ELSEVIER

Journal of Chromatography B, 664 (1995) 241–246

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

# *In vitro* toxicity assays for dye ligands used in affinity chromatography

P. Santambien[a], S. Sdiqui[b], E. Hubert[b], P. Girot[a], A.C. Roche[b], M. Monsigny[b], E. Boschetti[a,*]

[a] *BioSepra SA, 35 Avenue Jean Jaurès, 92390 Villeneuve-la-Garenne, France*
[b] *CBM–CNRS, 45045 Orleans, France*

**Abstract**

Some reactive textile dyes have been used for years as biomimetic ligands in protein purification. There has been reluctance, however, to use these dyes on a large scale for therapeutically applicable proteins for fear of possible dye leakage and consequent contamination. Therefore, toxicological data are necessary to quantify the level of this hazard. This study deals with a series of *in vitro* toxicity investigations with eukaryotic cells (growth, polyploidy, etc.) and with prokaryotic cells (*Escherichia coli*) for genotoxic studies. Both approaches demonstrated a lack of or slight toxicity for Reactive Blue 2 and Reactive Red 120 and their derivatives over the range 10–62.5 μg/ml in several assays.

## 1. Introduction

Among the most popular ligands used in affinity chromatography for protein purification, biomimetic dyes play a growing role. Their attractiveness is connected with their wide availability and their chemical stability in extreme conditions of pH and ionic strength while displaying under optimized working conditions a good specificity for a number of proteins. Although frequently used on the laboratory scale, immobilized dyes are still rarely used on a large scale for the purification of biopharmaceuticals. This paradoxical situation can be explained by the potential risk of leakage of dye ligands with consequent contamination of purified biologicals. Even though efforts have been made in order to reduce this risk by using more stable chromatographic matrices and linkages, and by the availability of very sensitive immunochemical techniques to detect trace levels of these dyes [1,2], almost no data exist on their toxicity. In this paper, results of *in vitro* toxicity studies concerning two major affinity dyes, Reactive Blue 2 and Reactive Red 120, are discussed.

## 2. Experimental

### 2.1. Chemicals

Reactive Blue 2 and Reactive Red 120 (also called Cibacron Blue F3GA and Procion Red HE3B, respectively) were obtained from Sigma (St. Louis, MO, USA). Dye derivatives were specifically prepared by Vilmax (Buenos Aires,

* Corresponding author.

0378-4347/95/$09.50 
*SSDI* 0378-4347(94)00351-3

Argentina). All other pure chemicals and biologicals were obtained from Aldrich (Brussels, Belgium) and Sigma, respectively. Genotoxic assay kits were purchased from Beak Consultants (Brampton, Canada).

### 2.2. Culture of HeLa cells

HeLa cells (ATCC: CCL2) were suspended at a density of $3 \cdot 10^5$ cells/ml in a DMEM medium containing 10% of foetal bovine serum. The cell suspension was then distributed in microtitration plate wells in 0.1-ml aliquots and incubated for 4 h at 37°C in the presence of 5% $CO_2$. Dye solutions (1 mg/ml) were prepared in water–dimethyl-sulfoxide (90:10). They were then diluted to 0.5 mg/ml with culture medium and added to cell cultures to final concentrations ranging from 0.122 and 250 $\mu$g/ml.

Cultured cells were incubated for 24 h at 37°C in the presence of 5% of $CO_2$ and the dyes were eliminated by repeated washings with a sterile phosphate-buffered saline solution. A 0.1-ml volume of a 0.2% Crystal Violet solution in ethanol was then added to each well and, after extensive washing, the cells were dried under an air stream. To the dry wells, 0.5-ml aliquots of a 0.3% sodium dodecyl sulfate aqueous solution were added and the wells were agitated vigorously to solubilize the cell material. The absorbance of the samples was measured at 570 nm. Absorbance values were proportional to the number of cells.

### 2.3. Culture of human MRC-5 cells and polyploidy determination

MRC-5 cells (human fibroblasts, ATCC:CCL 171) were grown in 25-$cm^2$ flasks in the presence of 7.5% of foetal bovine serum in DMEM medium. The initial cell density was $1.3 \cdot 10^4$ cells per flask; two passages per week were performed in the absence and in the presence of 10 $\mu$g/ml of the studied dyes. Each subculture was submitted to a trypsinization step to collect and count the cells. All experiments were carried out in triplicate.

After six passages, MRC-5 cells were cultured once more on microscopic slides for a time corresponding to the cell division time. Mitosis was then blocked in the metaphase stage and the cells were submitted to hypotonic shock to disperse the chromosomes. Each slide was stained using the standard Giemsa procedure. Selection of mitoses for chromosome counting was effected microscopically. About 500 mitoses per slide were examined and polyploidy cells counted on five different slides.

### 2.4. Dye internalization studies in cultured cells

HeLa cells were cultured as described above. They were then added to 10 and 100 $\mu$g/ml dye dissolved in the culture medium and incubated for 1 h at 37°C. After washing, the cells were fixed with 1% formaldehyde for 1 h at 4°C. The cell walls were then permeabilized with a solution of 0.2% saponin in physiological saline phosphate buffer containing 1% bovine albumin for 15 min at 25°C. They were then incubated in the presence of specific antisera to the dyes. After repeated washings to eliminate excess of reagent, adsorbed antibodies were revealed with fluorescein-labelled anti-immunoglobulins G under the same conditions. Localization of dyes inside the cells was revealed by confocal microscope observation. 1,4-Diazacyclo[2.2.2]-octane (1%) in physiological buffer diluted with 50% glycerol was used to avoid photoattenuation of the fluorescence [3].

### 2.5. Genotoxic studies

Genotoxic activity of the dyes and derivatives was determined using the SOS-Chromotest. This method involves a culture of *Escherichia coli* strain containing $\beta$-galactosidase gene controlled by an SOS system which controls the cell division inhibition [4]. In the presence of genotoxic products during *E. coli* culture, the SOS system is activated and $\beta$-galactosidase enzyme released. The extent of activation is proportional to the genotoxic power of the studied molecules and their concentration. Bacterial culture in

exponential growth was diluted tenfold with the culture medium and 0.6-ml aliquots were transferred into wells of a microtitration plate. To each well, 20 $\mu$l of dye solution in a mixture of nine volumes of water and one volume of dimethyl sulfoxide were added. The dye concentration varied between 3 and 39 $\mu$g/ml. After incubation for 2 h, the $\beta$-galactosidase activity was measured.

As standard genotoxic compounds, 2-aminoanthracene and quinoline N-oxide were used in parallel. As the dyes could possibly inhibit all protein synthesis generating false-negative results, alkaline phosphatase activity was also measured. Moreover, considering that the native dyes themselves are not genotoxic, this does not prove that their possible metabolites are not toxic. To avoid this potential problem, a preliminary metabolic activation of dyes was also made in parallel using liver S-9 fraction extract [5].

## 3. Results and discussion

In a previous study [6], it was observed that the dyes leached from chromatographic supports do not necessarily have the same chemical structure as the native dyes. In this work, we extended the toxicity investigations to dye derivatives, as shown in Fig. 1. Special attention was focused on the dye derivatives containing a carboxy terminal spacer because they have been identified as major released molecules in strongly alkaline conditions starting from Trisacryl dye derivatives [6].

One of the most important *in vitro* toxicity tests is the behaviour of living cells in culture in the presence of investigated molecules. In this work, a preliminary dose–effect study was made on HeLa cells in the presence of various dyes at different concentrations for 24 h. Fig. 2 shows that the presence of dyes did not influence the cell viability at dye concentrations up to 62.5

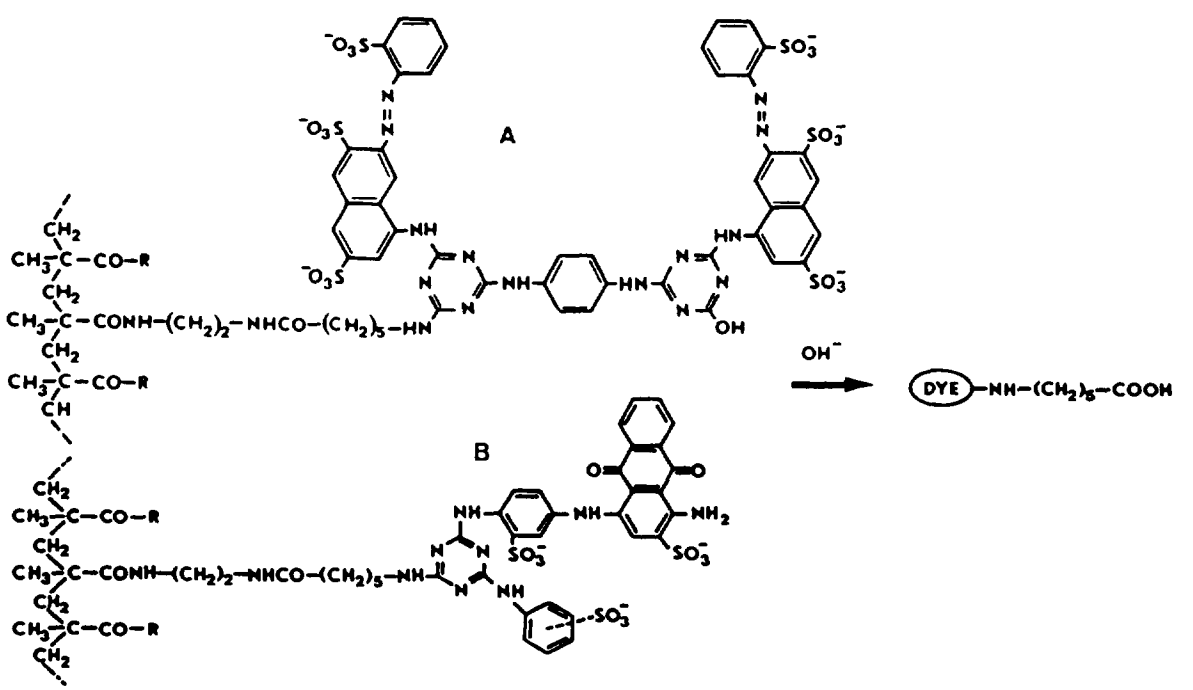


Fig. 1. Structure of studied immobilized dyes on a synthetic support and released dyes after strong alkaline degradation. (A) Reactive Red 120; (B) Reactive Blue 2.

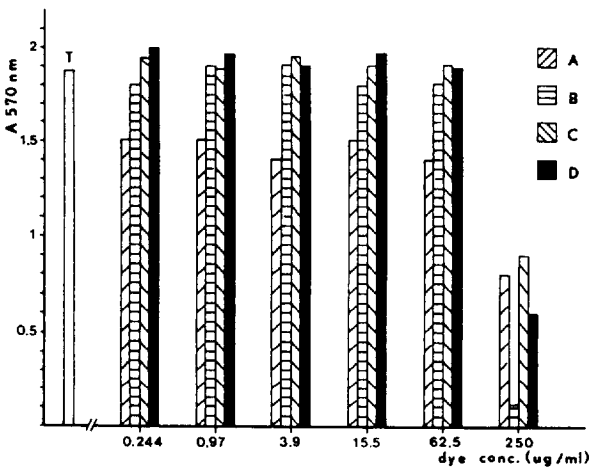


Fig. 2. Estimate of the amount of viable HeLa cells (absorbance at 570 nm) in the presence of various amount of dyes during 24 h. (A) native Reactive Blue 2; (B) native Reactive Red 120; (C) carboxy derivative of Reactive Blue 2; (D) carboxy derivative of Reactive Red 120. T = standard culture in the absence of dyes.

μg/ml. At 250 μg/ml, the cell density was considerably diminished. This was apparently not related to toxicity but rather to inhibition of cell adhesion. It should be noted, however, that a dye concentration as high as 62.5 μg/ml was not toxic in the assay, and that such a high level of leakage never occurs in liquid chromatography [1,2], where the highest leakage found in extreme conditions is around 1–2 μg/ml.

MRC-5 cell culture in the presence of dyes for an extended period of time (21 days) confirmed that dyes had no influence on the cell growth (see Fig. 3) and morphology. The cell generation (or the cell division time) in the presence of dyes was similar to that of a standard culture and the behaviour of MRC-5 cells was the same (cell shape, dimensions and adhesion). According to the described experimental protocol, a chromosome analysis was effected on a large number of metaphases to determine the induction of polyploidy (see Table 1). With an average of about 2000 metaphases per culture, the polyploidy level was in the range of that observed from the control culture. Polyploidy levels of 3.4%, considered as the limit of toxicity were never observed; experimental data were in the range 1.3–2% versus 1.33% for the standard culture.

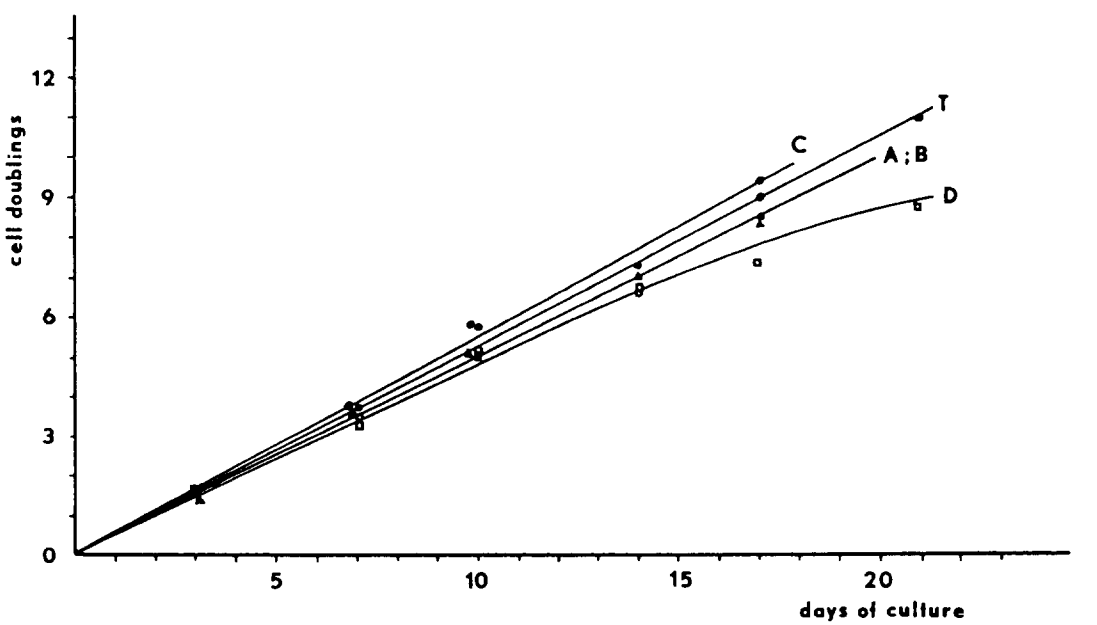


Fig. 3. Growth curves of MRC-5 human cells *in vitro* over 21 days in standard conditions (see Experimental) in the presence of (A) 10 μg/ml of Reactive Blue 2, (B) 10 μg/ml of Reactive Red 120, (C) 10 μg/ml of carboxy derivative of Reactive Blue 2 and (D) 10 μg/ml of carboxy derivative of Reactive Red 120. T represents the standard growth curve in the absence of dyes.

Table 1
Induction of polyploidy in MRC-5 cells in culture

| Dye | No. of slides | No. of metaphases | No. of polyploid cells | Polyploidy (%) |
|---|---|---|---|---|
| Control | 5 | 2552 | 34 | 1.33 |
| Reactive Blue 2 (RB 2) | 5 | 2584 | 51 | 1.58 |
| Reactive Red 120 (RR 120) | 5 | 2530 | 40 | 1.58 |
| COOH-RB 2 | 5 | 1745 | 34 | 1.94 |
| COOH-RR 120 | 5 | 2572 | 35 | 1.36 |

As regards genotoxic activity of the studied dyes, no secretion of β-galactosidase was induced in *E. coli* in the presence of dyes at concentrations between 3 and 39 μg/ml. Known genotoxic products, such as 2-aminoanthracene and quinoline N-oxide showed, as expected, a rapid increase in genotoxicity between 3 and 39 μg/ml.

The absence of enzyme activity, and consequently of the toxicity of the dyes, was not related to inhibition of protein synthesis, since alkaline phosphatase activity was still present as in the controls (see Fig. 4). As suggested [2], metabolic activation was also ineffective in producing toxicity.

Dye-related mutagenicity has been reported in a limited number of papers in the last decade.

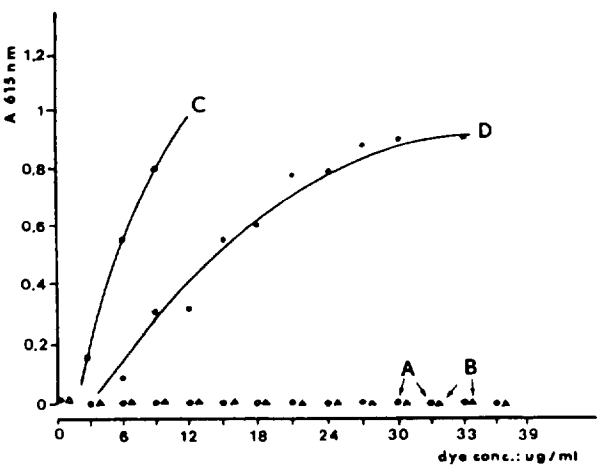


Fig. 4. Determination of the genotoxic effect of the dyes and their derivatives. (A) native blue and red dyes; (B) carboxy derivatives of blue and red dyes; (C) quinoline N-oxide; (D) 2-aminoanthracene.

Brown and Brown [7] demonstrated that the mutagenicity potential of anthraquinone dyes (Reactive Blue 2 belongs to this group) is related to the presence of $NO_2$ groups. However, the presence of OH groups can induce a certain level of mutagenicity, as in the case of anthragallol and purpurine (three OH groups) and of anthrafurine (two OH groups). Anthraquinone compounds containing sulfonate groups and/or amino groups did not show mutagenicity [8]. More recently, Przybojewska et al. [9] showed the absence of mutagenicity of Alcian Blue 62, which is structurally close to Reactive Blue 2.

Azo dyes such as Reactive Red 120 represent a very large group of dyes used in the textile and food industries and could possess a certain degree of mutagenicity, as discribed [5]. However, other reports [8] showed that Procion Red H3B (structurally identical with Reactive Red 120, the latter being a dimer) is totally devoid of mutagenic activity. According to the results presented above, it can be stated that the studied dyes are well tolerated by cells in culture and seem not to be toxic, at least in the frame of this study. Nevertheless, in order to know more about the possible interaction with living cells, we extended our investigations to the possible penetration of dyes into the cell cytoplasm and to their local accumulation. Results from confocal microscopy showed that dyes can cross the cell walls in regular culture only when their concentration was at least 100 μg/ml and internalization of studied dyes seemed to be independent of their chemical nature. Below this concentration, all dyes remained outside the cell walls. When dyes penetrated into the cell cytoplasm,

they were concentrated in well individualized vesicles; no diffuse images were apparent and dyes never penetrated the cell nucleus.

## 4. Conclusions

Within the limits of this study, our conclusion is that the two major dyes used as ligands in affinity chromatography are not toxic. This statement can also be extended to some derivatives generated by sorbent deterioration consequent upon a strong alkaline treatment (e.g., Blue Trisacryl). This study contributes to the knowledge of the biological behaviour of immobilized dyes after extensive work in the area of dye leakage and of immunodetection [1,2]. Highly sensitive assays permit one to determine if traces of dye ligands are released into the column effluents and toxicity data allow one to establish whether these concentrations are toxic or not. This approach, developed specifically for dye affinity chromatography, should also be extended to a number of other chromatographic media, and to other materials in contact with critical biological preparations.

## Acknowledgements

This work was partially supported by a grant from the French Ministry of Research and Technology as a part of a Eureka project (EU-384). We thank Dr. Mazza and Dr. Outumuro of Vilmax for their continuous support and for supplying dyes and dye derivatives.

## References

[1] P. Santambien, P. Girot, I. Hulak and E. Boschetti, *J. Biophys. Biochem. Methods*, 24 (1992) 285.
[2] P. Santambien, I. Hulak, P. Girot and E. Boschetti, *Bioseparation*, 2 (1992) 327.
[3] G.D. Johnson, R.S. Davidson, K.C. MacNamee, G. Russel, G. Goodwin and E.J. Holborow, *J. Immunol. Methods*, 55 (1982) 231.
[4] P. Quillardet, O. Huisman, R. D'Ary and M. Hofnung, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 5971.
[5] B.N. Ames, W.E. Durston, E. Yamasaki and F.D. Lee, *Proc. Natl. Acad. Sci., U.S.A.*, 70 (1973) 2281.
[6] E. Boschetti, O. Bertrand, P. Girot and P. Santambien, in A. Faure and P. Doinel (Editors), *Protein Purification Technology*, GRBP, Grenoble, 1992, p. 91.
[7] J.P. Brown and J.R. Brown, *Mutat. Res.*, 40 (1976) 203.
[8] S. Venturini and M. Tamaro, *Mutat. Res.*, 68 (1979) 307.
[9] B. Przybojewska, B. Baranski, E. Spiechowicz and K. Sitarck, *Toxicol. Lett.*, 40 (1988) 183.