Anais da Academia Brasileira de Ciências
ISSN: 0001-3765
aabc@abc.org.br
Academia Brasileira de Ciências
Brasil

Busso, Cleverson; Castro-Prado, Marialba A. A.
Cremophor EL stimulates mitotic recombination in uvsH//uvsH diploid strain of Aspergillus nidulans
Anais da Academia Brasileira de Ciências, vol. 76, núm. 1, março, 2004, pp. 49-55
Academia Brasileira de Ciências
Rio de Janeiro, Brasil

Anais da Academia Brasileira de Ciências (2004) 76(1): 49-55
(Annals of the Brazilian Academy of Sciences)
ISSN 0001-3765
www.scielo.br/aabc

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

CLEVERSON BUSSO and MARIALBA A.A. CASTRO-PRADO

Universidade Estadual de Maringá, Departamento de Biologia Celular e Genética
Av. Colombo 5790, 87020-900 Maringá, PR, Brasil

Manuscript received on June 13, 2003; accepted for publication on October 8, 2003;
presented by LUCIA MENDONÇA PREVIATO

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 recombinagenic potential of chemical compounds is of the utmost importance. In our research genotoxicity of cremophor EL has been studied by using an *uvsH//uvsH* 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 recombinagenic 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, antineoplasm 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 antineoplasm 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-

Correspondence to: Castro-Prado M.A.A.
E-mail: maacprado@uem.br

TABLE I

Genotype and origin of strains.

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

Mutant alleles give the following phetotypes (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 antineoplasm 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.

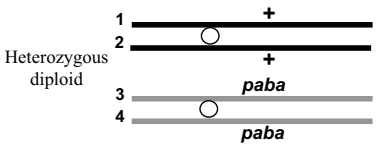
Evaluation of Drug Toxicity

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



No Crossing-over		Crossing-over in the centromere-*paba* interval	
Chromatid Segregation	Diploid genotypes	Chromatid Segregation	Diploid genotypes
1 + 3	*paba+* // *paba*	1 + 3	*paba+* // *paba+*
1 + 4	*paba+* // *paba*	1 + 4	*paba+* // *paba*
2 + 3	*paba+* // *paba*	2 + 3	*paba* // *paba+*
2 + 4	*paba+* // *paba*	2 + 4	*paba* // *paba* *
Segregation after haploidization	4 *paba+* : 4 *paba*		4 *paba+* : 2 *paba*

(*) 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 recombinagenesis test (Franzoni et al. 1997). Conidia of each haploid sector were transferred 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 recombinagenesis; 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.

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

ACKNOWLEDGMENTS

Thanks are due to Dr. Heloisa Helena Rodrigues de Andrade (Universidade Federal do Rio Grande do

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
paba +	73	1.97	71	2.21	58	1.66	90	3.10
paba	37		32		35		29	
bi +	70	1.75	66	1.78	64	2.21	92	3.41*
bi	40		37		29		27	

D1, D2 and D3, diploid strains obtained after treatment of the B211//A837strain 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
paba +	73	1.97	136	3.77*	75	2.20	200	3.03
paba	37		36		34		66	
bi +	70	1.75	120	2.30	73	2.02	199	2.97*
bi	40		52		36		67	

D4, D5 and D6, diploid strains obtained after treatment of the B211//A837strain 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).

Sul) and Carmem Boto Querol (Universidade Estadual de Maringá) for supplying the cremophor EL; to Mrs. Sonia A. de Carvalho and Mrs. Luzia A. S. Regasse for technical assistance. Cleverson Busso is the holder of a PIBIC/CNPq fellowship.

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 recombinagênico 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 *uvsH//uvsH* de *Aspergillus nidulans*. Neste fungo as células vegetativas comumente repousam no período G2 do ciclo celular, facilitando a ocorrência da recombinação mitótica. O efeito recombinagênico 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.

REFERENCES

BARRETT JC. 1993. Mechanisms of multistep carcinogenesis and carcinogen risk assessment. Environ Health Perspect 100: 9-12.

BÉGIN ML, ELLS G AND HORROBIN DF. 1988. Polyunsaturated fatty acid-induced cytotoxicity against tumor cells and its relationship to lipid peroxidation. J Natl Cancer Inst 80: 188-194.

BERGEN GG AND MORRIS NR. 1983. Kinetics of the nuclear division cycle of *Aspergillus nidulans*. J Bacteriol 156: 155-160.

BREIER A, DROBNÁ Z, DOCOLOMANSKÝ P AND BARANCÍK M. 2000. Cytotoxic activity of several unrelated drugs on L1210 mouse leukemic cell sublines with P-glycoprotein (PGP) mediated multidrug resistance (MDR) phenotype. A QSAR study. Neoplasma 47: 100-106.

BURTON AF. 1991. Oncolytic effects of fatty acids in mice and rats. Am J Clin Nutr 53: 1082-1086.

BUSSO C, CHIUCHETTA SJR, BAPTISTA F AND CASTRO-PRADO MAA. 2001. *uvsH//uvsH* diploid strain favors an efficient method to evaluate the recombinagenic effect of chemical and physical agents in *Aspergillus nidulans* (Ascomycetes). Acta Scientiarum 23: 603-607.

CHIUCHETTA SJR AND CASTRO-PRADO MAA. 2002a. Vincristine induces somatic segregation, via mitotic crossing-over, in diploid cells of *Aspergillus nidulans*. Biol Res 35: 31-38.

CHIUCHETTA SJR AND CASTRO-PRADO MAA. 2002b. Recombinagenic effect of Cryptolepine in *uvsH+// uvsH+* and *uvsH//uvsH* diploid strains of *Aspergillus nidulans*. Folia Microbiol 47: 516-520.

DE LA TORRE RA, ESPINOSA-AGUIRRE JJ, CORTINAS DE NAVAS C, IZQUIERDO T AND MORON F. 1994. Genotoxic activity of mebendazole in *Aspergillus nidulans*. Mutat Res 305: 139-144.

FISHEL R AND KOLODNER RD. 1995. Identification of mismatch repair genes and their role in the development of cancer. Curr Opin Genet and Dev 5: 382-395.

FJÄLLSKOG M-L, FRII L AND BERGH J. 1993. Is Cremophor EL, the solvent for paclitaxel, cytotoxic? Lancet 342: 873.

FRANZONI MGM, CASTRO-PRADO MAA AND GEBARA JS. 1997. On the recombinagenic activity of Norfloxacin in a diploid strain of *Aspergillus nidulans*. Cytologia 62: 39-45.

FRICHE E, DEMANT EJF, SCHESTAD M AND NISSEN NI. 1993. Effect of anthracycline analogs of photolabelling of p-glycoprotein by ^{125}I-iodomycin and ^{3}H-azidopine: relation to lipophilicity and inhibition of daunorubicin transport in multidrug resistant cells. Br J Cancer 67: 226-231.

GELDERBLOM H, VERWEIJ J, NOOTER K AND SPARREBOOM A. 2001. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. Eur J Cancer 37: 1590-1598.

GOLDMAN GH, MCGUIRRE SL AND HARRIS SD. 2002. The DNA damage response in filamentous fungi. Fungal Genet Biol 35: 183-195.

HJERTVIK M, ERIXON K AND AHNSTRÖM G. 1998. Repair of DNA damage in mammalian cells after treatment with UV and dimethyl sulphate: discrimination between nucleotide and base excision repair by their temperature dependence. Mutat Res 407: 87-96.

HOFFMAN H. 1984. Polyoxythylenglycerol triricinoleat 35 DAC 1979. Pharm Zeit 129: 1730-1733.

IWANEJKO L, COTTON C, JONES G, TOMSETT B AND STRIKE P. 1996. *nuvA*, an *Aspergillus nidulans* gene involved in DNA repair and recombination, is a homologue of *Saccharomyces cerevisiae RAD18* and *Neurospora crassa uvs-2*. Microbiology 142: 505-515.

KUNZ BA, BARCALY BJ AND HAYNES RH. 1981. Phenomenology and genetic control of mitotic recombination in yeast. Ann Rev of Genet 15: 57-80.

MAHER VM, BHATTACHARYYA NP, MAH MC, BOLDT J, YANG JL AND MCCORMICK JJ. 1993. Mutations induced by 1-nitrosopyrene and related compounds during DNA replication in human cells and induction of homologous recombination by these compounds. Res Rep Health Eff Inst 40: 41-51.

NOWELL P. 1976. The clonal evaluation of tumour cell populations. Science 194: 23-28.

NYGREN P, CSÓKA K, JONSSON B, FRIDBORG H, BERGH J, HAGBERG H, GLIMELIUS B, BRODIN O, THOLANDER B AND KREUGER A. 1995. The cytotoxic activity of Taxol in primary cultures of tumor cells from patients is partly mediated by Cremophor EL. Br J Cancer 71: 478-481.

OSMAN F, TOMSETT B AND STRIKE P. 1993. The isolation of mutagen-sensitive *nuv* mutants of *Aspergillus nidulans* and their effects on mitotic recombination. Genetics 134: 445-454.

PIRES LTA AND ZUCCHI TMAD. 1994. A new method to

detect potential genotoxic agents using mitotic crossing over in diploid strains of *Aspergillus nidulans*. Bras J Gen 17: 371-376.

PREISLER V, CASPARY WJ, HOPPE F, HAGEN R AND STOPPER H. 2000. Aflatoxin B1-induced mitotic recombination in L5178Y mouse lymphoma cells. Mutagenesis 15: 91-97.

RAMEL C, CEDERBERG H, MAGNUSSON J, VOGEL J AND NATARAJAN AT. 1996. Somatic recombination, gene amplification and cancer. Mutat Res 353: 85-107.

ROPER JA. 1952. Production of heterozygous diploids in filamentous fungi. Experientia 8:14-15.

SENGSTAG C. 1994. The role of mitotic recombination in carcinogenesis. Crit Rev Toxicol 24: 323-353.

STERN C. 1936. Somatic crossing-over and segregation in *Drosophila melanogaster*. Genetics 21: 625-730.

VAN DE VATE C AND JANSEN GJO. 1978. Meiotic recombination in a duplication strains of *Aspergillus nidulans*. Genet Res 31: 29-52.

WIJNHOVEN SW, SONNEVELD E, KOOL HJ, VAN-TEIJLINGEN CM AND VRIELING H. 2003. Chemical carcinogens induce varying patterns of LOH in mouse T-lymphocytes. Carcinogenesis 24: 139-144.

WOODCOCK DM, JEFFERSON S, LINSENMEYER ME, CROWTHER PJ, CHOJNOWSKI GM, WILLIAMS B AND BERTONCELLO I. 1990. Reversal of the Multidrug Resistance Phenotype with Cremophor EL, a common vehicle for water-insoluble vitamins and drugs. Cancer Res 50: 4199-4203.