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Defining EMS and ENU dose–response relationships using the *Pig-a* mutation assay in rats

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ABSTRACT

In recent years, experimental evidence has accumulated that supports the existence of sublinear dose–response relationships at low doses of DNA reactive mutagens. However, creating the in vivo data necessary to allow for a more detailed dose–response modeling with the currently available tools might not always be practical. The purpose of the current work was to evaluate the utility of the *Pig-a* gene mutation assay to rapidly identify dose–response relationships for direct acting genotoxicants. The induction of mutations in the peripheral blood of rats was evaluated following 28 days of exposure down to low doses of the direct acting alkylating agents ethyl methane sulfonate (EMS) and ethylnitrosourea (ENU). Using statistical modeling based on the 28-day studies, a threshold for mutation induction for EMS was estimated to be 21.9 mg/kg, whereas for the more potent ENU, the threshold was estimated to be 0.88 mg/kg. Comparing mutation frequencies from acute and sub-chronic dosing indicated less than additive dose–response relationships, further confirming the possibility of a threshold dose–response relationship for both compounds. In conclusion, the work presented provides evidence that the *Pig-a* assay might be a practical alternative to other in vivo mutation assays when assessing dose–response relationships for direct acting mutagens and that an experimental approach using fractionated dosing could be used to substantiate a biological mechanism responsible for the observation of a sublinear dose–response relationship.

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1. Introduction

The dose–response relationship associated with the genotoxic and carcinogenic effects of a compound that directly interacts with DNA has been thought to be linear, even with very low doses of a genotoxic agent inducing increases in mutations in a strictly additive fashion. An underlying assumption associated with this hypothesis is that DNA repair capabilities, detoxification pathways and other forms of protection all function in a linear manner regardless of the burden of exposure and associated DNA damage. The conservative assumptions underlying a linear dose–response relationship are the basis of the threshold of toxicological concern (TTC), which has been recommended by the Committee for Medicinal Products for Human Use (CHMP) as an allowable exposure limit for a genotoxic impurity in a drug substance [1]. This daily lifetime limit of 1.5 μg/day is expected to pose a negligible excess cancer risk to patients. The TTC was derived by linearly extrapolating carcinogenic effects determined at high (and often toxic) doses to derive a dose of a genotoxic substance associated with an excess lifetime cancer risk of 1×10^{-5}.

However, recent studies suggest that low doses of direct acting genotoxic agents may exhibit a sublinear dose–response and a threshold below which no increase in genotoxicity is observed [2–7]. Of greatest interest to the current work are a number of recent investigations that provide convincing evidence for a nonlinear dose–response for the genotoxic effects of the alkylating agent ethyl methane sulfonate (EMS). Observation of a threshold dose for EMS induced genotoxicity has been reported from in vitro studies [2] and compelling evidence of an in vivo threshold for EMS induced genotoxicity (25 mg/kg/day) has been reported based on elaborate dose–response investigations in mice [3,4]. The primary purpose of these in vivo studies was to support a risk assessment for patients who ingested EMS as a contaminant in Viracept tablets [8]. The design of the studies was robust in that there were many dose groups near the anticipated threshold dose, seven animals were included per dose group (larger than typical) and multiple tissues were evaluated for mutagenicity. Further, ethylvaline adducts of hemoglobin were measured to assess exposure in the animals. Profound increases in adducts were observed at low doses of EMS, which showed no evidence of genotoxicity, indicating that DNA repair (or some other protective mechanism) was preventing the

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induction of mutations. Additional evidence in support of a threshold was observed by comparing the mutagenic response observed in animals treated for 28 days with low doses of EMS to animals exposed to acute high doses of EMS. Whereas a high acute dose of EMS (350 mg/kg) induced an increase in mutations, fractionating the same dose of EMS over 28 days (12.5 mg/kg/day) completely abolished the mutagenic effect [3,4].

The data generated for these recent in vivo studies were also used to derive a compound specific permissible daily exposure (PDE) limit for EMS as a genotoxic impurity in a drug substance [9]. Prior to these investigations there were insufficient data to derive a PDE for EMS, and therefore the TTC limit (1.5 μg/day) would need to be applied. Based on the approach recommended in the CHMP guideline for establishing exposure limits for genotoxic impurities with sufficient evidence of a threshold [1], the investigators utilized the procedure outlined in ICH Q3C Guidance on Residual Solvents [10] to derive a PDE of 104 μg/day for EMS.

In theory, the notion of utilizing experimental data to establish allowable limits for genotoxic impurities in pharmaceuticals is very attractive. Data driven decision making rather than pragmatic decision making (current TTC approach) would focus lowest level impurity control and measurement to those compounds presenting the greatest risk to human health. The challenge is to find a way to generate the necessary data in a manner that can be practically applied and implemented. The scope of the in vivo work recently reported for EMS would not be practical to generate on a routine basis during the normal course of drug development.

Recently, a flow cytometric *Pig-a* assay for measuring gene mutation in the RBCs of rats has been developed [11,12]. The endogenous gene, phosphatidylinositol glycan complementation group A (*Pig-a*) codes for a cytoplasmic member anchor protein that holds proteins to the surface of cells. In this assay, RBCs with mutant *Pig-a* are readily differentiated from wild type cells by fluorescent labeling of CD59. Whereas wild type cells are labeled with CD59 antibody, mutant cells, deficient in anchor proteins, remain unlabeled. The assay has several attributes which make it attractive as a potential tool to characterize dose–response relationships associated with genotoxic substances. First, it has been suggested that mutations induced in long-lived hematopoietic precursor cells persist and allow for the detection of an accumulation of mutations in the peripheral blood cells with repeat dosing [13,14]. Therefore, even weak mutagens or weakly mutagenic exposures should be detected with repeat dosing. Further, the assessment of mutation induction in peripheral blood cells allows an investigator to serially sample animals before, during and after exposure. Doing so allows one to characterize the relationship between accumulating dose and mutant frequency. Finally, in comparison to in vivo transgenic mutation assays, the *Pig-a* assay does not require use of costly animals, and both sample preparation and quantitation of mutants are much simpler and faster.

The purpose of the current work was to evaluate the utility of the *Pig-a* assay to rapidly differentiate linear and sublinear dose–response relationships for direct acting genotoxic agents. Dose–response relationships were characterized for two alkylating agents, EMS and ethylnitrosourea (ENU) and evaluated for linearity. Furthermore, the frequencies of *Pig-a* mutants induced from high acute doses were compared to that induced in animals treated with lower doses for 28 days. Comparison of the mutant frequencies induced from equivalent cumulative exposures should allow one to determine if some process (e.g. DNA repair) is more effective at preventing mutation induction at low doses, and if so, substantiate that an underlying biological mechanism is responsible for the observation of a sublinear dose–response curve.

2. Methods

2.1. Reagents

Ethyl methane sulfonate (EMS, CAS No. 62-50-0), N-ethyl-N-nitrosourea (ENU; CAS No. 759-73-9, 62% activity) and PBS were purchased from Sigma–Aldrich and distilled water was purchased from Gibco Inc. EMS, a liquid, was formulated daily in distilled water, and ENU was formulated in PBS (pH 6.0; the amount of water and stabilizer in the ENU preparation was taken into account when calculating the concentrations). Anticoagulant, balanced salt solution, anti-CD59-phychoerythrin and SYTO® 13 dye were supplied as Prototype MutaFlow® Pig-a Mutation Assay Kits by Litron Laboratories, Rochester, N.Y.

2.2. Animals

6–8-week-old male Sprague-Dawley (Crl:CD[SD]) rats (~150–200 g) from Charles River were used in all experiments. The animals received pelleted certified rodent diet and were provided purified municipal drinking water *ad libitum*. Animals were housed individually in hanging polycarbonate shoe box cages in a room with relative humidity of 50 ± 20%, temperatures of 70 ± 5 °F and a 12 h light/dark cycle. All animal care and experimental procedures were conducted in compliance with the U.S. Animal Welfare Act and the ILAR guide (1996).

2.3. Dose levels and treatment schedule

Initial dose levels and route of administration were selected based on literature information on the maximum tolerate dose (MTD) for the compound. All treatments in all studies were administered by oral gavage in a dose volume of 10 ml/kg. A summary of all studies in this manuscript including dose levels, treatment durations and blood collection is presented in Table 1.

In the EMS 28-day study, five animals per group were dosed by oral gavage once a day with EMS (6.25, 12.5, 25, 50, and 100 mg/kg) or distilled water for 28 consecutive days. In the acute EMS study, five animals per group with the exception of the high dose group, which contained 8 male rats, received a single dose of EMS (100, 175, 350, and 700 mg/kg) or distilled water. Animals were euthanized by CO_2 asphyxiation following the last time point taken.

In the first ENU study, 10 male animals per group were dosed by oral gavage once a day with ENU (2.5, 5, and 10 mg/kg) or PBS (pH 6.0) for 28 consecutive days. In the second ENU study, five male animals per group were dosed by oral gavage once a day with ENU (0.043, 0.25, 0.5, 1, and 15 mg/kg) or PBS for 28 consecutive days. In the acute ENU study, five male animals per group received a single dose of ENU (1.2 and 28 mg/kg) or PBS by oral gavage at a dose volume of 10 mL/kg. Animals were euthanized by CO_2 asphyxiation following the last time point taken.

2.4. Blood harvest

All blood harvest occurred via jugular venipuncture using a 1-ml syringe with a 25-gauge needle. Approximately 150–200 μl of blood was harvested and transferred to BD microtainer tubes with lithium heparin to prevent coagulation. For the first ENU 28-day study blood was harvested on Days −1, 15, 31, and 57. Blood was harvested on Days −1, 15, 29, and 57 for the second ENU study. For the EMS 28-day study blood was harvested on Days −1, 15, 29, 55, and 105 and on Days −1, 15, and 29 for the acute EMS study.

2.5. Flow analysis

Cell processing, staining and flow cytometric procedures followed methods described by Phonethepswath et al. [14]. Briefly, all blood samples were processed

Table 1
Summary of studies conducted.

Study	Rats per group	Dose levels (mg/kg)	Treatment duration (days)	Blood collection times
EMS 28-day study	5	0, 6.25, 12.5, 25, 50, 100	28	Days −1, 15, 29 and 55
EMS single dose study	5[a]	0, 100, 175, 350, 700	1	Days −1, 15, 29 and 57
ENU 1st 28-day study	5	0, 2.5, 5, 10	28	Days −1, 15, 31 and 57
ENU 2nd 28-day study	5	0, 0.043, 0.25, 1, 15	28	Days −1, 15, 29 and 57
ENU single dose study	5	0, 1.2, 3.5, 7, 28	1	Days −1, 15, 30 and 57

[a] 8 rats were assigned to the 700 mg/kg treatment group in case excessive toxicity occurred.

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