Gene homozygosis and mitotic recombination induced by camptothecin and irinotecan in *Aspergillus nidulans* diploid cells

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ABSTRACT

Mitotic recombination is a process involved in carcinogenesis which can lead to genetic loss through the loss of heterozygosity. The recombinogenic potentials of two anticancer drugs topoisomerase I inhibitors, camptothecin (CPT) and irinotecan (CPT-11), were evaluated in the present study. The homozygotization assay, which assess the induction of mitotic recombination and gene homozygosis, as well as the heterozygous A757//UT448 diploid strain of *Aspergillus nidulans* were employed. The three non-cytotoxic concentrations of CPT (3.5 ng mL^{-1}, 10.5 ng mL^{-1} and 17.4 ng mL^{-1}) were found to induce both mitotic recombination and gene homozygosis. CPT treatment produced three diploids homozygous, for nutritional and conidia color genes, and Homozygotization Indices (HI) significantly different from negative control. On the other hand, only the highest CPT-11 concentration tested (18 µg mL^{-1}), corresponding to the maximal single chemotherapeutic dose, produced HI values higher than 2.0 and significantly different from negative control HI values. The recombinogenic effects of both topoisomerase I blockers were associated with the recombinational repair of DNA strand breaks induced by CPT and CPT-11. The anticancer drugs CPT and CPT-11 may be characterized as secondary malignancies promoters in cancer patients after chemotherapy treatment.

Key words: anticancer drugs, homozygotization assay, homologous recombination, secondary malignancies.

INTRODUCTION

DNA topoisomerase I (Topo I) is an eukaryotic nuclear enzyme that catalyzes relaxation of both positively or negatively charged DNA during multiple cellular process, such as DNA replication, recombination and transcription. The relaxing action of Topo I occurs by nicking a DNA single-strand and enabling the broken strand to rotate around the Topo I – bound DNA strand. Once the DNA is relaxed, the Topo I reseals the original nick by reversing its covalent binding (Pommier 2006).

Topo I is the cellular target of the anticancer drug camptothecin (CPT), a plant alkaloid isolated in the 1960s from the bark of the Chinese tree *Camptotheca acuminate*. Despite the potent antitumor activity of CPT observed among gastrointestinal cancer patients, the severe adverse effects of this Topo I poison revived the interest in research on CPT analogs as anticancer drugs. The water-soluble CPT derivative, irinotecan (CPT-11), which also exerts its antitumour activity by binding to the enzyme Topo I, has been reported to have effective clinical activity against several types of human malignancies, especially colorectal

cancer (Moertel et al. 1972, Pommier 2006, Punt and Koopman 2008).

CPT and its derivative CPT-11 bind to the Topo I DNA complex, preventing the next DNA re-ligation step. The action of both Topo I inhibitors on the cleavage complex results in the accumulation of the reversible ternary complex consisting of Topo I-camptothecin-DNA. The persistence of the drug-induced cleavage complexes is essential for optimum cytotoxicity of the Topo I inhibitors. This occurs because a fraction of the drug-induced cleavage complexes is converted into DNA double-strand breaks (DSB) upon collision with replication forks. DSB triggers cell cycle S and G2-M phases arrest, and may be repaired mainly through the homologous recombination repair pathway. The cytotoxicity of Topo I poisons has in fact been related to defects in cell cycle checkpoint pathways and DNA repair (Tanizawa et al. 1995, Arnaudeau et al. 2001, Huang et al. 2008, Pommier et al. 2010).

Homologous mitotic recombination (HR) is a crucial process for both faithful DNA replication in vertebrate cells and DSB repair pathway. HR occurs by the exchange of genetic material either between sister chromatids or homologous chromosomes. Whereas inter-sister HR restores the DNA sequence just as it was before the injury, inter-homologue HR induces the loss of heterozygosity (LOH) of parental markers. Inter-homologue HR may be a prerequisite for the development of tumors, such as hereditary retinoblastoma, in which it is estimated that inter-homologue HR undertakes the loss of the wild-type retinoblastoma allele in approximately 40% of the tumors. The role of HR in cancer development was also demonstrated in some cancer-prone hereditary diseases like Bloom and Werner syndromes (Saintigny et al. 2002, Payne and Hickson 2009, Moynahan and Jasin 2010).

Heterozygous diploid strains of the filamentous fungus *Aspergillus nidulans* have been used to evaluate the recombinogenic potential of several chemical compounds (Domingues Zucchi et al.

2005, Cardoso et al. 2010, Santos et al. 2012). *A. nidulans* is considered a model system for the mitotic crossing-over study because its cells spend the greater part of their cycle in the G2 phase. Since chromosomes are in duplicate at this phase, they significantly favor mitotic recombination (Bergen and Morris 1983, Castro-Prado et al. 2009, Sant'Anna et al. 2009).

Because inhibitors of DNA synthesis and inducers of DNA strand breaks have been described as the most potent inducers of homologous recombination in mammalian cells (Arnaudeau et al. 2000) and taking into account the DNA fragmentation caused by the Topo I inhibitors in human cells (Pommier 2006), this present study investigates the recombinogenic potential of CPT and CPT-11 for their ability to induce gene homozygosis and mitotic recombination in heterozygous diploid cells. In order to achieve our goal, a diploid strain of *A. nidulans*, which is heterozygous for several nutritional markers, as well as the homozygotization assay (Pires and Zucchi 1994), previously used to characterize the recombinogenic potential of several anticancer agents, such as cisplatin and cytosine arabinoside (Miyamoto et al. 2007), were employed.

MATERIALS AND METHODS

STRAIN AND CULTURE MEDIA

The master strains A757, with yellow conidia, and UT448, with white conidia, were used to form the diploid A757//UT448 strain of *A. nidulans* (Roper 1952) (Table I). Since diploid strain is heterozygous for five nutritional markers, it may grow in Minimal Medium (MM), consisting of Czapek-Dox medium, supplemented with 1% (w/v) glucose. On the other hand, when growing in the Complete Medium (CM), as previously described by Miyamoto et al. (2007), the diploid strain may originate auxotrophic mitotic segregants. Supplemented Medium (SM) consists of MM supplemented with all the

nutritional requirements of the strains which form the diploid (Table I), except one in each medium type. Solid Medium contains 1.5% (w/v) agar.

TABLE I
Genotype and origin of *A. nidulans* strains.

Strains	Genotype	Origin
A757	*yA2* (I), *methA17* (II), *pyroA4* (IV).	FGSC*
UT448	*riboA1* (I), *pabaA124*(I), *biA1*(I), *AcrA1* (II), *wA2*(II).	Utrecht, Holand.

Requirements for: riboflavin = *ribo*A1, p-aminobenzoic acid = *paba*A124, biotin = *bi*A1, methionine = *meth*A17, pyridoxine = *pyro*A4. Conidia color: white = *wA2*; yellow = *yA2*. *AcrA1*, resistance to acriflavine. *FGSC = Fungal Genetic Stock Center, University of Kansas Medical Center, Kansas, USA. (I) = linkage group I, (II) = linkage group II, (IV) = linkage group IV.

DRUG TREATMENT

(S)-(+)-camptothecin (CPT, CAS # 7689-03-4, $C_{20}H_{16}N_2O_4$, FW 348.4, Sigma-Aldrich Co, St. Louis Mo, USA) dissolved in NaOH (10%), and irinotecan hydrochloride (CPT-11, CAS # 100286-90-6, $C_{33}H_{38}N_4O_6.HCl$, FW 623.14, Sigma-Aldrich Co, St. Louis Mo, USA) dissolved in NaOH (4%), were added to molten MM. NaOH was *per se* neither visibly cytotoxic nor recombinogenic for the diploid strain (results not shown). Non-cytotoxic CPT concentrations, 3.5 ng mL^{-1}, 10.5 ng mL^{-1} and 17.4 ng mL^{-1}, that induced micronucleus in Chinese hamster ovary WBL cells (Kirpinic et al. 2005), and non-cytotoxic CPT-11 concentrations, 4.5 µg mL^{-1}, 9 µg mL^{-1} and 18 µg mL^{-1}, corresponding to the chemotherapeutic doses of CPT-11 (Campto$^{®}$) (Kašuba et al. 2010), were used in the present study. In the case of toxicity measurements, A757//UT448 diploid colonies' diameters were determined six days after incubation, at 37 °C. The growth rates in the presence (treatment) and in the absence (control) of the anticancer drugs were compared by One-Way Variance Analysis and by Bonferroni post-test, at p<0.05 (results not shown). The anticancer drug cisplatin (Pt(NH₃)₂Cl₂, FW 300.1, Sigma–Aldrich Co., St. Louis Mo, USA), previously

characterized as recombinogenic in human colorectal adenocarcinoma cells (Lin and Howell 2006) and in *A. nidulans* diploid cells (Miyamoto et al. 2007), was used as a positive control.

HOMOZYGOTIZATION ASSAY

Diploid colonies of A757//UT448 strain were obtained in petri dishes containing MM (negative control), MM + cisplatin (0.9 µg mL^{-1}, positive control), MM + CPT (3.5 ng mL^{-1}, 10.5 ng mL^{-1} and 17.4 ng mL^{-1}, treatment 1) and MM + CPT-11 (4.5 µg mL^{-1}, 9 µg mL^{-1} and 18 µg mL^{-1}, treatment 2) (Figure 1A-D). The petri dishes were incubated for six days at 37 °C and then visually inspected for diploid sectors arising on the original diploid strains' colonies. Diploids were purified on the MM, individually transferred to the CM dishes and then processed by spontaneous haploidization. Each diploid produced haploid mitotic segregants which were purified in CM and then had their mitotic stability evaluated in CM + benomyl (0.2 µg mL^{-1}). Benomyl, an haploidizing agent, is a strong spindle toxin, leading to disturbance in the mitotic segregation of chromosomes (Hüsgen et al. 1999). The mitotically stable haploid segregants at the final stage were the only ones selected for the recombinogenic test. Such segregants were individually transferred to different SM for their phenotypic analyses. The mitotic crossing-over causes homozygotization of heterozygous-conditioned genes. If CPT or CPT-11 induces mitotic crossing-over in the original diploid strain, only heterozygotes (+/- or -/+) or homozygotes (+/+) diploids will develop in the MM and the nutritional markers will segregate among the haploids in the proportion of 4$^+$ to 2$^-$. However, if the drug fails to induce crossing-over, the proportion will be 4$^+$ to 4$^-$. This is due to the fact that the initial selection process limits the growth of auxotrophic ($^-$/$^-$) diploids. The ratio of prototrophic to auxotrophic segregants is described by the Homozygosity Index (HI), in which an HI equal to or higher than 2.0 indicates the recombinogenic effect of the anticancer drugs (Figure 2). The recombinogenic potential of
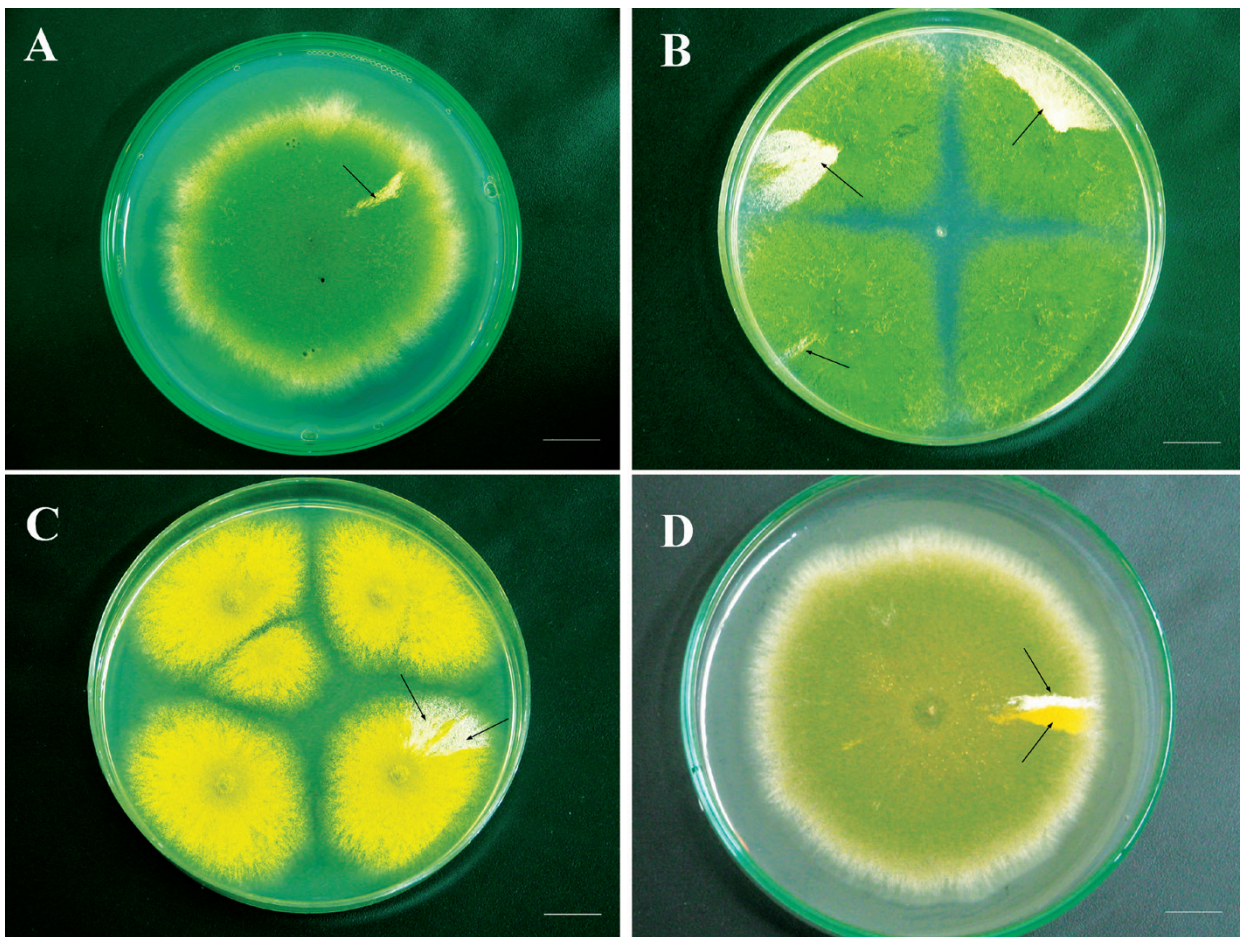
Figure 1 - Growth of A757//UT448 diploid strain in the absence of CPT or CPT-11 (control) **(A)**. Diploids obtained after treatment with CPT (17.4 µg/mL) **(B-C)** and CPT-11 (18 µg/mL) **(D)**. Arrows indicate the origin of mitotic segregants by the haploidization process. Bar = 5.0 mm.

CPT and CPT-11 was assessed by comparing the homozygotization indexes of the nutritional markers with Yates corrected Chi-square test, Contingency Table, $p < 0.05$.

RESULTS

A minimum of 455 mitotic segregantes were recovered from each CPT treatment (3.5 ng mL^{-1}, 10.5 ng mL^{-1} and 17.4 ng mL^{-1}) in CM, after haploidization. CPT treated diploids produced a higher number of prototrophic than auxotrophic segregants, leading to HI values which were higher than 2.0 and significantly ($p<0.05$) different from the negative control, for most of the analyzed markers (Table II).

All CPT concentrations produced unstable diploids which, although heterozygous for the *riboA1* and *pyroA4* genes, were homozygous for three other genes from linkage group I: *paba+, y* and *bi+*. The homozygous condition of the nutritional *pabaA124* and *biA1* genes was evidenced by the absence of auxotrophic segregants for these markers among the mitotic segregants derived from the unstable diploids, increasing HI values for genes *pabaA124* and *biA1* (Table II). Results indicate that such diploids are recombinant for the centromere-*paba* interval from linkage group I.

Nine diploid segregants were recovered after treatment of the original diploid strain A757//

TABLE II

Homozygotization Index (HI) values for markers from UT448//A757 diploid strain after treatment with camptothecin (CPT) and irinotecan (CPT-11).

Markers[a]	negative control[b]		positive control[c]		camptothecin						irinotecan					
					3.5 ng mL^{-1}		10.45 ng mL^{-1}		17.4 ng mL^{-1}		4.5 µg mL^{-1}		9 µg mL^{-1}		18 µg mL^{-1}	
	NS[d]	HI	NS[d]	**HI**	NS[d]	**HI**	NS[d]	**HI**	NS[d]	**HI**	NS[d]	**HI**	NS[d]	**HI**	NS[d]	**HI**
ribo+	129	1.4	194	1.6	368	1.6	313	**2.2***	460	**2.2***	254	1.0	297	1.2	444	**2.0***
ribo	92		120		225		142		211		248		247		227	
paba+	126	1.3	209	**2.0***	487	**4.6***	387	**5.7***	548	**4.5***	256	1.0	297	1.2	445	**2.0***
paba	95		105		106		68		123		246		247		226	
bi+	129	1.4	190	1.5	484	**4.4***	391	**6.1***	552	**4.6***	248	1.0	284	1.1	436	1.9
bi	92		124		109		64		119		254		260		235	
pyro+	112	1.0	289	**11.6***	323	1.2	319	**2.3***	425	1.7	250	1.0	267	1.0	367	1.2
pyro	109		25		270		136		246		252		277		304	

[a] *ribo* = riboflavin; *paba* = *p*-aminobenzoic acid; *bi* = biotin; *meth* = methionine and *pyro* = pyridoxine. [b] Negative control, diploids did not treated with cisplatin, CPT nor CPT-11. [c] Positive control, diploids treated with cisplatin (0.9 µg mL^{-1}. [d] Total number of mitotic segregants. *Significantly different from negative control (Yates Corrected Chi Square, Contingency Table, $p < 0.05$).
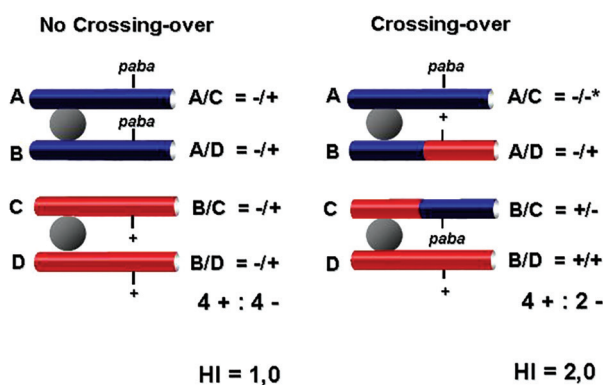

Figure 2 - Origin of heterozygous (+/- and -/+) and homozygous (+/+) diploids caused by mitotic crossing-over between *paba* gene and centromere. (*) Do not grow in MM (Pires and Zucchi 1994).

UT448 with 4.5 µg mL^{-1}, 9 µg mL^{-1} and 18 µg mL^{-1} CPT-11 concentrations. However, only the highest concentration tested (18 µg mL^{-1}), corresponding to the maximal single chemotherapeutic dose (Kašuba et al. 2010), produced HI values higher than 2.0 and significantly ($p < 0.05$) different from the negative control HI values. The genetic markers from linkage groups I and IV of the diploids obtained with CPT-11 middle (9 µg mL^{-1}) and low (4.5 µg mL^{-1}) concentrations produced HI values less than 2.0 similar to the negative control.

DISCUSSION

The present study evaluated the genotoxic profiles of two topoisomerase-inhibitors, CPT and CPT-11, using the homozygotization assay of *A. nidulans* (Pires and Zucchi 1994). Diploid segregants obtained after treatment of the original diploid A757//UT448 strain with CPT at 3.5 ng mL^{-1}, 10.5 ng mL^{-1} and 17.4 ng mL^{-1} exhibited homozygosis for the markers from *A. nidulans*'s linkage group I and HI values different ($p < 0.05$) from the negative control. On the other hand, concerning the recombinogenic potential of CPT-11, a significant increase in the HI values was observed only following treatment with the highest CPT-11 concentration (18 µg mL^{-1}), corresponding to the maximal single therapeutic dose (Kašuba et al. 2010). The lowest CPT-11 concentrations, corresponding to the recommended monotherapy (9 µg mL^{-1}) and combined therapy (4.5 µg mL^{-1}) doses (Kašuba et al. 2010) were not recombinogenic under current experimental conditions. Contrasting results were obtained by Kašuba et al. (2010) who demonstrated a higher cytotoxic effect of CPT-11 at low (9 µg mL^{-1}) rather than at high (18 µg mL^{-1}) concentrations in Chinese hamster V79 cells.

Although CPT-11 is a prodrug, poorly active against Topo I, its active metabolite, 7-ethyl-10-

hydroxycamptothecin (SN-38) is one of the most potent Topo I inhibitors. Previous studies comparing the molecular and cellular pharmacology of various CPT derivatives suggest that the CPT-11 clinical activity depends on its hydrolysis to SN-38 by human liver carboxylesterase (Tanizawa et al. 1994). Therefore, the recombinogenic effect of CPT-11, observed in the present study only at the highest concentration, may be a result of a low conversion of CTP-11 to SN-38 in *A. nidulans* diploid cells.

Taking into account that a fraction of the drug-stabilized Topo I cleavage complexes is converted into DNA damage upon collision with replication forks (Pommier et al. 2010, Huang et al. 2008, Arnaudeau et al. 2001), the recombinogenic effect of CPT and CPT-11, demonstrated here, may be associated with the recombinational repair of DNA strand breaks induced by these Topo I blockers.

Evidence that HR is required for the DSB repair after exposure to CPT comes from studies reporting on the high sensitivity to CPT exhibited by mutant cells for enzymes related to recombination repair: mammalian mutant cells for XRCC3 and Werner syndrome patients' cells, which are deficient in RecQ helicase (Agrelo et al. 2006, Ferrara and Kmiec 2004, van Waardenburg et al. 2004).

HR is an important mechanism involved in carcinogenesis which leads towards genetic loss through LOH when the recombinant sister chromatids segregate in mitosis to different daughter cells. HR is potentially able to induce the loss of the functional allele of a tumor suppressor gene in previously heterozygous cells. LOH in dermal neurofibromas has been shown to be frequently caused by mitotic recombination, resulting in homozygosity of the *NF1* tumor suppressor gene mutant allele (*NF1$^{-/-}$*). In addition, mitotic recombination within the region of 17q harboring the *NF1* gene was observed in 46% of plexform neurofibromas (Moynahan and Jasin 2010, Steinmann et al. 2009, Serra et al. 2001).

The recombinogenic effects of CPT and CPT-11, demonstrated here, as well as the ability of drugs to induce chromosomal aberrations (Sortibrán et al. 2006, Palitti et al. 1993) and DSB (Huang et al. 2008, Pommier 2006, Arnaudeau et al. 2001) imply in a possible risk of cancer patients developing secondary malignancies after chemotherapy with topoisomerase-inhibitors. In the normal cells of such patients, HR may induce aberrant genomic rearrangements, which may act as the primary step in the two-step model of carcinogenesis (Bishop and Shiestl 2002). Additionally, in pre-malignant cells, HR may induce genetic loss by LOH (Moynahan and Jasin 2010). Therefore, the clinical use of chemotherapeutic agents that induce HR and DNA fragmentation, such as CPT and CPT-11, must be weighed against the risk of the development of second malignances.

Numerous reports have related the effect of high-dose chemotherapy and the pathogenesis of second neoplasms. Treatment-related factors are presumed to be responsible for the elevated risk of myelodysplastic syndrome, lung cancer, non-Hodgkin's lymphoma and acute myeloid leukemia in patients treated with chemotherapy for distinct primary malignancies, such as hematologic and non-hematologic malignancies and Hodgkin's lymphoma (Swerdlow et al. 2011, Godley and Larson 2008, Leone et al. 2007). These studies show that there is a need for careful long-term monitoring of patients receiving chemotherapy for a primary condition, for the early detection and treatment of secondary cancers (Freeman et al. 2012, Papanikolaou et al. 2011, Swerdlow et al. 2011, Yamada et al. 1999).

RESUMO

A recombinação mitótica é um processo com participação na carcinogênese e com capacidade de induzir perda

genética através da perda da heterozigosidade. Os potenciais recombinagênicos de duas drogas anticancerígenas, camptotecina (CPT) e irinotecan (CPT-11), caracterizadas como inibidores da DNA-Topoisomerase I, foram avaliados no presente estudo, utilizando-se o ensaio de homozigotização, que avalia tanto a ocorrência da recombinação mitótica quanto a indução de homozigose, e a linhagem diplóide heterozigota de *Aspergillus nidulans*, A757//UT448. Três concentrações não-citotóxicas de CPT ($3,5$ ng mL^{-1}, $10,5$ ng mL^{-1} e $17,4$ ng mL^{-1}) induziram tanto recombinação mitótica quanto homozigose gênica. Os tratamentos com CPT produziram diplóides homozigotos para genes nutricionais e de coloração de conídios, bem como Índices de Homozigotização (HI) significativamente diferentes do controle negativo. Em contraste, somente a maior concentração de CPT-11 utilizada (18 μg mL^{-1}), correspondente à dose única máxima utilizada em protocolos de quimioterapia, produziu valores de HI significativamente diferentes do controle negativo. Os efeitos recombinogênicos de ambos bloqueadores de Topoisomerase I foram associados ao reparo recombinacional de quebras no DNA induzidas pelos compostos CPT e CPT-11. Os antineoplásicos CPT e CPT-11 podem ser caracterizados como promotores de malignidades secundárias em pacientes com câncer após tratamento quimioterápico.

Palavras-chave: anticancerígenos, ensaio de homozigotização, recombinação homóloga, malignidades secundárias.

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