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The Viracept-EMS case: Impact and outlook

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ABSTRACT

This perspective first considers the potential impact of the Viracept-EMS case in the framework of the current understanding of the low-dose effects of DNA-reactive chemicals and the approaches used to estimate health risks from genotoxins occurring as impurities in pharmaceutical products or as contaminants in the environment or workplace. It also presents an outlook on the nature of additional research building upon the Viracept-EMS case to test assumptions underlying thresholded dose–response relationships and to establish biologically based risk assessment models in lieu of default models for DNA-reactive compounds.

1. Introduction and background

The Viracept-EMS case encompasses the rapidly occurring course of events recounted by Müller (in press) in this special issue, extending from the first reports by HIV-infected patients (May 2007) of a bad smell in blister-pack tablets of the protease inhibitor, Viracept (nelfinavir), to the Press Release by the European Medicines Agency (EMEA) (24 July 2008) stating that "Studies assessed by the EMEA indicate no increased risk of developing cancer for patients who have taken Viracept contaminated with ethyl mesilate" (EMEA, 2008). Events most pertinent to this commentary include (1) the quick discovery that ethyl mesilate, a well-studied monofunctional alkylating agent better known as ethyl methanesulfonate (EMS), was the source of the bad smell in Viracept tablets due to a production accident (June 2007), (2) the completion of key "follow-up measures" by Roche that built in pivotal experiments assessing the dose–response for chromosome and gene mutations in mice exposed to EMS [or a potent positive control mutagen and alkylating agent, ethylnitrosourea (ENU)] and evaluating cross-species *in vitro* and *in vivo* exposure to EMS for extrapolation to humans (September 2007 until April 2008), (3) peer review and discussion of the new and existing non-clinical data by established experts in the toxicology (30 May 2008) and clinical (10 June 2008) arenas, including whether the data demonstrated that significant thresholds existed between actual patient exposures and exposures where DNA damage are likely to have occurred, and (4) the responsive interactions between Roche and the EMEA and the regulatory actions of the EMEA leading to the above mentioned Press Release. As discussed below, the Viracept-EMS case will likely become a landmark in the development of low-dose *in vivo* mutagenicity data for toxicology and the advancement of approaches for assessing cancer risk and setting safety standards for DNA-reactive chemicals occurring as contaminants in pharmaceutical products or in the environment.

Monofunctional alkylating agents are a group of genotoxic chemicals that form adducts via the reaction of electrophilic moieties with nucleophilic centers in DNA (Miller and Miller, 1966; Beranek, 1990; Swenberg et al., 1990; Lee et al., 1992; Gocke et al., in press-a,b). Simple alkylating agents can react with DNA at more than a dozen different sites, mostly at oxygen and nitrogen atoms (Singer and Grungerger, 1983), with the relative reaction rates depending upon the nucleophilicity of the reaction site, the Swain–Scott substrate constants (*s*-values), and steric factors (Ehrenberg and Hussain, 1981). Alkylating agents with high *s*-values, such as the classical experimental compounds ethylene oxide (EO; $s = 0.96$) and methyl methanesulfonate (MMS; $s > 0.83$), are 'soft' electrophiles with high nucleophilic selectivity that react primarily at the N7 position of guanine and N3 position of adenine via bimolecular (S_N2) mechanisms. In contrast, chemicals with low *s*-values like ENU ($s = 0.26$) and methylnitrosourea (MNU; $s = 0.42$) are 'hard' electrophiles that have reduced selectivity and react more efficiently at both oxygens and nitrogens in DNA bases

Abbreviations: DDR, DNA damage response(s); EMEA, European Medicines Agency; EMS, ethyl methanesulfonate; ENU, ethylnitrosourea; EO, ethylene oxide; EPA, U.S. Environmental Protection Agency; FDA, U.S. Food and Drug Administration; *Hprt*, hypoxanthine–guanine phosphoribosyltransferase; LNT, linear nonthreshold; MGMT, O^6-methylguanine–DNA methyltransferase; MMS, methyl methanesulfonate; MNU, methylnitrosourea; *Pig-A*, phosphatidylinositol glycan complementation group A; TTC, Threshold of Toxicological Concern.

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by unimolecular (S_N1) mechanisms. EMS ($s = 0.67$) acts by mixed S_N1/S_N2 mechanisms that predominately lead to GC to AT transitions attributed to mispairing of O^6-ethylguanine with thymine during DNA replication (Jansen et al., 1995). Genotoxic alkylating agents generally are assumed to possess a linear dose–effect relationship for carcinogenic risk, so it has been assumed that there is no 'safe dose'; however, a limited number of recent *in vitro* and *in vivo* studies in addition to the Viracept-EMS case are beginning to challenge this assumption. In a key *in vitro* study using human lymphoblastoid cells, Doak et al. (2007) showed that EMS and MMS gave non-linear dose–response curves for hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene mutations and chromosomal damage, with a range on non-genotoxic low doses, whilst ENU and MNU gave linear dose–response curves for both endpoints over a similar dose range.

Synthesis and/or formulation of pharmaceuticals can generate genotoxic impurities present at low levels in the final drug products that may impose a safety concern (Müller et al., 2006; Jacobson-Kram and McGovern, 2007; Munro et al., 2008). For instance, strong acid/alcohol interactions during the process of drug salt formation may produce various alkylating agents including nearly two dozen alkyl halides, esters of alkyl sulfonic acids (mesilates, e.g., EMS and MMS), esters of aryl sulfonic acids (besilates and tosylates), and esters of sulfuric acid (Sobol et al., 2007). The genotoxicity of EMS and MMS has been well characterized in *in vitro* and *in vivo* test systems, while the majority of the other genotoxic impurities have been tested only in the Salmonella reversion assay and in *in vitro* assays to measure clastogenicity and DNA deletions (Sobol et al., 2007). However, the chemical structure of these compounds suggests that most are S_N2 alkylating agents, with fewer having mixed S_N1/S_N2 activities. Thus, the outcome of the Viracept-EMS case and follow-up studies may ultimately provide better guidance for managing risk from other genotoxic impurities in pharmaceuticals.

This commentary first provides a brief perspective on the potential impact of the Viracept-EMS case in the context of current knowledge of low-dose effects of DNA-reactive chemicals and the models used to assess health risks from genotoxic contaminants in pharmaceuticals or the environment. It also offers an outlook on future work building upon the Viracept-EMS case to test and confirm certain assumptions and hypotheses underlying the threshold risk assessment for EMS toxicity (Müller et al., submitted for publication) that will act as a cornerstone in developing biologically based risk assessment approaches for genotoxic agents mutually acceptable to both the pharmaceutical/chemical industry and regulatory authorities worldwide.

2. Impact

Historically, it has been generally assumed that a single molecule of a genotoxin like EMS or ENU could produce a mutation that ultimately developed into a tumor in a random fashion, and, thus, any exposure carried a cancer risk no matter how small the dose (Butterworth and Bogdanffy, 1999; Haber et al., 2001). The no threshold assumption of the 'single hit, single target' hypothesis for DNA-reactive agents was originally a science policy decision intended as a cautious approach to public health protection, and served as the basis of the application of the linear nonthreshold (LNT) model as a default to assess cancer risk from environmental or occupational exposure to genotoxins (Wiltse and Dellarco, 1996). Because low levels of DNA-reactive chemicals may be present in some pharmaceuticals, a different approach using the "Threshold of Toxicological Concern" (TTC) concept (Kroes et al., 2004) was used to develop a regulatory guideline to define an acceptable exposure level of genotoxic impurities in pharmaceuticals (EMEA, 2006). This guideline thus accepts that at certain defined levels of human exposure, genotoxins can present no significant risk. The regulatory assumption of no discernable threshold for DNA damage by genotoxic agents has been the subject of repeated albeit seemingly fruitless challenges over many years as emerging knowledge has shown that cancer is a mechanistically complex disease, and cells possess DNA damage response and repair processes providing protective effects at low-dose genotoxin exposures (Loeb, 1989; Calabrese and Cook, 2005; Cook and Calabrese, 2006; Bartek et al., 2007b). The Viracept-EMS case represents the first challenge that has the power to effect fundamental changes in regulatory practices for assessing health risk and setting exposure standards for genotoxins.

The guidelines for limits on genotoxic impurities in pharmaceuticals and carcinogen risk assessment of chemicals in the environment have evolved over the past decade. The TTC approach for establishing a generic human exposure threshold value for low levels of impurities resulting from manufacturing and formulation of pharmaceutical products is based upon extrapolation of toxicity data from an available database to a chemical compound for which the chemical structure is known, but no or limited toxicity data is available (reviewed in Müller et al., 2006; Jacobson-Kram and McGovern, 2007; Munro et al., 2008). Agencies regulating environmental chemicals have developed differing approaches; for example, the Guidelines for Carcinogen Risk Assessment from the U.S. Environmental Protection Agency (EPA) suggest that the most appropriate model(s) for risk extrapolation be used to incorporate the existing understanding of mode of action, with a preference for biologically based dose–response models (EPA, 2005). Incorporation of mode of action information into dose–response assessment reduces uncertainty in cross-species risk extrapolation. Without such information, the EPA guidelines (2005) recommended a LNT default from the point of departure identified in the range of observed data to the origin. Currently, there is a limited but increasing number of cases for specific carcinogens where mode of action data have been developed to support deviation from linearity, such as $alpha_{2\mu}$-globulin and renal neoplasia in the male rat, regenerative cell proliferation and nasal tumors in formaldehyde-exposed rats, and sustained necrosis and regenerative cell proliferation and liver cancer in mice gavaged with chloroform (Butterworth and Bogdanffy, 1999; Andersen et al., 2000; Haber et al., 2001). The same is true for particular genotoxins that do not directly interact with DNA or cause specific changes in animal physiology. Examples of mechanisms of genotoxicity that may lead to non-linear or thresholded dose–response relationships include interaction with the spindle apparatus of cell division leading to aneuploidy, topoisomerase inhibition, inhibition of DNA synthesis (Henderson et al., 2000), and physiological perturbations (e.g., induction of erythropoeisis, hyper- or hypothermia) (Tweats et al., 2007); this is recognized in the EMEA guideline on genotoxic impurities (EMEA, 2006).

In the case of DNA-alkylating agents that may include the bulk of potential genotoxic chemical carcinogens, there is growing interest in the generation of low-dose mutation data and dose–response models that may support deviation from the LNT extrapolation procedure. A recent framework analysis to examine mode of action data for several DNA-alkylating agents, and the default assumption that cancer can be expected to be linear at very low doses, concluded that biomarkers of exposure are usually linear at low doses, whereas induction of gene mutations by some chemicals may not be linear in the low-dose region where mutant frequencies approach spontaneous background levels (Swenberg et al., 2008). There is a small but emergent literature on low-dose *in vitro* mutagenesis induced by alkylating agents, suggestive of low-dose thresholds, but there are still major gaps in our knowledge, particularly *in vivo* (Sofuni et al., 2000; Jenkins et al., 2005; Doak et al., 2007; Swenberg et al., 2008; Walker et al., submitted for publication).