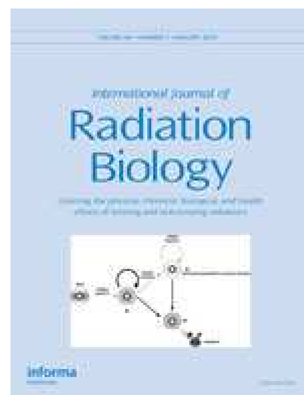

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LETHAL AND MUTAGENIC INTERACTIONS BETWEEN γ-RAYS, CISPLATIN AND ETOPOSIDE AT THE CELLULAR AND MOLECULAR LEVELS.

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Complete List of Authors:	Lillo, Olga; Fac. of Medicine, Lab.Radiobiology. Dpt.of Biophysics Bracesco, Nelson; Fac. of Medicine, Lab.Radiobiology. Dpt.of Biophysics Nunes, Elia; Fac. of Medicine, Lab.Radiobiology. Dpt.of Biophysics

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LETHAL AND MUTAGENIC INTERACTIONS BETWEEN γ-RAYS, CISPLATIN AND ETOPOSIDE AT THE CELLULAR AND MOLECULAR LEVELS.

Authors

*)**Lillo, Olga. MD. Assistant Professor**. Lab. Radiobiology. Dpt. of Biophysics. Faculty of Medicine. Universidad de la República.

Bracesco, Nelson. MSc. Assistant Professor. Lab. Radiobiology. Dpt. of Biophysics. Faculty of Medicine. Universidad de la República.

Nunes, Elia. MD. PhD. Professor in charge. Lab. Radiobiology. Dpt. of Biophysics. Faculty of Medicine. Universidad de la República.

*)**Corresponding author:** Olga Lillo

Faculty of Medicine, Dpt. Biofisica. Univ.de la Republica (UR)

Gral. Flores 2125, CP11800. Montevideo. URUGUAY.

Tel: 00598 2 9243414 int 3601

Fax: 00598 2 9248784

Email: lucefa@adinet.com.uy

Running title: Interaction between γ-rays, cisplatin and etoposide

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ABSTRACT

Purpose: We analyzed the lethal and mutagenic interactions between γ-rays, cisplatin(Pt) and etoposide(E), three agents used in tumour chemoradiotherapy. Corresponding results at cellular and molecular levels could provide additional elements on involved mechanisms and, on antitumor activity and toxicity in combined cancer treatments.

Materials and methods: The yeast *Saccharomyces cerevisiae* SC7K(*lys2-3)* (auxotrophic for lysine) was used as eukaryotic model. Exponential growing cells were exposed to the mentioned agents, as single and combined treatments. Lethal and mutation interaction equations were determined as a function of doses according to quantitative models (Ager and Haynes,1990). DNA double-strand breaks were evaluated immediately after treatments, through pulsed-field electrophoresis and laser densitometry.

Results: All three agents induced significant mutant frequency. The γ+Pt+E combination determined maximal lethal and mutagenic synergism, followed by γ+Pt and γ+E combinations. Meanwhile, Pt+E combination showed lethal additivity and very low mutagenic synergism. The highest DNA degradation observed immediately after treatment corresponded to the γ+Pt+E, followed by γ+Pt and γ+E. Regarding single treatments maximal DNA fractionation corresponded to γ-exposure.

Conclusions: Synergistic lethal and mutagenic interactions indicate crosstalk between non-homologous end joining, homologous recombination and postreplicative repair pathways. Pt+E additivity indicate independence of involved repair pathways. Furthermore, the quantification of interactive events may be an additional suitable tool in tumour therapy planning.

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INTRODUCTION

Combined treatments using ionizing radiations (IR) and chemical agents are widely used in oncology treatments. The concurrent chemoradiotherapy is a treatment modality in several solid cancer localizations (e.g. head and neck, lung, esophagus, anus) (Milas and Cox, 2003), being the agent interactions a hot topic. At the molecular level, the interactions between agents depend on nature and site of damage as well as on the induced DNA-repair pathways.

In our present work we analyzed the combined lethal and mutagenic effect of IR (γ-rays), cisplatin (Pt) and etoposide (E). These agents are used in the concurrent chemoradiotherapy of certain tumors. It is known that γ-rays can induce, depending on absorbed dose, DNA single- and double-strand breaks, base loses and their oxidative modifications at the nano- to picometer level (Coleman, 2001). Cisplatin can induce Pt-DNA adducts, DNA interstrand and intrastrand cross-links, and DNA-protein cross-links (Zamble and Lippard, 1995; McA'Nulty and Lippard, 1996; Gallagher et al., 1997; Manic et al., 2003). Furthermore cisplatin interacts with glutathione, the most important natural radioprotector (Eastman, 1987). Etoposide induce double-strand breaks (DSB) at DNA level by stabilization of the DNA-topoisomeraseII covalent complex and interfering with the ability of the enzyme to ligate cleaved nucleic acid molecules (Degrassi et al., 2004**;** Nitiss, 2009, for reviews). In eukaryotic cells, the processing of double-strand breaks induced by ionizing radiations takes place mainly through homologous recombination (HR) and non homologous end joining (NHEJ) (Siede et al., 1996; Friedl et al., 1998; Nunes et al., 2008; Shrivastav et al., 2008; Huertas, 2010). Abasic sites and base modifications are processed mainly by base excision repair (BER), nucleotide excision repair (NER) and mismatch

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repair (MMR). In case of spatial accumulation, these lesions can further be processed by recombination (Friedberg et al., 2006, for review). Pt lesions are processed in part by the above mentioned excision repair pathways, but also by recombination and postreplicative repair systems in unicellular organisms and in human cells (Gallager et al., 1997; Salvo et al., 1998; Chang and Cimprich, 2009). It is known that the Rad6 protein (Rad: radiation repair) determines, through monoubiquitination of the proliferating cell nuclear antigen (PCNA), a highly mutagenic translesion synthesis (TLS) and that PCNA poliubiquitination results in error-free TLS (Hartwell et al., 1997; Bergink and Jentsch, 2009; Chang and Cimprich, 2009). Importantly, rad6 mutant yeast resulted to be the most sensitive to Pt-compounds, in a screening performed with mutant strains belonging to the three known epistasis groups (Salvo et al., 1998; Motegi et al., 2006). The primary functions of topoisomerase II are unrelated to DNA repair, nevertheless the stabilization of the DNA-topoisomeraseII complexes by etoposide could alter genome stability and DNA-repair efficiency, inducing alternative pathways in damage processing (Degrassi et al., 2004; Cline and Hanawalt, 2006; Nitiss 2009). Furthermore, lesions induced by all three used agents determine checkpoint induced division delays (Lowndes and Murguia, 2000; Nyberg et al., 2002).

The additive, synergistic or antagonistic cellular effects of the combination of physical and chemical genotoxic agents depend on the nature of the agents, corresponding doses, and metabolic momentum of the biological target (i.e. the physicochemical characteristics of the cellular microenvironment, the cell cycle phase, and the genetic background). On the basis of quantitative models (Ager and Haynes 1990), it is possible to assess the degree of lethal and mutagenic interaction between two or more DNA damaging agents. At the molecular level, it is important to analyze the corresponding regulatory networks

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including interactions between checkpoints, DNA-repair and apoptosis-pathways. In fact, the induction of apoptosis in human cells by each single genotoxic agent or by their interaction depends on sophisticated regulatory mechanisms involving mitochondrial and nuclear signals, sensors, transduction cascades, and effectors (Hartwell and Kastan, 1994; Lowndes and Murguia, 2000; Nyberg et al., 2002; Friedberg et al., 2006, for reviews; Park et al., 2008; Auclair, 2008). Importantly, several components in mammalian cells have remarkable structural and functional similarities in yeast cells regarding especially DNA repair genes and check point networks (Hartwell et al., 1997; Nitiss, 1998; Rhind and Russell, 2000; Nyberg et al., 2002; Putnam et al., 2005; Huertas, 2010).

The purpose of present work was to analyze the interactions between γ-rays, cisplatin and etoposide at the cellular and molecular levels, using *Saccharomyces cerevisiae* as eukaryotic model. Several studies have been performed on lethal and mutagenic effects of double combinations using IR either with Pt or E at clinical and cellular levels. Here we used mathematical models to measure the magnitude of lethal and mutant interactions of double and triple agent combinations. Importantly, regarding the lethal (Pt,E) interaction there are contradictory results in the literature (Bonner et al., 2007). We also studied the mutant interactive effects induced by the three mentioned genotoxic agents in different combinations, as well as corresponding DSB induction.

The quantification of interaction between antineoplastic agents could provide additional insight in tumour therapy strategies.

MATERIAL AND METHODS

-Yeast strain: we used the haploid strain of *Saccharomyces cerevisiae* SC7K(*lys2-3*), auxotrophic mutation affecting the coding of Lysine in the locus 2-3 (Nunes et al., 1984),

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derived from SC7K (Saccharomyces cerevisiae #7K, wild type strain) (Benathen and Beam, 1977).

-Media: YPD broth: 1% yeast extract (US Biological, Cleveland, Ohio, USA), 2% bactopeptone (US Biological, Cleveland, Ohio, USA) and 2% dextrose (Sigma, St. Louis, Illinois, USA). YPDA (solid medium): YPD broth + 2% agar (US Biological, Cleveland, Ohio, USA). OM (omission medium): 0.67% YNB (yeast nitrogen base [Difco, Detroit, Michigan, USA]), 2% dextrose (Sigma, St. Louis, Illinois, USA), 2% agar (US Biological, Cleveland, Ohio, USA).

-**Radiation conditions:** The used source was ^{60}Co Theratron 780 equipment (Theratronics, Ottawa, Ontario, Canada). The mean dose rate was 200cGy/min. The absorbed dose (D) was: 40Gy≤D≤120Gy. Dosimetry was performed by international standards (International Atomic Energy Agency, 2002).

-Treatments and procedures

Samples of cell culture in exponential phase were irradiated into nutrient medium (Lillo et al., 1997). Unirradiated control cells and irradiated samples were immediately cooled after gamma exposure, and treated (at 33ºC during 40 min) either with cisplatin (Delta FarmaSA, Buenos Aires, Argentina) (0.3-3mM) and/or with etoposide (Farmaco Uruguayo, Montevideo, Uruguay), (0.1-2mM). Chemical agents were kindly provided by Chemotherapy Department at the Instituto Nacional del Cáncer-Uruguay. Part of the cell samples were exposed to single agents in the same conditions. Thereafter the treated samples and respective controls were centrifuged, washed in distilled water (in order to remove the agents) and plated either on YPDA or OM (at 30ºC).

Survival and mutation frequencies were determined by the colony forming ability of plated cells. The relative frequency of survivors (S) was determined after plating on YPDA (three

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plates per point, 200 cells/plate). The number of revertants to prototrophy (*lys2-3* → LYS), was determined after plating on five plates of OM ($5x10^7$ cells/plate).

-Mathematical analysis

According to Ager and Haynes (1990), the surviving probability P(0) can be estimated by: $S(x) = e^{-Hk(x)}$, where H_k is the number of lethal events and x represents the dose for a given agent X.

S(x) is determined by Ns/No, where Ns is the number of surviving cells and No is the number of surviving cells in untreated samples (controls).

The expected number of lethal events is: $- \ln S(x) = H_k(x)$.

In case of two agents combination (X,Y): $-\ln S(x,y) = H_k(x) + H_k(y) + h(x,y)$,

where H(x) and H(y) correspond to the expected number of lethal events produced independently as a function of doses x and y of the agents X and Y, respectively (Ager and Haynes,1990). h(x,y) is the number of lethal events induced by the interaction X,Y.

The combined effect of two (X,Y) or three agents (X,Y,Z) will be synergistic, additive or antagonistic, depending on the positive, zero or negative sign, of h(x,y) or h(x,y,z), respectively (Ager and Haynes, 1990).

Mutation frequency is defined as M(x):

$M(x) = Y(x) / S(x)$, where Y(x) is the mutant yield (Y= Nmut/No). Nmut: number of mutant cells (revertants).

$M(x,y) = M(x) + M(y) + m(x,y)$ (Ager and Haynes, 1990)

In the case of our present work, and for the three used agents,

$M(\gamma,p,e) = M(\gamma) + M(p) + M(e) + m(\gamma,p,e)$,

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where m(γ,p,e) is the number of mutagenic events produced by the interaction between the doses γ, p and e, of γ-rays, Pt and E agents respectively (Lillo et al., 2007).

The interaction functions can be used to quantify the degree of interaction between two or more genotoxic agents, depending on corresponding dose product. Thus, h(x,y) and m(x,y) values indicate the magnitudes of the (γ,p) or (γ,e) interactions for cell killing and mutation, respectively.

Note that the drug "cisplatin" is symbolized "Pt", and the "dose of cisplatin" is symbolized as "p". The same nomenclature was used for the drug etoposide (E) and the "dose of etoposide" (e).

Statistical analysis of experimental data: each mean value presented in the figures represents the result of at least three independent experiments; 95% confidence intervals, calculated from Binomial distribution, are included in the figures.

-DNA double-strand breaks (DSB) determination.

After exposure to the mentioned agents or their combinations, nuclear DNA was immediately isolated in agarose plugs after enzymatic treatment with lyticase and proteinase K (Sigma, St Louis, Illinois, USA) and submitted during 28h to pulse field electrophoresis (TAFE; Gene Line II, Beckman, Fullerton, CA. USA). Analysis of chromosomal DNA bands was performed with a LKB laser densitometer (633nm, UltroScan XL, Pharmacia) of the gel photograph negatives (Polaroid film type 665). The laser densitograms show the absorbance intensity as a function of the DNA migration distance in the electrophoresis. The DNA breakage (DSB) can be quantified by the relative decrease in absorbance intensity of the individual chromosomal bands observed in the

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treated sample as compared to respective control and the corresponding increase of absorbance between bands (smear) (Friedl et al., 1998; Keszenman et al., 2000).

RESULTS

Cell samples of the SC7K(*lys2-3*) strain were exposed to γ-irradiation during the exponential growth phase (see Material and Methods). Figure 1 shows surviving fractions (S), as a function of the absorbed dose. For combinations, the absorbed dose (D) of 120 Gy determining a surviving fraction S= 0.26±0.03 was selected. This survival level corresponded to an iso-effect dose of two standard radiotherapy fractions of 2 Gy, in human cell lines (Coogle, 1983). The surviving fractions (S) to single and combined treatments are shown in Figure 2. Survivals to different Pt concentrations and to the concurrent γ+Pt exposure are shown in panel 2a. E and γ+E surviving values can be observed in 2b. Figure 2c shows the surviving fraction (S) to Pt+E combination and to γ+Pt+E. For combinations, the fixed γ, Pt and E doses were selected on the basis of similar observed surviving fractions (120 Gy and 1mMolar respectively).

In order to better characterize the lethal interactions, the corresponding interactive functions were represented in linear-linear plots (Ager and Haynes, 1990) in Figure 3. The lethal interactions between γ-rays and the single used chemical agents are shown in 3a. Clearly the h(x,y) values increased as a function of corresponding dose product for both combinations γ+Pt and γ+E. h(γ,e) exhibited an approximately linear course, meanwhile h(γ,p) had two components of different slopes (in the assessed dose product range). The Pt+E combination (Figure 3b) resulted in no clear lethal interaction. The h(p,e) function was independent of the selected dose product, suggesting molecular independence between the involved repair pathways. Furthermore, Figure 3b shows the γ+Pt+E triple combination

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determining maximal observed lethal interaction. The h(γ,p,e) and h(p,e) values are in the same plot in order to better compare the data. Regarding mutation frequency (M) (Figure 4), etoposide induced the lowest values as single agent or in combinations, meanwhile γ+Pt+E combination induced the maximal ones (Figure 4b). The corresponding m(γ,Pt,E) interaction values are shown in Table I.

Regarding double combinations, γ+E showed a lower mutagenic interaction as compared to γ+Pt (Figures 4a and 5a) and m(p,e) function values (Figure 5b) were significantly lower than those corresponding to m(γ,p) or m(γ,e).

Figure 6 shows the chromosomal DNA electrophoresis pictures immediately after treatments, and corresponding densitograms. The magnitude of induced DSB is indicated by the relative decrease in absorbance peaks of the individual chromosomal bands in each treated sample, as compared to respective control, and the corresponding increase of absorbance between bands (smear). Regarding single treatments, either cisplatin or etoposide induced very low DSB. Pt+E double combination, as well as γ-rays as single agent, determined moderate DNA degradation. The highest DNA degradation corresponded to γ+Pt+E (maximal smear, between chromosomal bands and at the right side of the pictures), followed by γ+Pt and γ+E.

DISCUSSION

The main purpose of this work was to perform a quantitative analysis of the interactive lethal and mutagenic effects of ionizing radiations and two antineoplastic drugs on yeast cell populations. Even unicellular organisms lack angiogenesis, inflammatory and endocrine factors, as well as transformation and metastasis events (as observed “in vivo” in patients and other mammals), Saccharomyces cerevisiae has proven to be a very useful

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model organism to study regulatory toggle switch networks involving DNA repair pathways and cell cycle checkpoints (Friedberg et al. 2006, for review). Furthermore, statistical analysis using cell samples of 10^7 – 10^9 cells per millilitre results in a very high precision in survival and mutation frequency estimations. Thus, results at different organization levels, using different biological systems, are important to integrate our knowledge on a complex topic as the interaction of therapeutic agents in combined treatments. The experimental data of this study were interpreted on the basis of mathematical models (Ager and Haynes, 1990), and taking into account current data on nature and processing of corresponding DNA damages.

The observed lethal and mutagenic interactions, as measured by h(x,y,z) and m(x,y,z) equations, after different concurrent treatments with γ-rays, cisplatin and etoposide, are compatible with the processing of induced DNA-lesions by different repair pathways, with different error probabilities, sharing some common steps (Nunes et al.,1984; Nyberg et al., 2002; Bracesco et al., 2007).

Synergistic lethal interactions observed after double and triple agent combinations that included gamma rays indicate the probability of interaction of NHEJ, HR- and postreplicative- repair pathways (Figures 2 and 3). Misrepaired or unrepaired DNA damage can elicit genomic rearrangements, chromosome breakage or cell death. The synergistic mutant interactions, in the presence of gamma rays can be explained by the alternative induction of error-prone components of the mentioned pathways plus the error-prone components of excision repair pathways (mainly MMR) which are extensively independent of the three ones mentioned above. Cisplatin has also additional oxidative effects due to glutathione cross-linking, determining an increase of free radicals induced by

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ionizing radiations. Hence, a complex network with different competitive fluxes and nodal points can determine division delay and DNA repair, or cell death (Lowndes and Murguia, 2000; Nyberg et al., 2002; Bracesco et al., 2007; Nunes et al., 2008), and could explain lethal and mutagenic synergistic interactions observed in case of γ+Pt, γ+E and γ+Pt+E treatments (Figures 2-5).

On the other side, the lethal additivity and the low mutagenic interaction observed in case of Pt+E combination (Figures 2c, 3b, 4b, and 5b) can be explained by independent error-prone processing of induced damage, most probably error-prone excision repair (MMR), NHEJ and postreplicative error-prone repair (Keszenman et al., 2005; Bracesco et al., 2007). The contribution of NHEJ in the repair of DNA damage produced by etoposide in *Saccharomyces* has been previously determined (Malik et al., 2006). Other authors showed an increase in the global genomic repair of pyrimidine dimers induced by ultraviolet irradiation in topoisomerase II mutant yeast strains, suggesting the induction of nucleotide excision repair and recombination repair pathways (Malik and Nitiss, 2004; Cline and Hanawalt, 2006). In accordance to these authors, we observed a relatively low mutation frequency in topoisomerase II deficient yeast mutants when exposed to Pt and γ+Pt (unpublished results). Further analysis of obtained revertants should be performed in order to asses the mutations in the LYS locus of the revertants as well as acquired resistance to each used agent or their combinations.

Regarding induced lesions at the molecular level, it is known that DSB trigger checkpoint responses and impaired DSB repair associates to radiosensitivity and genomic instability by eliciting lethal events, genomic rearrangements and chromosome fragmentation. Here, as expected, maximal DSB induction corresponded to γ-rays, either as single agent or in

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combination with Pt and E. Regarding Pt and E effects, a very low DNA fragmentation was observed in our experimental conditions (Figure 6). Interestingly, pulsed field DNA electrophoresis performed immediately after treatments showed low differences between γ-rays acting alone or in γ+Pt and γ+E double combinations. The highest DNA degradation (maximal smear between the absorbance peaks), although moderate, corresponded to the γ+Pt+E triple combination. These facts indicate that the different lethal and mutation synergisms observed depend on differential processing of damage through different repair pathways during incubation in nutrient media after treatment. Additivity or synergism arises, from the alternative processing of DNA lesions, either by independent pathways or by pathways sharing common steps, respectively (i.e. HR, NHEJ and TLS, depending on cell cycle phase). It was demonstrated that the balance between HR and NHEJ depends on cell population age, cell cycle phase, ploidy and different cell genotypes of a single species (Shrivastav et al., 2008; Nunes et al., 2008; Huertas, 2010)".

The (γ,p), (γ,e) and (γ,p,e) lethal and mutagenic synergisms observed in present work are in accordance to their appropriate antitumor activity previously shown at the clinical level, counteracted by the observed risk of long term loco-regional second tumors. In addition, the absence of lethal interaction and the very low mutagenic interaction observed between cisplatin and etoposide, could explain the adequate tolerance to the systemic toxicity during the concurrent γ+Pt+E or Pt+E clinical treatments. Present method can be useful to analyse interactive events induced in human cell lines, especially in tissues that strongly suffer from the action of agents used in different chemoradiotherapy combinations. The study of the correlation between the degree of lethal and mutagenic interaction at the human cellular

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level and results of antitumor activity and toxicity at the clinical level could provide a critical basis regarding concurrent treatments.

More insight into the molecular interaction of the antineoplastic agents is a challenge for the rationale in chemoradiotherapy. Moreover, the quantitative assessment of the interaction between the agents to be used in patients could be useful, as an additional tool, in selecting the best chemoradiotherapy for each one (personalized treatments).

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Figure legends

Figure 1. Fractional Survival (S) as a function of γ-rays absorbed dose in Gray (Gy). Log-linear plot. Error bars indicate 95% binomial confidence intervals (five independent experiments).

Figure 2. Bar diagrams showing Fractional Survival (S) of the cells submitted to different Drug Doses in miliMolar (mM). Pt molecular weight: 300; E molecular weight: 588.6. Panel a: Pt acting alone at the mMolar concentrations indicated at the bottom (black bars) and as combined to γ-rays (120 Gy) (grey bars). Panel b: E acting alone at the concentrations indicated in the figure (black bars) and as combined to γ-rays (120 Gy) (grey bars). Panel c: E acting alone for increasing concentrations (bottom) (black bars); Pt (1mM)

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plus E (light grey bars); γ-rays (120 Gy) plus Pt (1mM) plus E (dark grey bars). Error bars indicate 95% binomial confidence intervals (five independent experiments).

Figure 3. Lethal interaction values (h) as a function of corresponding dose product. Pt molecular weight: 300; E molecular weight: 588.6. A) h(γ,p) (•), h(γ,e) (o). B) h(p,e) (•), h(γ,p,e) (o). The top abscissa values ($[ug/ml]^2$ x Gy) are those from the bottom multiplied by 120 Gy. Error bars indicate 95% binomial confidence intervals (five independent experiments).

Figure 4. Mutation frequency (M) as a function of Drug Dose. Log-log plot. The spontaneous mutation frequency was 1.98×10^{-6} ($\pm 5 \times 10^{-7}$). Panel a: M(γ) produced by 120Gy was 2.8×10^{-5}(■). M(E) (•) and M(Pt) (▼) as a function of respective concentrations. The combined treatments are: γ+E (o) and γ+Pt (∇). Panel b: M(E) (•), M(Pt+E) (o), and M (γ+Pt+E) (▼). The mutation frequency produced by 1mM of Pt was 2.8×10^{-5}(■). Error bars indicate 95% binomial confidence intervals (three independent experiments).

Figure 5. Mutagenic interaction (m[x,y])/10^4 survivors, as function of dose product. Panel a: m(γ,p) (•), m(γ,e) (o). Panel b: m(p,e) (•). Error bars indicating 95% binomial confidence intervals (three independent experiments) are of the size as the plotted symbols.

Figure 6. Top pictures correspond to the chromosomal DNA pulsed field electrophoresis gel photographs. Bottom diagrams show laser absorbance profiles of each electrophoresis: Left (from top to bottom): control sample, Pt, E, Pt+E.

Right (from top to bottom): γ-rays, γ+Pt, γ+E, γ+P+E.

D(γ-rays): 120 Gy. [Pt]: 1 mM. [E]: 1 mM.

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The peak on the right side of each absorbance profile indicates the accumulation of DNA fragments of low molecular weight induced by each treatment (absent in control sample). Note that the smear (between chromosome bands and at the right side of the pictures) is maximal for the triple γ+P+E combination.

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Table I. Mutagenic interaction values for double and triple agent combinations.

E [mM]	m(γ,e)	m(p,e)	m(γ,p,e)
0.5000	$0.38x10^{-4}$	$3.4x10^{-5}$	$0.02x10^{-2}$
1.0000	$2.86x10^{-4}$	$4.9x10^{-5}$	$2.2x10^{-2}$
2.0000	$13.6x10^{-4}$	$10x10^{-5}$	$8.7x10^{-2}$

Conflict of Interest Notification

There is no current, neither potential, conflict of interest related to the present manuscript.

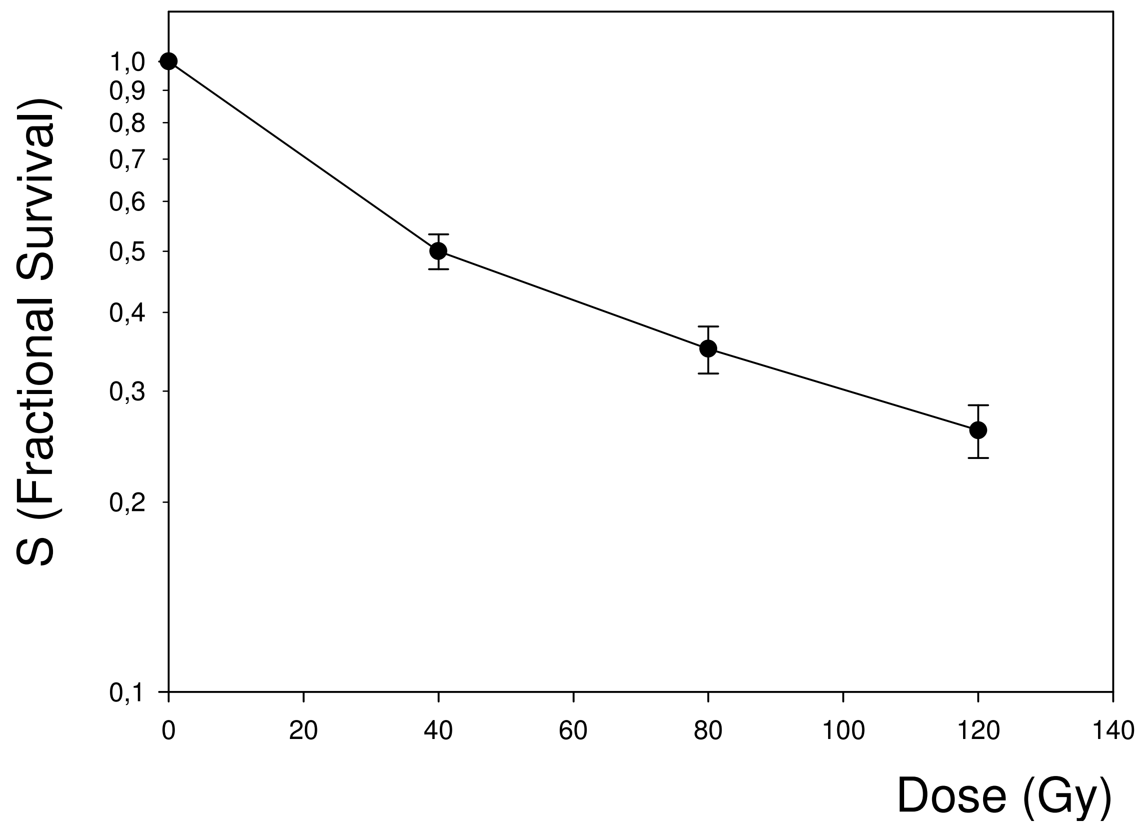

Figure 1

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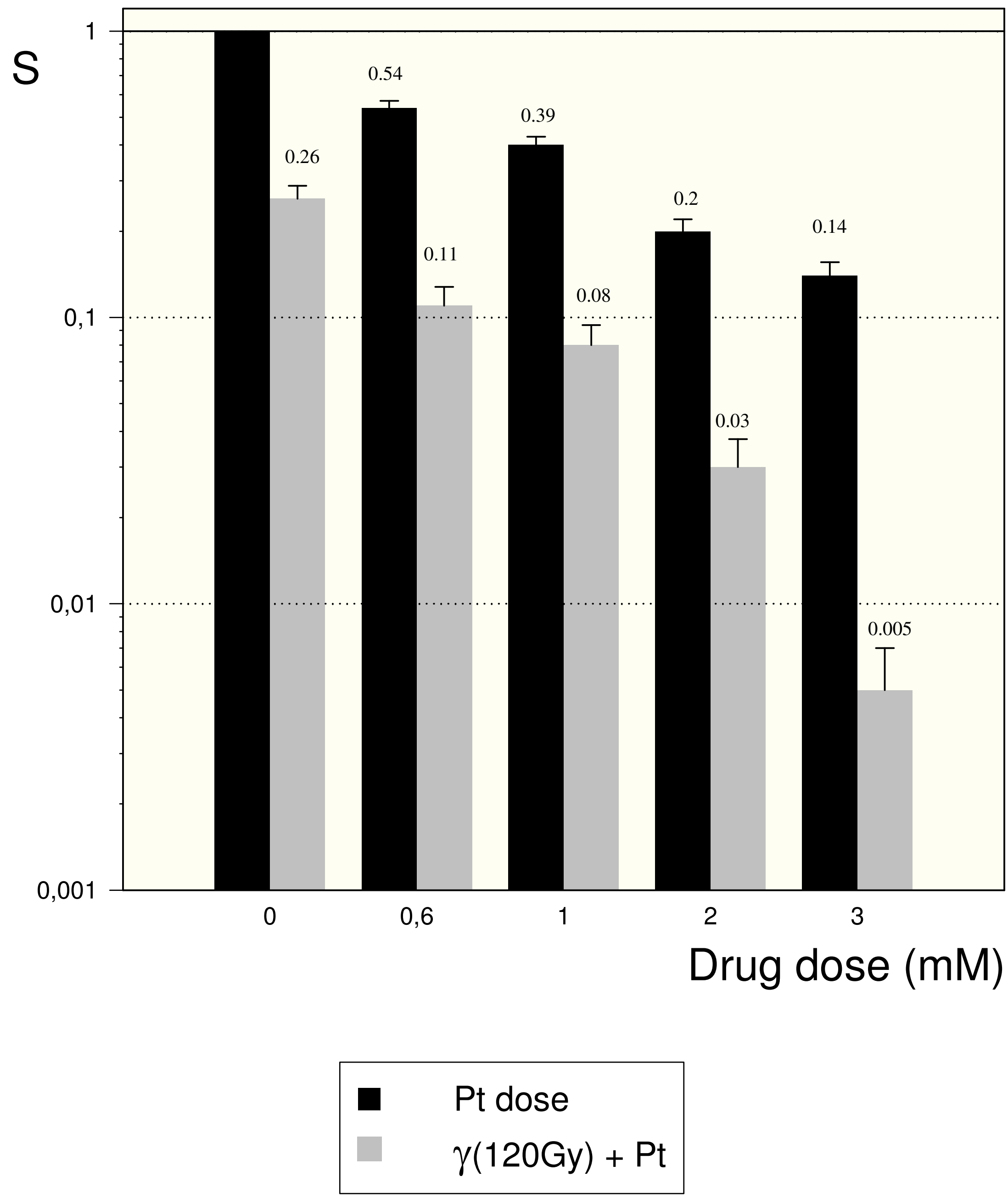

Figure 2a

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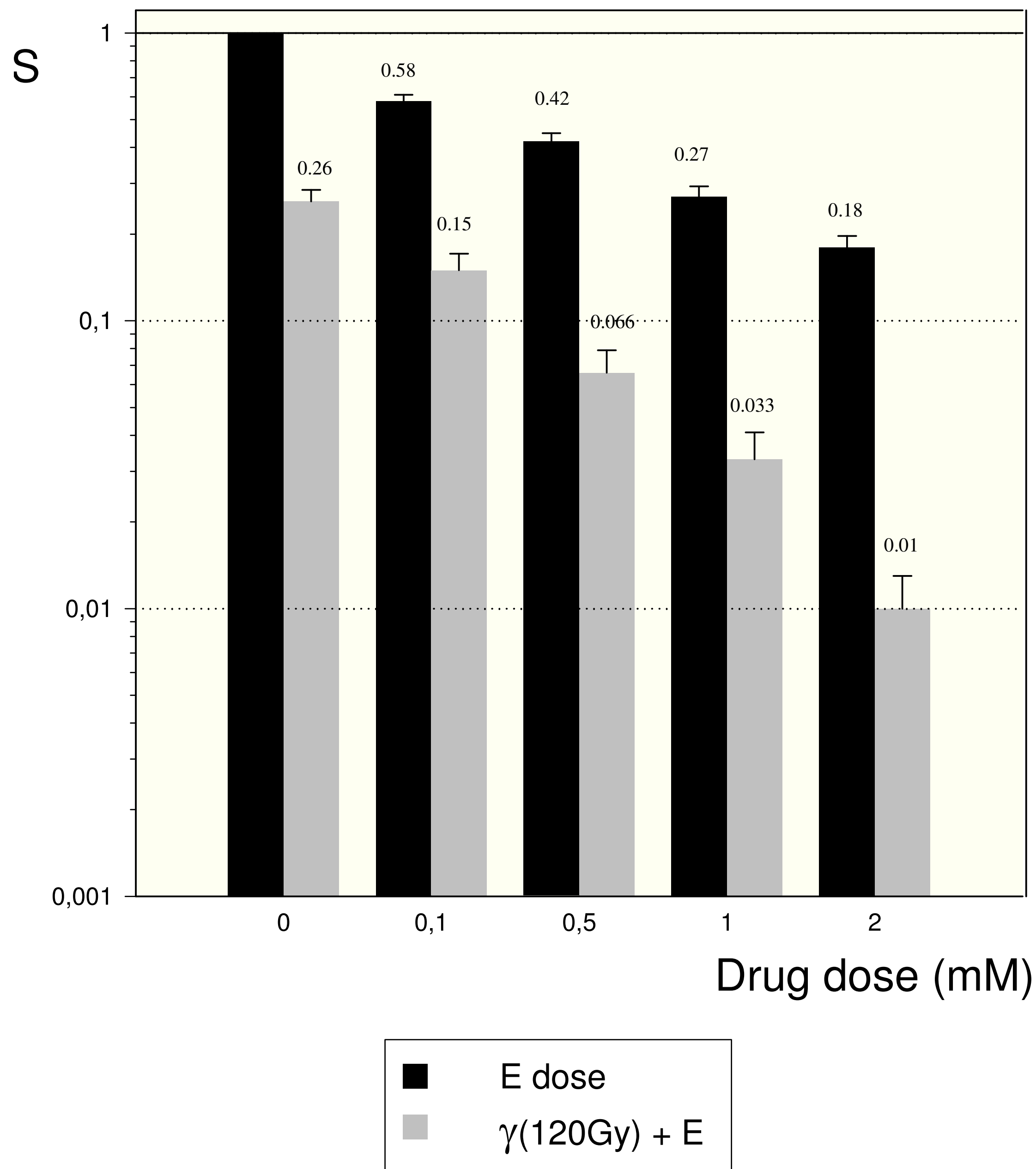

Figure 2b

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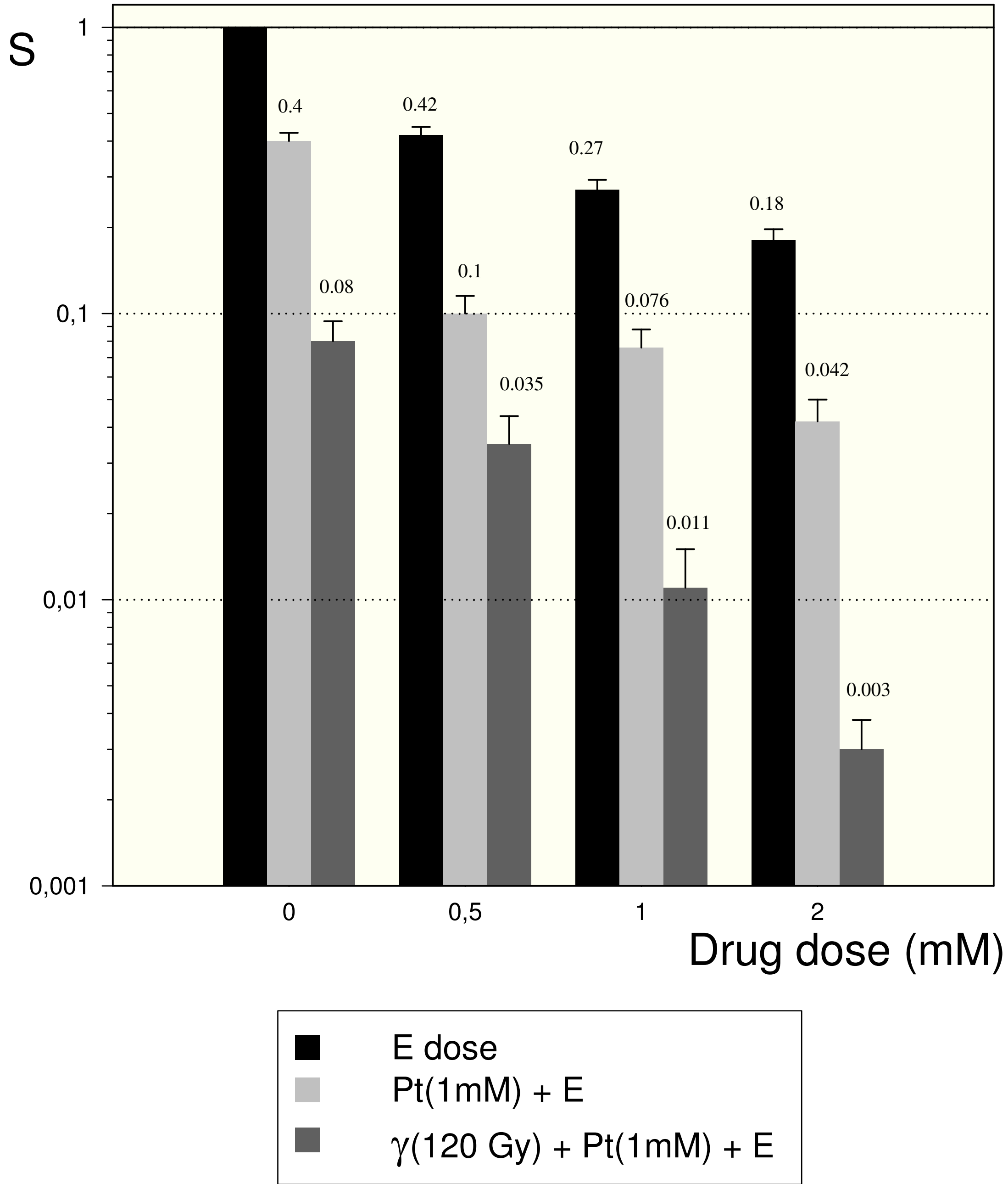

Figure 2c

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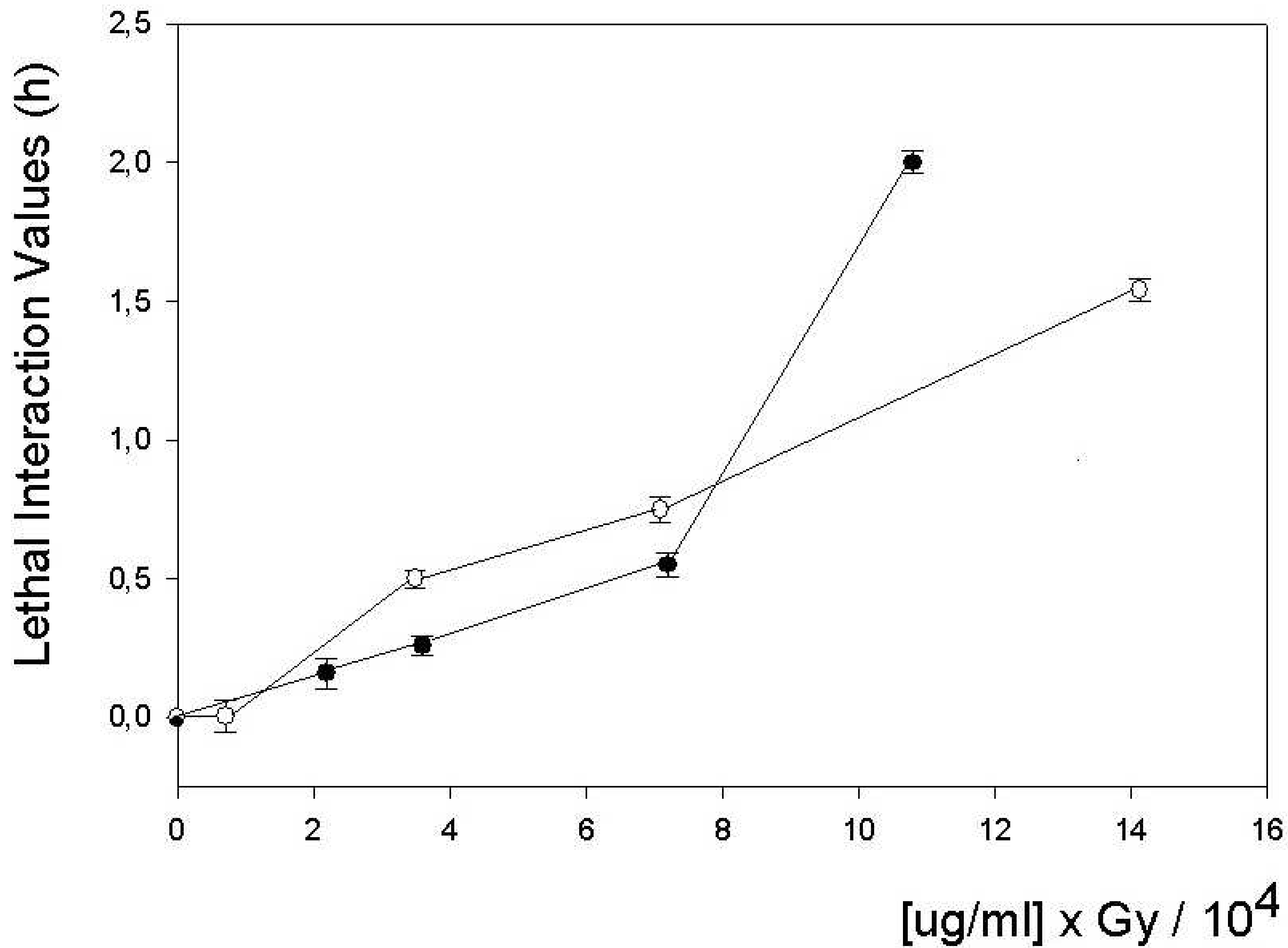

Figure 3a

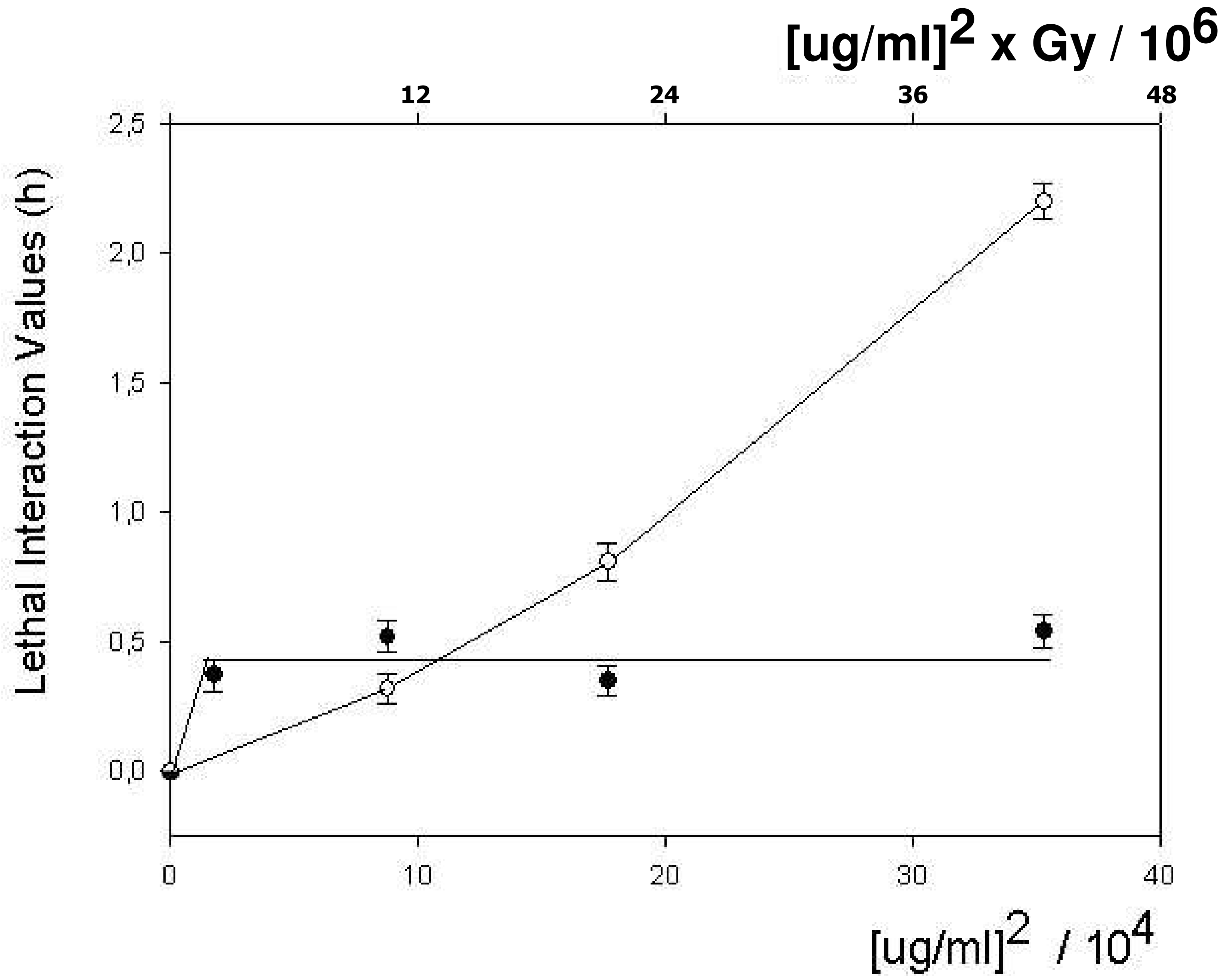

Figure 3b

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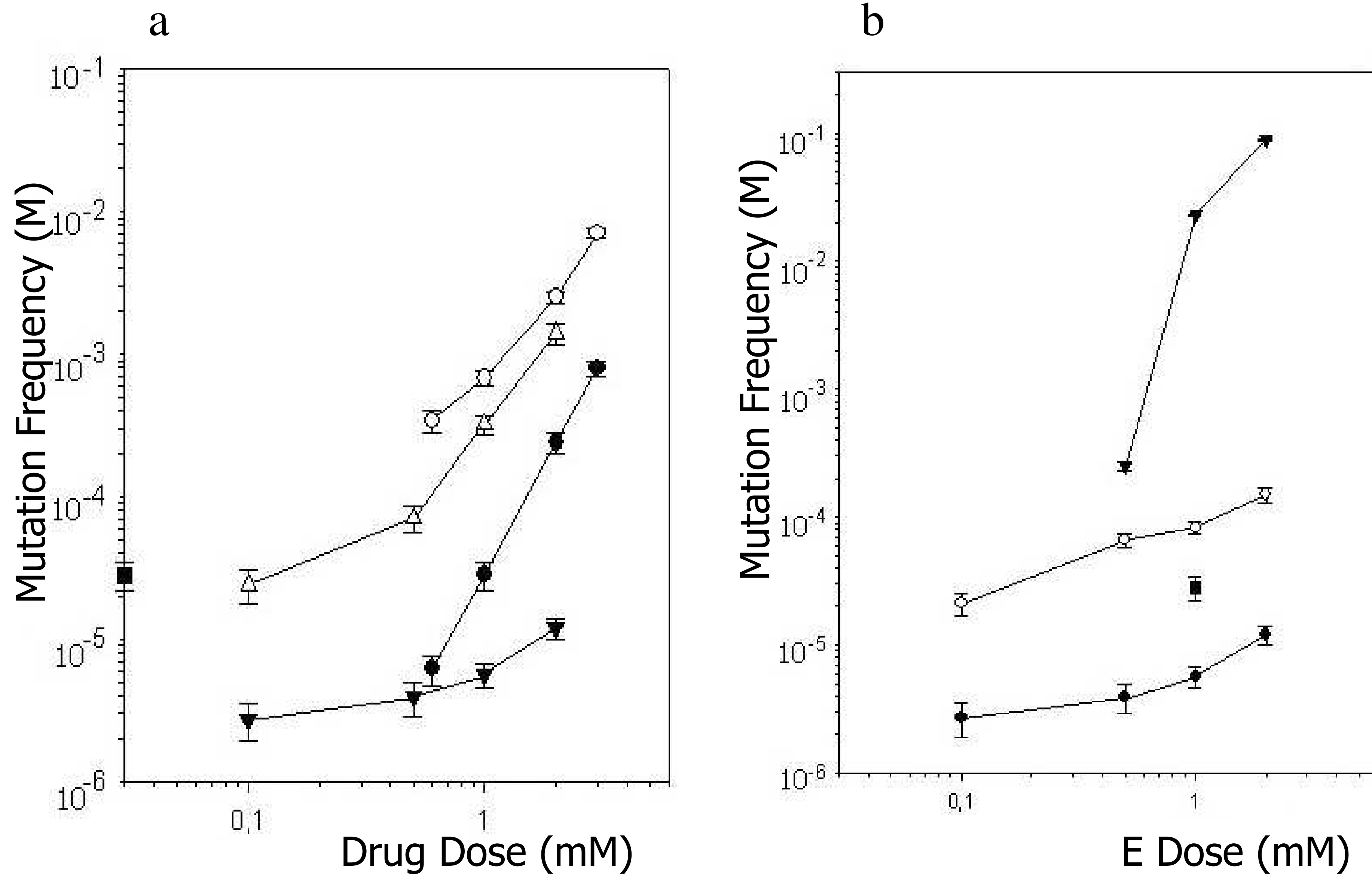

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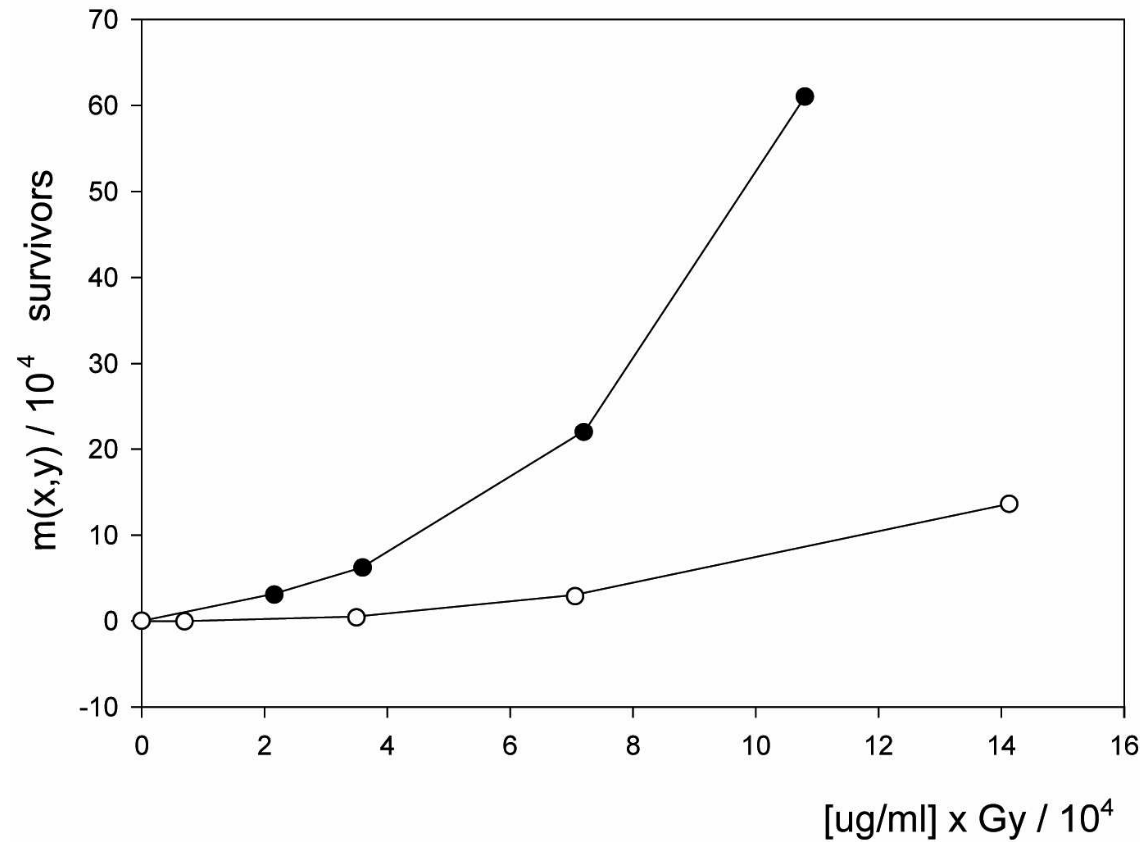

Figure 5a

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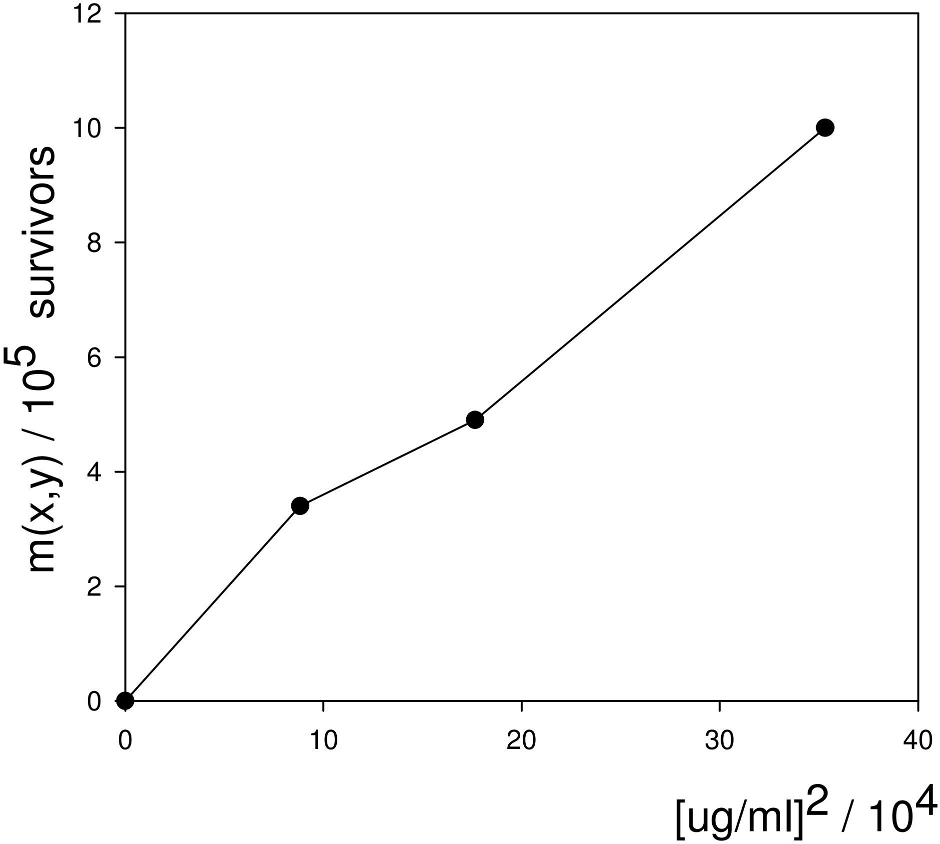

Figure 5b

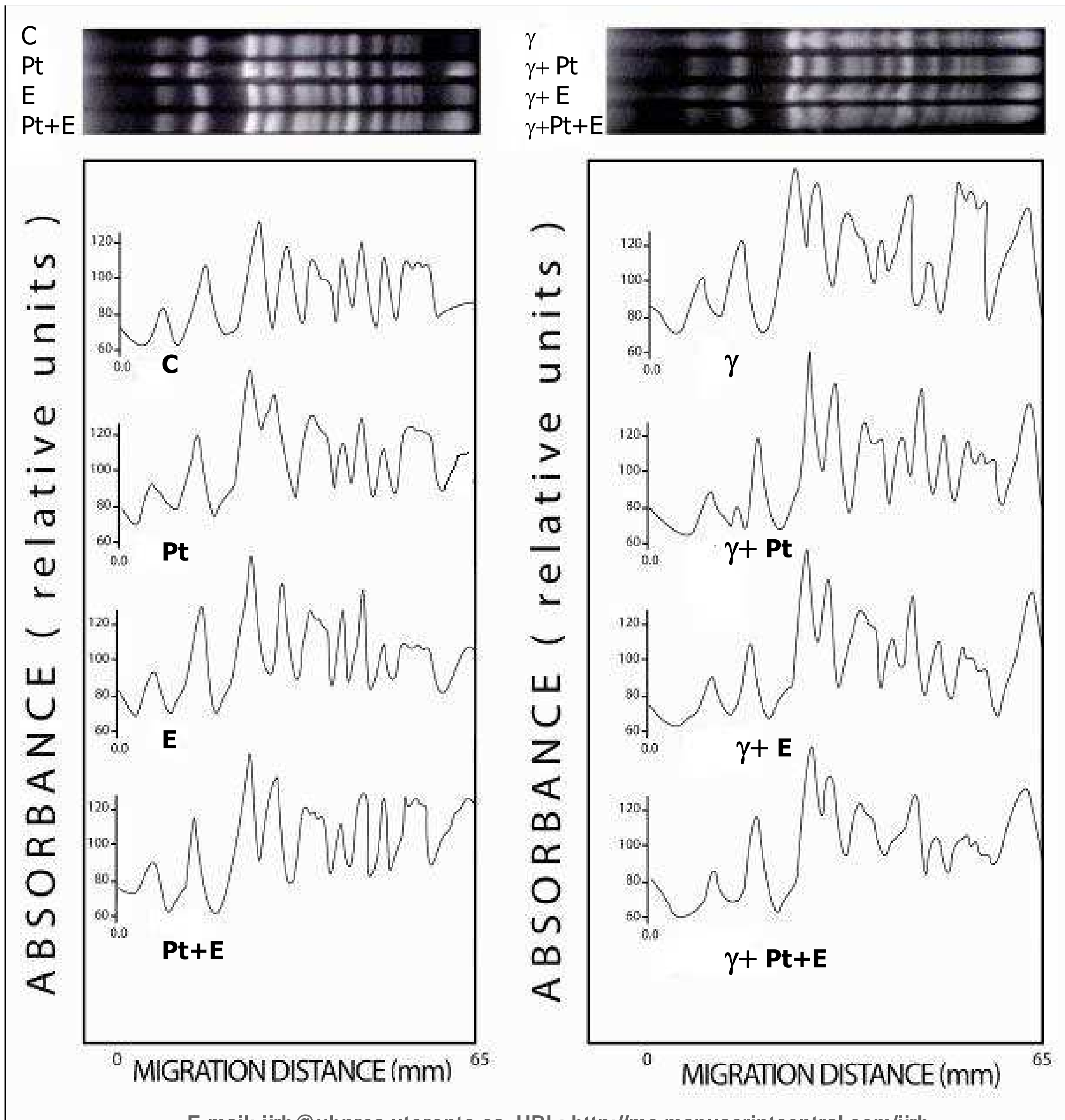

Figure 6