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Antiviral activity of tiazofurin and mycophenolic acid against Grapevine Leafroll-associated Virus 3 in *Vitis vinifera* explants

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Abstract

The ability to control plant viral diseases with chemicals has great potential value for agriculture, but few chemicals are available to date due to the difficulty in obtaining effective drugs. IMP dehydrogenase is an enzyme which catalyzes the conversion of inosine 5′-monophosphate to xanthosine 5′-monophosphate in the *de novo* purine nucleotide synthetic pathway, and is considered a sensitive target for antiviral drugs. Two IMPDH inhibitors, tiazofurin (TR) and mycophenolic acid (MPA), were tested for their inhibitory effect on *Grapevine leafroll-associated virus* 3 (GLRaV-3) in *in vitro* grapevine explants. TR administration produced plantlets characterized by negative ELISA readings. No PCR products were obtained from these samples. This was confirmed by the absence of viral particles. MPA was essentially ineffective against GLRaV-3 replication in Sangiovese explants. This is the first report of GLRaV-3 eradication in grapevine explants following TR administration.

Keywords: *Vitis vinifera* explant; GLRaV-3; Tiazofurin; Mycophenolic acid

1. Introduction

Chemical procedures, one of the experimental approaches for restoring parts of infected plants to health, have been evaluated for their ability to interfere with virus replication in host tissue. So far, few chemicals have been found to eliminate or substantially reduce replication of phytoviruses (Griffiths et al., 1990) as compared to the broad range of therapeutic chemicals available against human viruses.

With regard to inhibitors of the inosine monophosphate dehydrogenase (IMPDH) group, two types of compounds endowed with antiviral activity have been described (Jager et al., 2002; De Clercq, 2002): synthetic nucleoside inhibitors, such as tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) (TR) and non-nucleoside inhibitors, such as mycophenolic acid (6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methyl-hex-4-enoic acid) (MPA). IMP dehydrogenase is an enzyme which catalyzes the conversion of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP), an essential step in the biosynthesis of purine nucleotides. By blocking the conversion of IMP to XMP, IMPDH inhibitors bring about a reduction in the supply of the precursors of RNA and DNA synthesis (Franchetti et al., 1996).

TR is a synthetic C-nucleoside, an analogue of Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), which exerts its action on IMPDH activity through its active metabolite thiazole-4-carboxamide adenine dinucleotide (TAD), an analogue of the cofactor nicotinamide adenine dinucleotide (NAD) formed from TR monophosphate via its 5′-phosphate (Franchetti et al., 1996; Weber et al., 1996; Jager et al., 2002). TR has progressed to phase IIII clinical trials in patients with acute leukemia (Jayaram et al., 2002).

MPA, a very potent non-nucleoside IMPDH inhibitor, is produced by fermentation of several *Penicillium* species. The compound binds to IMPDH after NADH is released but before XMP is produced (Link and Straub, 1996). Like TR, MPA blocks the production of necessary precursors for RNA and DNA synthesis (Kitchin et al., 1997). MPA is used clinically to prevent rejection of transplanted organs (Holt, 2002). MPA also has activity against several viruses such as hepatitis B virus (HBV) and dengue virus (DV) (Gong et al., 1999; Diamond et al., 2002).

GLRaV-3 belongs to the genus *Ampelovirus* (Martelli et al., 2002). It is characterized by a positive-sense ssRNA and organized into 13 ORFs which have been completely sequenced (Ling et al., 2000). Eight serologically distinct viruses, Grapevine Leafroll-associated Viruses (GLRaV-1 to 8),

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are associated with the Leafroll complex (Dovas and Katis, 2003). The complex is an economically important disease of grapevine, causing yield losses ranging from 3 to 68% and reductions of berry sugar concentration (Walter and Martelli, 1997).

Chemotherapy for generating virus-free grapevines has not been pursued; the few studies available in the literature focus mainly on Ribavirin, 2,4-dioxohexahydro-1,3,5-triazine (DHT) and ((*S*)-9-(2,3-dihydroxypropyl)adenine) (DHPA) (Monette, 1983; Barba et al., 1990; Panattoni and Triolo, 2003).

The aim of this research was thus to assess the antiviral action of TR and MPA against a widespread ssRNA virus: Grapevine Leafroll-associated Virus 3 (GLRaV-3) in *Vitis vinifera* L. cv Sangiovese explants.

2. Materials and methods

2.1. Sources of in vitro-material

In vitro grapevine explants were obtained from field-grown *V. vinifera* cv Sangiovese naturally infected by GLRaV-3. Selected plants were transferred to the insect-proof greenhouse and assayed by the ELISA test for several years, against *Grapevine leafroll associated ampelovirus* (GLRaV)-1 to 8; *Grapevine fleck maculavirus* (GFKV), *Grapevine fanleaf nepovirus* (GFLV), *Grapevine vitivirus A* (GVA), *Grapevine vitivirus B* (GVB), *Arabic mosaic nepovirus* (ArMV), at the appropiate time for each virus. ELISA was followed by RT-PCR assay discarding material with mixed infections, in order to use explants characterized by GLRaV-3 single infection.

An accession from the same variety, maintained in the greenhouse and known not to be infected by any of the above mentioned viruses, was used to generate the collection of healthy control explants.

Internodes were collected and surface sterilized before transfer to culture tubes with fresh Quorin–Lepoivre (1977) medium. All explants were maintained in a controlled environment chamber that assured maintenance of sanitary condition, with a temperature regime of $22 \pm 1\,^{\circ}C$, 16 h photoperiod, $50\ \mu E\ m^{-2}\ s^{-1}$, and transferred to proliferating medium at 30 day intervals. After an acclimatization period, the sanitary condition of each plantlet was confirmed by the ELISA test. Therapeutic drugs were added separately to the proliferation medium for treated replicates. At the same time, untreated explants were maintained on drug-free proliferation medium.

2.2. Antiviral drugs

TR (Kirsi et al., 1983) was kindly provided by Prof. Jayaram (Indiana University School of Medicine, Indianapolis, Indiana); MPA (Allison and Eugui, 2000) was purchased from Sigma–Aldrich (St. Louis, MO).

Drugs were hydrated in stock solution and, immediately prior to use, ultra-filtrated and added to proliferation medium. The experimental design involved drug administration for three consecutive subcultures, for a total treatment duration of 90 days.

A preliminary screening on healthy explants submitted to 30 days of administration at several concentrations was carried out to determine drug-induced phytotoxicity (Table 2). To define the phytotoxic levels, the number of dead explants out of total treated explants was counted at the end of the first subculture for each dose.

2.3. Virus detection by ELISA

The ELISA test was performed according to the method described by Clark and Adams (1977). Tissue samples from healthy (HC) and infected (IC) explants were used as negative and positive controls, respectively.

Polyclonal antibodies to GLRaV-1 to 8, GFKV, GFLV, GVA, GVB, ArMV (Agritest kit) were used in ELISA tests with a sample dilution ratio 1:10.

After each treatment (30 days), surviving explants were counted. The apical portion was then transferred to fresh supplemented medium, and the residue was assayed with the ELISA test. Absorbance at OD_{405} nm was recorded by photometry (Titertek multiskan).

Readings were normalized as R value (OD-treated explant/OD-HC), identifying the $R = 2$ threshold which distinguishes the positive response *versus* the negative response (Monette, 1983).

2.4. Biomolecular assay by RT-PCR tests

Total RNA was extracted from grapevine samples using components of the Plant RNeasy kit from Qiagen according to the methods described by MacKenzie et al. (1997). Approximately 0.2 g tissue was ground in liquid nitrogen, then homogenized with 2 ml of lysis buffer composed of 4 M guanidine isothiocyanate, 0.2 M sodium acetate pH 5.0, 25 mM EDTA, 2.5% (w/v) PVP-40 and 1% (v/v) 2-mercaptoethanol.

The homogenate was incubated for 30 min at 37 °C. One millilitre aliquot of the lysate was then transferred to a microcentrifuge tube, mixed with 100 μl of 20% (w/v) sarkosyl, and incubated for 10 min at 70 °C with shaking. The mixture was applied to a Qiashredder spin column (Qiagen) and centrifuged for 2 min at maximum speed in a microcentrifuge. After adding 0.5 vol ethanol to the cleared lysate, aliquots were loaded onto an RNeasy (Qiagen) column, and centrifuged for 30 s at $8000 \times g$. After washing the column with buffer RW1 (Qiagen), DNase I incubation mix (Qiagen) was applied to the column. RNA was eluted in 100 μl of RNase-free water and stored at −80 °C or used for RT-PCR.

For amplification of GLRaV-3 three primer pairs, designed to amplify fragments from the HSP-70 gene located in ORF 4, were used. For each primer sequence, annealing temperature adopted and amplicon size are shown in Table 1.

2.5. RT-PCR amplification

RNAs were detected using a one-step RT-PCR procedure based on the Qiagen One Step RT-PCR kit. A 50 μl reaction volume containing 5 μl of total RNA (about 500 ng) was used. The

Table 1
DNA primer pairs for RT-PCR amplification of GLRaV-3

Name	Sequence	Annealing temperature (°C)	Amplicon size (bp)	Nucleotide position (nt)
LC1[a]	cgctagggctgtggaagtatt	52	546	10979–11525
LC2[a]	gttgtcccgggtaccagatat			
C629[b]	gatgctttcgcgtatttcttg	54	300	11923–12223
H330[b]	cggcacgatcgtactttctaa			
Nolasco For[c]	tacagatacgattttgaatgga	52	322	11711–12033
Nolasco Rev[c]	ctgaaaaacgcgcttaaat			

Name, sequence, annealing temperature, amplicon size and nucleotide position are shown.
[a] Turturo et al. (2005).
[b] MacKenzie et al. (1997).
[c] Mansinho et al. (1999).

Table 2
Mortality observed for each drug, after 30 days administration for healthy *V. vinifera* cv Sangiovese explants at different concentrations

Drugs (μg/ml)	0	20	40	60	80	150	200	250
Mycophenolic acid (MPA)	0 (15)[a]	0 (15)	0 (15)	0 (15)	0 (15)	9 (15)	9 (15)	12 (15)
Tiazofurin (TR)	0 (15)	0 (15)	1 (15)	3 (15)	8 (15)	15 (15)	15 (15)	15 (15)

[a] Number of dead explants out of total treated healthy explants.

RT-PCR mixture contained 1× one step RT-PCR buffer, 400 μM of each dNTP, 0.6 μM of both forward and reverse primers, 2 μl of one step RT-PCR enzyme mix (a Qiagen enzyme blend containing OmniscriptTM, SensiscriptTM Reverse Transcriptases and HotStartTaqTM DNA polymerase). Amplification was carried out in a thermocycler (GenAmp PCR System 9700, PE Applied Biosystems). The cycling profile was as follows: a first step at 50 °C for 31 min (reverse transcription), followed by 15 min incubation at 95 °C and 35 cycles at 94 °C for 30 s, the appropriate annealing temperature (Table 1) for 45 s, then 72 °C for 60 s. The final extension step was at 72 °C for 5 min.

2.6. Analysis of amplified products

Aliquots (25 μl) of the PCR products were analyzed by electrophoresis on 1.5% agarose gel in 1× TBE buffer, stained with

Table 3
Mortality observed for MPA (A) and TR (B) administration, for infected *V. vinifera* cv Sangiovese explants at different concentrations, during the three subcultures (90 days)

Treatment days	(A) MPA (μg/ml)							
	0	20	40	60	80	150	200	250
30	0 (15)[a]	0 (15)	0 (15)	0 (15)	0 (15)	7 (15)	10 (15)	11 (15)
60	0 (15)	0 (15)	0 (15)	0 (15)	0 (15)	5 (8)	5 (5)	4 (4)
90	0 (15)	0 (15)	0 (15)	0 (15)	0 (15)	2 (3)	–	–
Treatment days	(B) TR (μg/ml)							
	0	20	40	60	80	150	200	250
30	0 (15)[a]	0 (15)	2 (15)	3 (15)	10 (15)	15 (15)	15 (15)	15 (15)
60	0 (15)	0 (15)	3 (13)	0 (12)	5 (5)	–	–	–
90	0 (15)	0 (15)	4 (10)	2 (10)	–	–	–	–

[a] Number of dead explants out of total treated infected explants.

Table 4
Therapeutic efficacy, expressed as ELISA-negative assays, of different concentrations of MPA and TR administrations, at varying times on infected *V. vinifera* cv Sangiovese explants.

	MPA 250 mg/l		TR 80 mg/l		TR 60 mg/l	
Treatment days	ELISA-negative	(%)	ELISA-negative	(%)	ELISA-negative	(%)
30	2/4[a]	(50%)	3/5[a]	(60.0)	3/12[a]	(25.0)
60	–	–	–	–	6/12	(50.0)
90	–	–	–	–	4/10	(40.0)

[a] Number of ELISA-negative/assayed explants.

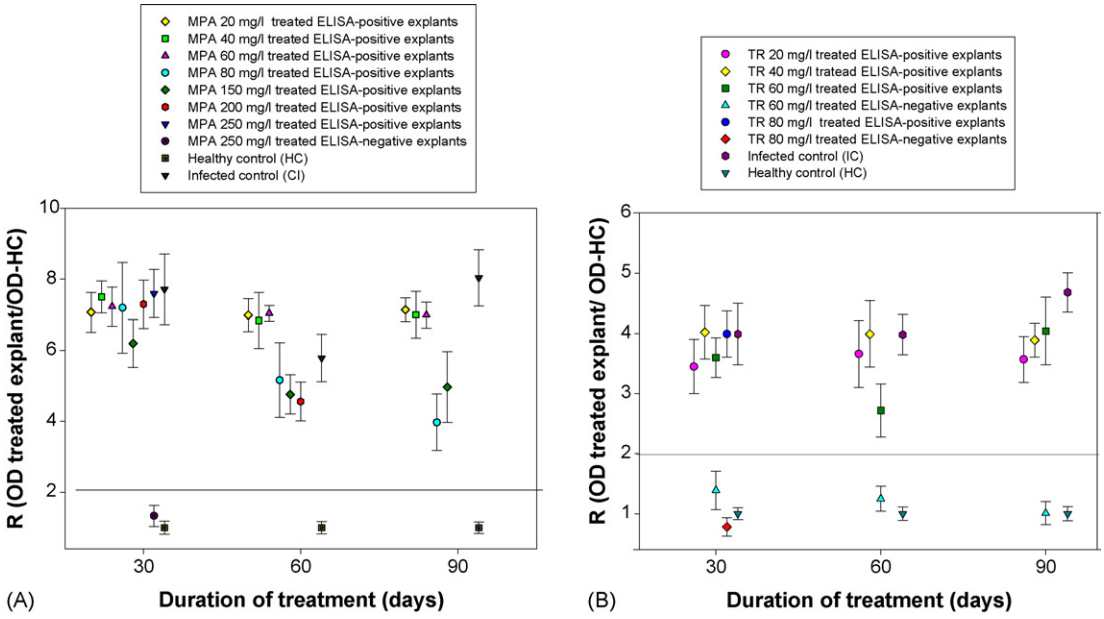

Fig. 1. ELISA test after 30, 60 and 90 days of MPA administration (A) and TR administration (B) on *in vitro V. vinifera* cv Sangiovese/GLRaV-3. *R* represents the mean value obtained by separating ELISA-negative from ELISA-positive data for each subculture, $R = 2$ as threshold value distinguished the positive response vs. negative response. Bars referred to standard deviation.

10 μg/ml ethidium bromide and photographed over an UV transilluminator. Fragment size was determined by comparison with DNA molecular markers (Invitrogen and MBI Fermentas).

3. Results

3.1. Drug toxicity on explants

No mortality on untreated healthy control explants was observed due to the micropropagation technique.

The lack of bibliographic information on the phytotoxicity of these two drugs made it necessary to consider a wide range of concentrations, although the highest concentrations showed elevated toxicity, suggesting probable difficulty in completing the 90 days of therapy.

The mortality values recorded for explants propagated on medium supplemented with the two drugs at different concentrations are indicated in Tables 2 and 3, referring to healthy and infected plantlets, respectively. No appreciable differences between the two groups were observed.

3.1.1. MPA treatment

No toxic effect on MPA-treated healthy explants was detected up to 80 μg/ml concentration. At the three highest doses, marked plantlet decline was observed, resulting in 60.0, 60.0 and 80.0% mortality, respectively, at the end of 30 days of administration (Table 2).

Infected explants treated at 20, 40, 60 and 80 μg/ml concentrations also showed no drug-induced phytotoxicity. In contrast, mortality levels of 46.6, 66.6 and 73.3% were recorded after 30 days at the doses of 150, 200 and 250 μg/ml, respectively (Table 3A), thus exhibiting values close to the healthy samples.

The 150 μg/ml concentration allowed treatment to be continued up to the third cycle, although due to a marked increase in mortality only one viable explant was obtained.

Administration of 200 μg/ml did not allow the five plantlets surviving after 30 days of treatment to remain viable up to the third treatment cycle. It was therefore necessary to interrupt treatment after 60 days.

The 250 μg/ml dose manifested elevated drug-induced phytotoxicity, causing treatment to be stopped after 60 days on supplemented medium.

3.1.2. TR treatment

In healthy explants TR administration induced a slight phytotoxic effect as early as the 40 μg/ml dose (6.6% mortality). Elevated values were recorded at higher doses, reaching 20.0% at 60 μg/ml, 53.3% at 80 μg/ml and 100% at the highest doses (Table 2).

Thus, at the 20 μg/ml concentration no mortality was detected in infected plantlets, allowing the entire therapeutic cycle to be completed (Table 3B). At 40 and 60 μg/ml mortality occurred after no more than 30 days of administration, with values of 13.3 and 20.0%, which rose to 26.6 and 33.3%, respectively, at the end of the third subculture.

The 80 μg/ml dose resulted in 66.6% mortality after 30 days of administration, rising to 100% mortality in the subsequent cycle.

3.2. Effects of chemotherapy on GLRaV-3

ELISA readings (*R*) of treated and untreated infected explants were examined at the end of each therapeutic period. All data represent the mean value obtained by separating ELISA-negative from ELISA-positive data for each subculture.

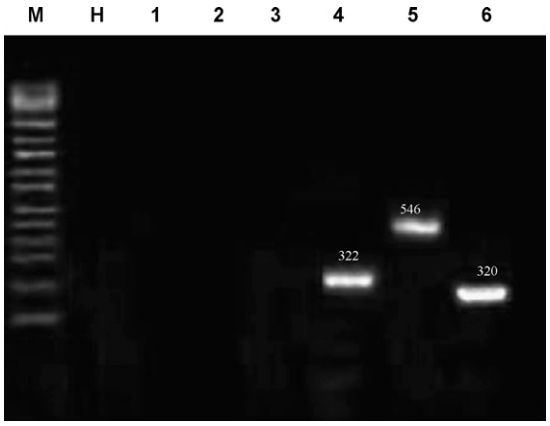

Fig. 2. Analysis of RT-PCR products from TR (80 mg/l) treated *in vitro* Sangiovese/GLRaV-3. Lane H, healthy control; lanes 1–3, TR treated ELISA-negative explants; lanes 4–6, infected control; lane M, DNA markers (MBI Fermentas).

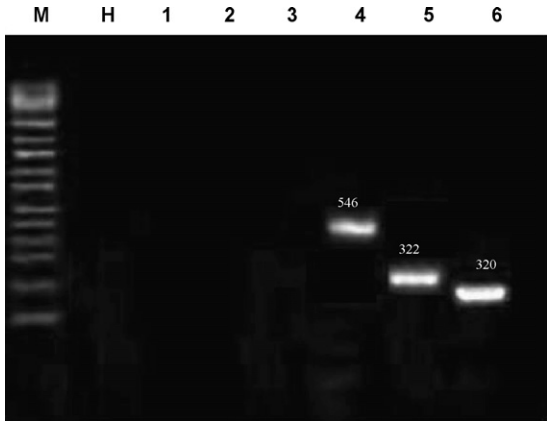

Fig. 3. Analysis of RT-PCR products from MPA (250 mg/l) treated *in vitro* Sangiovese/GLRaV-3. Lane M, DNA markers (MBI Fermentas); lane H, healthy control; lanes 1–3 MPA treated ELISA-negative explants; lanes 4–6, infected control.

3.2.1. MPA treatment

As shown in Fig. 1A, MPA was ineffective against GLRaV-3 in *V. vinifera* cv Sangiovese explants up to 200 μg/ml concentration, although OD values of treated plantlets decreased during the later stages of treatment, without ever reaching OD values of healthy plantlets.

Only 250 μg/ml MPA made it possible to obtain ELISA-negative explants after 30 days of administration, but the trial could not be completed due to the low survival rate of plantlets (Table 4).

3.2.2. TR treatment

Fig. 1B shows *R* values for TR treated explants. At 60 μg/ml concentration, up to 30 days, plantlets were characterized by absence of differences in optical density between treated ELISA-negative explants and healthy samples (25%), and also showed no OD differences between treated ELISA-positive explants and the infected control. After 60 days, 50.0% of treated explants showed OD values lower than the threshold, and at the end of therapy (90 days) similar data were found.

3.3. Biomolecular assay

RT-PCR amplification was carried out in order to determine the health status of ELISA-negative explants. All primer pairs were tested for the ability to detect GLRaV-3, using as template RNAs from untreated *in vitro* samples, along with RNAs from the MPA and TR treated plantlets. Agarose gel electrophoretic analysis of RT-PCR reactions revealed the presence of three DNA bands, of the expected size (546, 322, 300 bp), for the GLRaV-3 IC explants, referring to each primer pair.

No PCR products were obtained from the ELISA-negative samples, in any combination of primers, for both drug treatments, thereby confirming the absence of viral particles, in agreement with the results of the immunoenzymatic assay (Figs. 2 and 3).

4. Discussion

The genomic similarity between some phytoviruses and some animal viruses has prompted the suggestion that compounds which have shown efficient results against animal viruses should also be screened for efficacy against plant viruses. Tiazofurin and mycophenolic acid belong to the class of IMPDH inhibitors that include drugs currently being investigated in medical antiviral research programs.

Our results showed no toxic effects on grapevine explants after MPA administration up to 80 μg/ml, and up to 20 μg/ml in TR plantlets. A dose-dependent toxicity was recorded for higher drug concentrations, resulting in 73.3% death rate at 250 μg/ml MPA and 100% death rate at 150 μg/ml TR administration. MPA had no detrimental effects on GLRaV-3, although only a weak virostatic action was achieved. The 250 μg/ml MPA for 30 days was the only concentration that made it possible to obtain GLRaV-3-free grapevine explants confirmed by RT-PCR, but the damaging effects produced on explants showed the impossibility of using this dose for therapy on grape. These results confirm the findings of the initial screening, in which elevated mortality levels were already detected after 30 days of treatment.

TR 60 μg/ml for 30 days was the lowest dose of this drug that produced a strong reduction in virus replication, below levels detectable by ELISA test. The immunoenzymatic assay confirmed the antiviral action throughout all treatment cycles, and RT-PCR established that TR was capable of eradicating this virus and achieved a substantial rate (50.0%) of GLRaV-3 free explants surviving *in vitro*. The health of treated explants, during the year after the healing treatment, confirmed the antiviral activity of TR against GLRaV-3.

With regard to 80 μg/ml, while this dose produced an appreciable rate (60.0%) of GLRaV-3-free explants, it caused rapid explant death, indicating that such a concentration cannot be utilized.

This is the first report on TR and MPA administration aimed at eradicating plant viruses, as the only chemotherapic experiment against GLRaV-3 was performed on a combination of meristematic cultures and Ribavirin treatment (Barba et al., 1990). Thus

in the present study, the biochemical mechanism underlying the antiviral action of the two drugs was not investigated. It is likely, however, that it involves the same metabolic pathway reported for IMPDH inhibitor activity against animal viruses (Franchetti et al., 1996; De Clercq, 2001; Jager et al., 2002). In future studies we will assess the effective mechanism of action of this drugs in plant tissue by addition of exogenous guanosine (Damonte et al., 2004). Therefore, this research should be regarded as a preliminary stage within a broader chemotherapy-based experimental approach for control of some of the major plant diseases. The positive results obtained with the use of TR on grapevine leave two important problems unresolved: (i) the theoretical presence of toxic residues in berries and (ii) the possibility that chemotherapic treatment may cause mutagenic alterations.

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