-2-AAF (dG-C8-AAF), and 3-(deoxyguanosin-N^2-y1)-2-AAF (dG-N^2-AAF). Because of the bulky N-acetyl group. dG-C8-AFF) distorts the DNA double helix significantly, and thus, is readily recognized by the DNA repair exzymes and is facilely repaired. Its formation does not correlate with tumor induction and cannot be employed as a biomarker of effect of 2-AAF. The adduct dG-N^2-AAF is not repaired and its quantity increases with extension of exposure time. However, dG-C8-AF is the only DNA adduct that has a half-life long enough to be detected in rat liver 2-3 weeks after feeding with 2-AAF. Therefore, dG-C8-AF can be a biomarker of effect of 2-AAF[11]. Another example is benzo [a] pyrene (BaP), which is the prototype for studying the chemical carcinogenesis of polycyclic aromatic hydrocarbons (PAHs), a class of genotoxic contaminants detected in the environment and in the food chain. Metabolism of BaP, generates Bap *trans*-7, 8-diol- *anti*-9, 10-epoxide as the ultimate metabolite. Reaction of this ultimate metabolite with DNA, both *in vitro* and *in vivo*, results in the formation of 10-(deoxyguanosin-N^2-yl)-7, 8, 9-trihydroxy-7, 8, 9, 10-tetrahydrobenzo [a] pyrene as the major DNA adduct. This DNA adduct has been used as biomarkers of exposure, susceptibility and effect [12-14].

PROTEIN ADDUCTS AS BIOMARKERS

Covalent binding of chemical carcinogens to proteins can potentially reveal precise information about exposure of the carcinogens. Consequently, protein adducts are ideal biomarkers for risk assessment. Because tumors arise from cells that undergone a permanent heritable change in their genetic material, binding of the chemical carcinogens with cellular DNA in the target tissues leading to the DNA is the critical step in the tumor formation. On the other hand, because protein is not the genetic material leading to tumor formation , protein adducts cannot be employed as biomarkers of effect. Nevertheless, if a relationship between the quantitative formation of protein adduct and the corresponding DNA adduct derived from the same chemical carcinogen can be established, this protein adduct can serve as a surrogate for DNA adduct, and thus can be a suitable biomarker of effect of this chemical carcinogen. An excellent review entitled "Protein adducts in the molecular dosimetry of chemical carcinogens" has recently been published by skipper and Tannenbaum [15]. A number of carcinogen-protein adducts have been prepared. A high degree of specificity is characteristic of the interactions between the activated forms of chemical carcinogens and proteins[15]. As described in the review [15], a good protein adduct used as a biomarker should at least include the following: (i) the adduct is chemically stable; (ii) the adduct does not influence the stability of protein; (iii) the adduct is accessible for epidemiological studies; and (iv) a relationship between the protein dose and DNA dose can be made. Both the hemoglobin and serum albumin adducts can meet all these criteria. Ehrenberg et al[16] reported the protein adduct of ethylene oxide in 1974 and this study represents the first report in the recognition of the potential of protein adducts to serve as surrogates for DNA adducts. Since then, a number of hemoglobin adducts and serum albumin adducts have been prepared. These include those derived form methyl methanesulfonate[17], aflatoxin B_1[6,7], fluoranthene[18], 4-aminobiphenyl [19], N-nitrosonomicotine[20], styrene and styrene 7, 8-oxide [21], diethylnitrosamine[22], Glu-p-1[23], and benzo[a] pyrene [24].

Protein adducts are usually characterized by high-resolution GC/MS [25]and immunoassay including radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA)[26]. A method involving removal of the protein moiety from the protein adduct followed with characterization of the remaining carcinogen moiety by GC/MS has also been developed [27].

There exist problems concerning the use of protein adducts as biomarkers. Human hemoglobin adducts have a lifespan of 120 days, and serum albumin adducts have a half-life of 20-25 days. Thus, for risk assessment, quantitative analysis of protein adducts sometimes is difficult. It is important to develop a long-term, or even a lifetime protein dosimeter. Currently, in most cases, it is difficult to characterize protein adducts by any conventional analytical methods, including mass spectrometry. Thus , it is timely and important to develop more advance methodologies for better characterization.

DNA ADDUCTS AS BIOMARKERS

Because DNA adducts are formed from covalent binding of the activated metabolites of chemical carcinogens with DNA, DNA adducts can provide inside of the mechanism by which the carcinogens induce tumors. Therefore, DNA adducts can be considered the best dosimeters to provide an indication of the relative risk of tumor induction. Currently, DNA adducts derived from different classes of chemical carcinogens have been prepared[14]. These include those from PAHs [14, 28, 29], arylamines [11], aflatoxins [30], nitrosamines [31], and nitro-PAHs [32].

While DNA adducts represent the most promising biomarkers for risk assessment, it requires highly sensitive analytical techniques for characterization and quantification. This is because the quantity of DNA adducts formed *in vivo* or *in vitro* is generally extremely small. DNA adduct levels in experimental animals administered with carcinogens are generally on the range of 10 - 100 pmoles/mg DNA, which is equivalent to 3 adducts per 10^5- 10^6 nucleotides[14]. The DNA adduct level expected based on human exposure is about 0.1 - 1 pmol/mg DNA, which is 100 times less than that from experimental animals. Thus, even after exposure to carcinogens in substantial amount, only about one carcinogen-modified DNA adduct per 10^6 to 10^8 nucleotides is formed. Furthermore, DNA accounts for only about 0.1% of a cell by weight. Therefore, only 1 mg of DNA can be obtained from 1 g of tissue. Thus, assuming a concentration of 1 pmol/mg DNA of DNA adduct is formed, only about 10 ng of DNA adduct will be obtained from 20 g of tissue. Clearly, this requires skillful techniques for separation of the adduct, and requires sophisticated methodology for identification and quantification of the DNA adducts.

Currently, there are three major techniques that are highly sensitive and can be utilized for efficient separation and quantification of DNA adducts. They are: (i) immunoassay; (ii) mass spectrometry; and (iii) ^{32}P-postlabelling.

IMMUNOASSAY

In the immunoassay, the monoclonal and polyclonal antibodies are prepared by dealing the carcinogen-modified DNA or carcinogen-nucleoside adducts as immunogens. The antibodies prepared are then used to identify and quantify the specific DNA adducts by either employing the RIA assay or the ELISA assay. The sensitivity of ELISA assay is about one adduct per 10^7 nucleotides. Although RIA assay is less sensitive, both ELISA and RIA assays are sensitive for efficiently assaying human DNA samples. Immunoassay is relatively inexpensive, and many samples can be analyzed simutaneously. However, immunoassay has disadvantages. The antibody prepared for a particular immunogen (modified DNA adduct) sometimes may not be specific, and therefore, may respond to a broad spectrum of DNA adducts. This will hamper accurate quantification. Another problem is the requirement of sufficient amount of modified DNA adduct used as an immunogen to prepare the corresponding antibody. As a consequence, it requires chemical synthesis to prepare the modified DNA adduct in sufficient quantity.

MASS SPECTROMETRY

For structural identification, carcinogen-modified DNA adducts are enzymatically digested into the corresponding nucleosides which ard analyzed by high resolution mass spectrometry and proton nuclear magnetic resonance spectroscopy. Technology in mass spectrometry has recently been developed rapidly and has facilitated in

identification of DNA adducts in extremely small quantity. In general, volatility, thermal and chemical stability, sensitivity and resolution capability are the major elements concerning the capability in detecting and quantifying a DNA adduct. While volatility, thermal stability, and chemical stability are the intrinsic characters of a chemical (DNA adduct), sensitivity and resolution capability involve the quality of the mass spectrometer used and the type and the quantity of the chemicals (DNA adducts) subjected for study. The modern highly sensitive mass spectrometric techniques have made significant progress, and have been utilized to analyze the carcinogen-modified DNA adducts in human samples. The developed methodologies at least include: (i) pre-separation of the components from a mixture; GC/MS, HPLC/MS, capillary LC/MS, zone electrophoresis (CZE); (ii) ionization techniques, electron impact, positive ion chemical ionization, negative ion chemical ionization, fast atom bombardment (FAB); continuous flow fast atom bombardment (CF-FAB), electrospray ionization (ESI), collision induced dissociation (CID); (iii) derivatization of the DNA adducts for mass spectral measurement; and (iv) removal of the nuclear base from the DNA adduct by a chemical reaction and mass spectral analysis of the remaining carcinogen moiety. Thus, based on the necessity, the mass spectrometric techniques employed for measurement of a DNA adduct can include at least the folllowing on-line separation-MS systems: Ms/MS, FAB/ MS, CFFAB /MS, CZE-CFFAB/MS, CZE-ESI/MS, capillary LC-CFFAB/MS, and capillary LC-ESI/MS. Currently, mass spectrometric techniques can detect one adduct per 10^8 nucleosides, and has a potential up to one adduct per 10^{11} nucleosides. Consequently, mass spectrometry has a potential for identification of low levels of DNA adducts in physiological samples.

^{32} P-POSTLABELING

^{32}P-Postlabeling is the most recently developed highly sensitive technique used for identification of carcinogen-modified DNA adducts [33]. This method is highly sensitive, can detect DNA adducts at a level of one carcinogen-modified adduct in 10^{10} nucleotides [34,35]. Another advantage of this method is that radioactivity is introduced after sample is collected from an *in vivo* or an *in vitro* system. The process of this method involves: (i) DNA adduct is first digested with DNase II and micrococcal endonuclease to the corresponding 3′-monophosphate nucleotide; (ii) conversion of the 3′-monophosphate nucleotide with [^{32P}] ATP catalyzed with polynucleotide kinase to the ^{32}P-labeled 3′5′-bisphosphate nucleotide; (iii) separation of the modified adducts and removal of the unmodified adducts by multidirectional thin-layer chromatography (TLC); and (iv) identification of the specific adduct by comparing the location (spot) with that of a standard, and quantification of the adduct by autoradiography [33-55]. If the DNA adduct is derived from a PAH, a so-called nuclease pl-mediated technique has been developed, which can efficiently enhance the detection sensitivity [34]. On the other hand, the n-butanol extraction technique can enhance the detection of DNA adducts derived from aromatic amines or nitro-PAHs [35].

^{32}P-Postlabeling technique is currently the most promising methodology for detection of DNA adducts at a low level. This method has now been widely employed. It can also be employed for detection of unknown DNA adducts. Nevertheless, this method also has its problems. While this method can identify an unknown adduct, quantification of an unknown adduct is impossible. Another problem is that multidirectional TLC is not efficient enough to separate DNA adducts from the sampes containing many adducts.

FUTURE PERSPECTIVES

Biomarkers are potential surrogates for human health risk assessment. It can also provide information concerning the mechanisms by which the toxic chemicals exert their adverse activities, including carcinogenicity, in humans. Biomarkers can monitor human exposure and effect of a toxic component present in the food chain, drugs, or in the environment. Thus, it is anticipated that development of sensitive biomarkers for

risk assessment will be one of the major research areas in medical science and in regulatory research. The ideal biomarkers to be developed should be non-invasive and interpretable in terms of human susceptibility to exposure or effect. DNA adducts will be the critical biomarkers to measure the effect of a carcinogenic chemical to human health. Currently, although several modern techniques have been successfully employed to identify and quantify DNA adducts, all encounter different types of problems. The most important problem is detection capability. It is important to improve the current analytical methodologies and to develop new methodologies for accurate detection and quantification of DNA adducts, protein adducts and other biomarkers.

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Determination of ML-1035 Enantiomers in Plasma by Chiral High Performance Liquid Chromatography

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ABSTRACT

ML-1035, is a gastroprokinetic agent structrually related to metoclopramide. Because ML-1035 contains an asymmetric chiral sulfoxide moiety, a chiral HPLC method was developed to separate and quantitate its R and S enantiomers in plasma. The ML-1035 enantiomers present in plasma were extracted with dichloroethane under alkaline conditions. The extract was evaporated to dryness and reconstituted in the mobile phase. The samples were chromatographed on a Chiralcel OD HPLC column with hexane: absolute ethanol (1% TEA) with a ratio of 1:1 (v/v) as the mobile phase. The enantiomers of the unchanged drug were quantified by fluorescence detection (ex: 310 nm, em: 350 nm).

The method provided a linear response for both enantiomers over a concentration range of 25 (limit of quantitation)to 2500 ng/ml with correlation coefficients of 0.9987 or greater. The interassay precision was 9.5% or less and the accuracy ranged form 93.9% to 103.4% of the theoretical value. This method was utilized in determining the plasma concentrations of the R and S enantiomers following oral and intravenous administration of R or S enantiomers to dogs. The method was also adapted to measure enantiomer levels from in vitro reaction mixtures so that the possibility of metabolic inversion could be assessed. The data suggest that no significant level of inversion between the enantiomers occurred either in vivo or in vitro.

Key Words: Enantiomers, Chiral Seperation, Plasma, HPLC.

INTRODUCTION

ML-1035 is a gastroprokinetic agent which is structurally similar to metoclopramide, the widely used antiemetic and stimulant of upper gut motility [1-3]. ML-1035 is a racemic mixture with its chiral center in the sulfoxide moiety (Figure 1). Therefore, the objectives of this study were to develop a sensitive and selective chiral chromatographic method for quantitating the R and S enantiomers of ML-1035 in plasma, and then to determine the pharmacokinetic profile of the enantiomers following oral and intravenous administration to dogs. Further, the possible inversion of the R and S enantiomers was also investigated both in vitro and in vivo.

MATERIALS AND METHODS

1. *Materials and Reagents*

HPLC grade methanol, hexane, and dichloroethane were purchased from Buridck and Jackson (Muskegon, MI). Ethyl alcohol (absolute) was purchased from Quantum Chemicals Corporation (Tuscin,