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# Cytotoxicity determination without photochemical artifacts

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**Abstract**

A study was performed to improve cytotoxicity determinations by eliminating flavin-mediated photosensitization from tests with KB cells, NCI-H69 cells, P-glycoprotein expressing KBC5-8 cells, MRP1-expressing H69AR cells, and A240286S human lung adenocarcinoma cells. Growth inhibition by *cis*-platin, doxorubicin, etoposide, gemcitabine, taxol, vincristine, vinblastine, and vinorelbine was determined under flavin-protecting conditions using flavin-free culture media with fetal bovine serum as the only source of flavins. As compared to conventional tests, the $IC_{50}$ values determined under flavin-protecting conditions reflected increased apparent drug cytotoxicities, and were flawlessly reproducible. Flavin-mediated photosensitization should, therefore, be strictly eliminated from in vitro experiments involving cytotoxic and other drugs.


*Keywords:* Chemosensitivity testing; KB cells; NCI-H69 cells; H69AR cells; $IC_{50}$; Riboflavin

## 1. Introduction

Cytostatic drug effect depends on drug concentration whose accurate determination is of key scientific and practical importance. Unfortunately, however, experience has shown that major problems arise if drug cytotoxicities are being investigated in cell cultures, particularly in the context of predictive testing of human tumor specimens. The problems addressed here are of such severity that related reports are commonly greeted with profound scepticism [1]. Indeed, the term 'guesstimates' has been used to ironically appreciate the state of the art in this area of research [2]. Cytostatic drug action is, however, well reproducible under experimental conditions in vivo. This suggested to us that factors inherent in the in vitro setting might be responsible for the poor reproducibility of cytotoxicity testing. Riboflavin-mediated photosensitization of the vinca alkaloids vinblastine, vincristine, and vinorelbine was identified as the reason behind both poor test reproducibility and

*Abbreviations:* CI, confidence interval; CIS, *cis*-platin; DOX, doxorubicin; ETO, etoposide; GEM, gemcitabine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); $IC_{20}$, $IC_{50}$, $IC_{80}$, 20, 50, 80 percent growth inhibitory concentration; MEM, minimum essential medium; PBS, phosphate buffered saline; RPMI, Roswell–Park Memorial Institute Medium; TAX, taxol; VCR, vincristine; VLB, vinblastine; VRL, vinorelbine.

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falsely decreased apparent cytotoxicities of these drugs in tests with cultures of Ehrlich–Lettré mouse ascites tumor cells [3]. Soon afterward, riboflavin-mediated photosensitization of doxorubicin was shown to be suppressed by adding anti-oxidants to the media [4]. More recently identified targets of flavin-mediated photosensitization in cell culture media include nitric oxide [5], L-DOPA/dopamine [6], and the expression of bcl-2 [7].

Riboflavin and/or its derivatives, riboflavin phosphate and flavin adenine dinucleotide, are present, in varying amounts, in cell culture media. Flavin-mediated photoreactions generate reactive oxygen species in the media [8]. The media contain considerable concentrations of amino acids such as tryptophan, methionine, tyrosine, and histidine which strongly enhance such photoreactions. Hence, upon exposure to visible or near-UV light, the media become abundant in reactive oxygen species [9] capable of triggering numerous deleterious effects. In addition to the photosensitization of drugs, flavin-mediated artifacts in cell cultures include phototoxicity which kills cells [10–12] by inducing apoptosis [13], and DNA damage leading to mutations [14].

As a means to prevent flavin-mediated photochemical artifacts in cell culture experiments, flavin-free culture media were introduced [15]. Here, we describe the application to several drug-sensitive and drug-resistant human tumor cell lines of such media to optimize cytotoxicity determinations for a variety of cytostatic drugs.

## 2. Materials and methods

### 2.1. Reagents and cytostatic drugs

Unless otherwise specified, commercial products were purchased from companies in Germany. Aqua ad iniectabilia (referred to as water) and Casyton were obtained from Braun/Melsungen and Schärfe System/Reutlingen, respectively. A fluorescein-conjugated monoclonal antibody P-glycoCHEK C219 from Centocor was obtained from Isotopen Diagnostik CIS GmbH, Dreieich. Anti-fade reagent 1,4-diazabicyclo [2.2.2]octane and bovine serum albumin fraction V were products of Sigma/Munich. Trypsin 1:250 was a product of Difco (Detroit, MI, USA). Taxol, vincristine, and colchicine were obtained from Sigma/Munich. Pharmaceutical preparations of each of the following were used: vinblastine and gemcitabine (Lilly Deutschland/Gießen), vinorelbine (Pierre Fabre/Freiburg), *cis*-platin (Medac/Hamburg), etoposide (Bristol-Myers, Beragena/Baden–Baden) and doxorubicin (Pharmacia and Upjohn/Erlangen). Compounds without specified origin were products of E. Merck/Darmstadt. Frozen aqueous stock solutions of the vinca alkaloids, *cis*-platin, gemcitabine and doxorubicin as well as methanolic stock solutions of taxol and etoposide were kept at −20 °C until use. Drug concentrations (except for *cis*-platin) were verified spectroscopically. During experiments, all dilutions of water soluble cytostatic drugs below 0.1 mM were made in water containing 0.2 mg bovine serum albumin fraction V per mL.

### 2.2. Riboflavin-free cell culture media and further photochemical precautions

The cell culture media MEM [16] and RPMI 1640 [17] were prepared, in powder form, by Biochrom/Berlin, according to the original formulation but without riboflavin [15], glutamine, sodium bicarbonate and phenol red. Sodium bicarbonate and HEPES (Carl Roth/Karlsruhe) were added to give final concentrations of 13.5 and 4.5 mM, respectively, before dissolving the powder in water. After pH adjustment to 7.38 and osmolality determination (MEM: $279 \pm 2$ mOsm/kg, RPMI 1640: $277 \pm 2$ mOsm/kg), the media were sterile filtered under 0.2 bar $N_2$ through hollow fiber filters (Spectrum, Laguna Hills, CA, USA). Media were stored in brown glass bottles (Schott/Mainz) at 8 °C. Glutamine (Biochrom/Berlin) was added from frozen stock immediately before use. No antibiotics were used in the present study. The culture media were completed by adding 10% (v/v) of fetal bovine serum (Integro/Zaandam, The Netherlands). Previous tests had shown that this serum preparation allowed sensitive cytotoxicity determinations [18]. To ensure flavin-protecting illumination conditions [3], serum was thawed, stored at 4 °C, and added to the media in the absence of all light sources except a 30 W SOX sodium discharge lamp (Philips/Hamburg). Such illumination prevailed throughout all subsequent procedures performed with