PERGAMON

European Journal of Cancer 39 (2003) 715–717

European Journal of Cancer

www.ejconline.com

Editorial Comment

The best of times. . .the worst of times

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Received 18 November 2002; accepted 11 December 2002

Times are good for the study of biological pacemakers. With the sequencing of the human genome has come a rapid increase in understanding of the complex function and control of 'clock genes'. In mammals, the master oscillator appears to be located in the suprachiasmatic nuclei (SCN) of the hypothalamus and to be entrained by light through neural pathways connecting the retina to the SCN, in part via a p42/44 mitogen activated protein kinase mechanism [1]. Semi-autonomous circadian pacemakers also exist in peripheral tissues. Approximately 2% of all genes in rat fibroblasts have been shown to have robust circadian rhythmicity in their expression [2,3].

Disruption of these rhythms may predispose to a number of diseases, including cancer. For example, it has been reported that mice deficient in the *mPer2* gene show a marked increase in tumour development, and that destruction of the SCN in mice is associated with accelerated growth of tumour xenografts [4,5]. Popular attention has been caught by reports that graveyard shift work is associated with an increased risk of breast cancer in women, and that an increased number of long-distance flights may be associated with an increased risk of prostate cancer in male pilots [6,7].

Interest in the importance of diurnal variation for human function is, however, far from new. Aside from the classic rhythms of secretion for cortisol and other hormones, it has been recognised for years that there are cycles both in biological parameters, such as blood pressure (which drops at night and peaks during mid-morning for normal subjects), and in the occurrence of pathological events, such as heart attacks (which are most frequent within the first few hours of awakening [8]). Many of these cycles have a potential role on drug effects. These include variability in target tissue sensitivity (e.g. variation in cell proliferation kinetics), drug absorption and distribution (e.g. variation in gastric acid production), drug metabolism (e.g. variation in hepatic blood flow or enzyme activity [9-11]), and drug excretion (e.g. nocturnal increase in urine acidity [12]).

Many anticancer agents with a variety of intracellular targets show clear periodic variations in both toxicity and efficacy based on the time of day (or, more correctly, time of light/dark cycle) of administration in animal models. These include celecoxib [13], doxorubicin, cisplatin, docetaxel [14], and 5-fluoro-2′-deoxyuridine (FUDR) [15]. Given the narrow therapeutic index of most antineoplastic drugs, the promise of being able to improve results by accounting for diurnal rhythms is tantalising to cancer clinicians, but attempts to implement the available information have been frustrating. Studies in mice that strictly control conditions including light cycle, feeding schedule, and food and drug intake do not necessarily clarify what parameters are most responsible for the diurnal variability in toxicity and/or efficacy observed. For example, fasting has been shown to alter circadian rhythms of acetaminophen lethality and glutathione levels in mice, even when the light/dark schedule remains fixed [16].

For no anticancer drug class has there been more effort directed to untangling the potentially circadian-related variation in toxicity and efficacy than for the fluoropyrimidines. 5-Fluorouracil (5-FU) is a widely used anticancer drug with a short half-life; over 80% of the drug is catabolised by the enzyme dihydropyrimidine dehydrogenase (DPD). In rats, the hepatic extraction rate of 5-FU peaks in the mid-light period (rest period for a rat), which corresponds to their time of peak liver DPD activity [17]. Porsin and colleagues [18] and others [19] confirm that liver DPD activity in mice peaks during the light period. However, results in humans have been exceedingly variable. Harris and

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doi:10.1016/S0959-8049(02)00866-3

colleagues [20] reported that 5-FU levels during a prolonged intravenous (i.v.) infusion are lowest at 11 p.m. (early rest period) and that peripheral blood mononuclear cell DPD activity was highest at about the same time. Other studies have reported 5-FU levels during protracted infusions to be lowest at 1 p.m. [21] or to have no consistent time of peak and trough between or within subjects [22]. Similarly, studies of the time course of DPD activity in human PBMC have reported wide inter- and intra-individual variation in the timing of the peaks and troughs in DPD activity [23].

5-FU administration to patients completely deficient in DPD activity results in severe toxicity, including mucositis, neutropenia neuropathy and death [24]. Fortunately, complete DPD deficiency is a rare event, but patients with very low DPD activity may also be at increased risk for severe 5-FU toxicity. Since 5-FU is widely used as adjuvant therapy for breast and colon cancer patients without detectable disease, there could be particular utility in identifying patients at risk for severe toxicity. Using measurements of DPD activity for predicting toxicity is currently not practical. The assay, which involves PBMC isolation and high performance liquid chromatography to quantitate the conversion of radioactive 5-FU to dihydrofluorouracil, is not suitable for general screening, and the range and variability of DPD activity over time make single measurements less useful. In one report, the intrasubject range of activities in six normal volunteers during a 24-h time span varied from 2.1- to 3.2-fold [23].

Recently, semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) methods for assaying *DPD* mRNA have been developed. These have been used for assaying DPD expression in small biopsies of tumours and normal tissues. As Porsin and colleagues [18] note, point measurements of *DPD* mRNA do not necessarily correlate with DPD enzyme activity [25]. This will probably be true in many cases, since there is control of enzyme activity at many points, including rate of protein synthesis, post-translational modifications such as phosphorylation, and rate of degradation [26]. If the variability (circadian or otherwise) of the DPD enzyme were controlled *only* at the post-transcriptional level, then PBMC mRNA levels for a given patient might be fairly constant over time and hence be useful as a screening test for patients with very low or absent DPD activity.

Porsit and colleagues [18] therefore used a mouse model to investigate whether there are diurnal fluctuations in liver *DPD* mRNA level (DPD expression). Their answer is yes. The fluctuations in mRNA level were, in fact, larger than the fluctuations in DPD activity, and the peak level of mRNA occurred at a different time (12 h later; early dark/wake period) than the peak DPD activity (early light/rest period). The authors conclude that mRNA expression therefore cannot be used to screen for DPD deficiency; a subject might well appear to have very low levels at a given time point, but have overall adequate enzyme activity. This conclusion is supported by the work of Shimizu and colleagues [27] who found, in a small number of mice measured at two time points, that liver *DPD* mRNA levels varied, being higher at 2 a.m. (dark period) than 2 p.m. (light period).

An alternate approach to screening for DPD deficiency is genotyping. Raida and colleagues [28] reported that 6 of 25 cancer patients with severe 5-FU-related toxicities had the exon 14-skipping G→A mutation in the *DPD* gene (DPD*2A). Five were heterozygous and one was homozygous. 2 of the heterozygous cases and the homozygous case died. 1.8% of Caucasions are heterozygous for this mutation [29]. While homozygotes do appear to be at risk for severe toxicity, the risks for heterozygotes are uncertain. Moreover, there are clearly many cases of severe toxicity unrelated to this particular mutation. Routine genetic screening is therefore not yet ready for clinical use [30]. At this time, cancer clinicians are left with the traditional, empirical approach to estimating drug sensitivity: administration of a trial dose and determination of the toxicity to the patient. Given the rapid pace of basic science advancement, it would be nice to have something better to offer.

What are the clinical questions with regard to circadian variation in drug-metabolising enzyme activity?

Are enzyme activities variable in humans? Clearly.

Do endogenous circadian pacemakers control this variation? Possibly, at least in some cases. However, endogenous pacemakers do not appear to be the largest cause of variability in DPD enzyme activity. Otherwise normal volunteers, who presumably have preserved circadian rhythms, should have more reproducible cycles of DPD activity.

Can we overcome this variability when trying to screen for enzyme deficiencies by assessing gene expression level rather than enzyme activity? Not in this case, and probably not in many cases. First, there may well be a rhythmic component to mRNA levels for many human enzymes, just as there are in mice. Moreover, when PBMC *DPD* mRNA expression was evaluated in humans, levels were very variable, just as for DPD activity and 5-FU levels. Raida and colleagues [31] evaluated PBMC *DPD* mRNA expression every three hours in humans, 10 patients with gastrointestinal carcinomas and preserved cortisol rhythms, and five healthy controls. In the cancer patients, *DPD* mRNA exhibited no consistent circadian rhythm, and the mean standard deviation was 25%. Normal subjects exhibited a peak of *DPD* expression at 5 a.m. and a trough at 2 p.m. when all of the data were pooled, but only two individuals showed circadian variation that could be fit to a cosine wave in a statistically significant manner.

Identification of genetic polymorphisms will likely eventually be a more productive means of recognising

subjects at risk for excessive chemotherapy toxicity than assaying mRNA levels. However, this fact does not diminish the possibility that there may be situations in which incorporation of information about diurnal variations in enzymatic activity or target tissue drug sensitivity for populations as a whole will allow optimisation of drug delivery.

Acknowledgements

Thanks to Drs Mark Ratain, Eileen Dolan and Suzanne Conzen for their helpful discussions.

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