



Cremophor EL stimulates mitotic recombination in *uvrH//uvrH* diploid strain of *Aspergillus nidulans*

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ABSTRACT

Cremophor EL is a solubilizer and emulsifier agent used in the pharmaceutical and foodstuff industries. The solvent is the principal constituent of paclitaxel's clinical formulation vehicle. Since mitotic recombination plays a crucial role in multistep carcinogenesis, the study of the recombinogenic potential of chemical compounds is of the utmost importance. In our research genotoxicity of cremophor EL has been studied by using an *uvrH//uvrH* diploid strain of *Aspergillus nidulans*. Since it spends a great part of its cell cycle in the G2 period, this fungus is a special screening system for the study of mitotic recombination induced by chemical substances. Homozygotization Indexes (HI) for *paba* and *bi* markers from heterozygous B211//A837 diploid strain were determined for the evaluation of the recombinogenic effect of cremophor EL. It has been shown that cremophor EL induces increase in mitotic crossing-over events at nontoxic concentrations (0.05 and 0.075% v/v).

Key words: *Aspergillus nidulans*, mitotic recombination, cremophor EL, Homozygotization Index, antineoplasms agents.

INTRODUCTION

The organic solvent cremophor EL (CEL) (polyoxyethyleneglycerol triricinoleate 35) is a viscous liquid produced by the reaction of castor oil (*Ricinus communis*) with ethylene oxide (Hoffman 1984). CEL is employed as a vehicle for the solubilization of a wide variety of hydrophobic drugs, including anesthetics, photosensitizers, sedatives, immunosuppressive agents and anticancer drugs (Gelderblom et al. 2001).

Without causing serious toxicity, in vitro cremophor EL reverts the Multidrug Resistance (MDR) phenotype in clinically executable concentrations. Multidrug resistance is a mechanism by which

cancer cells are able to survive diverse drugs in structurally unrelated groups. Overexpression of the multidrug transporter protein, also known as P-glycoprotein, has often been observed in MDR cells. This transmembrane protein is capable of pumping a wide variety of chemotherapeutic agents out of the cells, protecting them from the agent's toxic effects (Breier et al. 2000). Cremophor EL affects the metabolism of MDR cells, alters cell membrane properties, and impairs the P-glycoprotein function. Actually the solvent favors the action of antineoplasms drugs, such as paclitaxel, doxorubicin and vinblastine (Fjällskog et al. 1993, Friche et al. 1993, Woodcock et al. 1990).

The cytotoxic effect of CEL, found in tumoral human cells resistant to doxorubicin, has been as-

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TABLE I
Genotype and origin of strains.

Strain	Genotype	Origin
B211	<i>yA2; biA1; AcrA1; wA2; methA17; uvsH77; pyroA4; chaA1</i>	Busso et al. 2001
A837	<i>pabaA1; uvsH77; pyroA4; choA1; chaA1</i>	FGSC

Mutant alleles give the following phenotypes (corresponding genes between parentheses): requirements for 4-aminobenzoic acid (*pabaA1*), biotin (*biA1*), methionine (*methA17*), pyridoxine (*pyroA4*) and choline (*choA1*); yellow (*y*), white (*w*) and chartreuse (*cha*) coloring of conidia, respectively; resistance to acriflavine (*AcrA1*). FGSC – Fungal Genetic Stock Center, University of Kansas Medical Center, Kansas City, USA.

sociated with peroxidation of polyunsaturated fatty acids and with direct disturbing effect in the cell membrane (Nygren et al. 1995, Burton 1991, Bégin et al. 1988).

The transformation of normal human cells into cancer cells is a multistep process, while mitotic recombination is a mechanism involved in bringing about such transformation (Nowell 1976, Barrett 1993). In heterozygous cells bearing a mutant and normal alleles for a tumor suppressor gene, the somatic recombination may turn up to be a promoter of neoplasms by inducing homozygosis of the mutant tumor suppressor allele (Maher et al. 1993, Sengstag 1994).

Mitotic crossing-over has already been recorded in *Drosophila melanogaster*, *Aspergillus nidulans*, *Saccharomyces cerevisiae* and mammalian cells in culture, including human cells. It is currently thought to be a common occurrence process in diploid cells (Ramel et al. 1996, De La Torre et al. 1994, Maher et al. 1993, Kunz et al. 1981, Stern 1936).

The filamentous fungus *A. nidulans* is an excellent organism for studying mitotic crossing-over. This is chiefly due to two important factors: a) *A. nidulans*'s mitosis has many common characteristics with higher eukaryotes mitosis, and b) the fungus spends most of its vegetative cell cycle in G2 phase. At this phase, the presence of two copies of each chromosome favors the occurrence of mitotic crossing-over (Bergen and Morris 1983, Iwanejko et al. 1996).

Since several reports have suggested somatic

recombination in mechanisms leading to carcinogenesis and due to the fact that CEL is used as a solubilizer of hydrophobic drugs, such as antineoplastic agents, we decided to examine the ability of this solvent to induce mitotic recombination.

MATERIALS AND METHODS

STRAINS

The genotypes and origin of *A. nidulans* strains are provided in Table I. Diploid strain (B211//A837) (Busso et al. 2001) was prepared according to Roper (1952).

CULTURE MEDIA

Complete (CM) and minimum medium (MM) were prepared according to Van de Vate and Jansen (1978). Selective medium was prepared with MM and nutritional requirements of each strain. Solid medium was prepared with 1.5% agar; incubation for strain growth was done at 37°C.

EVALUATION OF DRUG TOXICITY

Conidia of diploid strain B211//A837 were inoculated in the plate center with MM + pyridoxine (control) and MM + pyridoxine + cremophor EL (0.05% and 0.075% v/v). Nine plates incubated at 37°C were used for each dose and for control. Diameters of colonies were measured after 24, 48, 72, 96 and 120 hours of incubation. Student's *t* test compared colony diameters with and without the drug.

TABLE II

Mitotic segregation of *paba* gene among haploid segregants derived from *paba+//paba* heterozygous diploid in the presence and in the absence of crossing-over.

		Chromosome I	
Heterozygous diploid	1		
	2		
Recombinant diploid	3		
	4		
		No Crossing-over	Crossing-over in the centromere- <i>paba</i> interval
Chromatid Segregation	Diploid genotypes	Chromatid Segregation	Diploid genotypes
1 + 3	<i>paba+ // paba</i>	1 + 3	<i>paba+ // paba+</i>
1 + 4	<i>paba+ // paba</i>	1 + 4	<i>paba+ // paba</i>
2 + 3	<i>paba+ // paba</i>	2 + 3	<i>paba // paba+</i>
2 + 4	<i>paba+ // paba</i>	2 + 4	<i>paba // paba *</i>
Segregation after haploidization	4 <i>paba+</i> : 4 <i>paba</i>		4 <i>paba+</i> : 2 <i>paba</i>

(*) Homozygous diploid (*paba // paba*) will not be chosen in MM + pyridoxine plates

CYTOLOGICAL ANALYSIS

Colonies of B211 strain were cultivated in dialysis membranes placed aseptically on the surface of petri dishes with CM and CM + 0.05% and 0.075% v/v of CEL. Incubation occurred for 30 h, at 37°C, and samples taken during 4-28 h period. Membranes were stained with cotton-blue-lactophenol and analyzed under an optic microscope.

EVALUATION OF THE RECOMBINAGENIC POTENTIAL (PIRES AND ZUCCHI 1994)

Conidia of the diploid strain were inoculated in MM + pyridoxine + cremophor EL (0.05% and 0.075% v/v) and incubated for 5 days at 37°C. Treatment produced visible diploid sectors, D1-D6, identified by their different morphology from the original diploid. Diploid sectors were submitted to spontaneous haploidization in CM after purification in MM + pyridoxine. Only haploid segregants were selected for recombination test (Franzoni et al. 1997). Conidia of each haploid sector were trans-

ferred to 25 defined positions in CM plates (master plates). After 48 hours of incubation at 37°C, colonies were transferred to selective media and the phenotypical analysis of the haploid segregants was carried out.

The treatment with CEL in MM + pyridoxine produces only heterozygous (+/- or -/+) or homozygous (+/+) segregants since the recessive ones (-/-) fail to grow in MM + pyridoxine (Table II). After haploidization of diploids D1-D6 the nutritional markers will segregate among the haploids in the proportion of 4+: 4-, if solvent fails to induce recombination; or 4+: 2-, if solvent induces crossing-over. Values of Homozigotization Indexes (HI) (the ratio between number of prototrophic and auxotrophic segregants) equal or above 2.0 evidence the recombinagenic effects of the substance under analysis (Pires and Zucchi 1994, Chiuchetta and Castro-Prado 2002a,b). Results were compared by Yates corrected Chi-square test.

HI for genes *paba* and *bi* were determined to

TABLE III

Time of conidiophore vesicle, metulae and phialides appearance and conidia production of B211 strain in the presence of cremophor EL 0.05 and 0.075% v/v.

Conidiophore structures and conidia production	Time (hs)		
	Control*	Cremophor EL	
		0.05% v/v	0.075% v/v
Vesicles	22	24	25
Metulae and Phialides	23	25	25
Conidia	25	26	26

*Development without cremophor EL.

evaluate diploid B211//A837 after treatment with cremophor EL.

RESULTS AND DISCUSSION

Aspergillus nidulans reproduces itself asexually by forming multicellular conidiophores and uninucleate spores called conidia. We have first studied the effects of CEL on mycelia growth of B211//A837 strain and on conidiophore morphology of B211 strain. Solvent was added to a pyridoxine-supplemented minimal agar medium to obtain final concentrations of 0.05% and 0.075% v/v. Both CEL concentrations had no effect on mycelia growth or on conidiophore morphology (results not shown). Only a slight delay in the timing for conidiophore formation of B211 strain was observed (Table III).

In this study, the B211//A837 diploid strain, homozygous for *uvsH* mutation, was used as a sensitive system for the evaluation of the genotoxic activity of CEL in nontoxic concentrations.

Various repair mechanisms are mobilized to restore the original DNA sequence when DNA damages occur. These mechanisms include base excision repair (BER), nucleotide excision repair (NER), mutagenic repair and post-replication repair (Goldman et al. 2002, Hjertvik et al. 1998, Fishel and Kolodner 1995). In *A. nidulans* the UV-sensitive mutants (*uvs*) have been classified in different epistatic groups such as: UvsB, UvsC, UvsF

and UvsI. The study of mitotic intergenic recombination in this filamentous fungus may be greatly facilitated by the use of UvsF group mutations: gene mutation *uvsH*, that operates in the post-replication repair pathway, is responsible for high frequencies of mitotic intergenic recombination in homozygous condition (Osman et al. 1993, Iwanejko et al. 1996).

It has been shown that CEL induces a statistically significant increase in mitotic crossing-over events in *A. nidulans*. The treatment of B211//A837 with CEL increased Homozygotization Indexes for *paba* and *bi* markers (Tables IV and V). This effect, nevertheless, was not dose-dependent.

It is believed that mitotic recombination involving heterozygous cells for a deleterious gene triggers carcinogenesis. This process leads towards homozygosis and subsequent expression of the malignant trait (Wijnhoven et al. 2003, Preisler et al. 2000).

Our data demonstrate the recombinogenic effect of CEL in *A. nidulans* when a sensitive strain is used to study mitotic crossing-over. Further analyses, using mammalian cells, may be conducted for a better understanding of the carcinogenic potential of this solvent.

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TABLE IV
Homozygosity Index (HI) of diploid B211//A837 exposed to 0.05% v/v of cremophor EL.

	Control		D1		D2		D3	
	n.seg.	HI	n.seg.	HI	n.seg.	HI	n.seg.	HI
<i>paba</i> +	73	1.97	71	2.21	58	1.66	90	3.10
<i>paba</i>	37		32		35		29	
<i>bi</i> +	70	1.75	66	1.78	64	2.21	92	3.41*
<i>bi</i>	40		37		29		27	

D1, D2 and D3, diploid strains obtained after treatment of the B211//A837 strain with 0.05% v/v of cremophor EL; n.seg., number of haploid mitotic segregants; (*) significantly different from control at $p < 0.05$ (Yates corrected Chi-square test, Statistic for Windows Program).

TABLE V
Homozygosity Index (HI) of diploid B211//A837 exposed to 0.075% v/v of cremophor EL.

	Control		D4		D5		D6	
	n.seg.	HI	n.seg.	HI	n.seg.	HI	n.seg.	HI
<i>paba</i> +	73	1.97	136	3.77*	75	2.20	200	3.03
<i>paba</i>	37		36		34		66	
<i>bi</i> +	70	1.75	120	2.30	73	2.02	199	2.97*
<i>bi</i>	40		52		36		67	

D4, D5 and D6, diploid strains obtained after treatment of the B211//A837 strain with 0.075% v/v of cremophor EL; n.seg., number of haploid mitotic segregants; (*) significantly different from control at $p < 0.05$ (Yates corrected Chi-square test, Statistic for Windows Program).

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RESUMO

Cremofor EL (CEL) é um solubilizante e emulsificante amplamente utilizado nas indústrias farmacêuticas e de gêneros alimentícios. É o principal veículo empregado nas formulações clínicas do antineoplásico paclitaxel. Considerando-se que a recombinação mitótica desempenha importante função no processo de carcinogênese, o estudo de substâncias químicas com potencial recombinação assume importância crucial, no sentido de se detectar aquelas que eventualmente possam atuar como promotoras de neoplasias. A genotoxicidade do cremofor EL foi estudada no presente trabalho, utilizando-se uma

linhagem diplóide *uvrH//uvrH* de *Aspergillus nidulans*. Neste fungo as células vegetativas comumente repousam no período G2 do ciclo celular, facilitando a ocorrência da recombinação mitótica. O efeito recombinação do CEL foi avaliado através da determinação dos Índices de Homozigotização para os marcadores nutricionais *paba* e *bi* do diplóide heterozigoto B211//A837. Os resultados demonstram que CEL é efetivo em induzir crossing-over mitótico em concentrações não tóxicas ao fungo (0.05 e 0.075% v/v).

Palavras-chave: *Aspergillus nidulans*, recombinação mitótica, cremofor EL, índices de homozigotização, agentes antineoplásicos.

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