Cancer Chemother Pharmacol (2010) 65:989–994
DOI 10.1007/s00280-010-1244-x

SHORT COMMUNICATION

DNA damage induced by the anthracycline cosmomycin D in DNA repair-deficient cells

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Received: 29 September 2009 / Accepted: 9 January 2010 / Published online: 28 January 2010

Abstract
Purpose Anthracyclines have been widely used as antitumor agents, playing a crucial role in the successful treatment of many types of cancer, despite some side effects related to cardiotoxicity. New anthracyclines have been designed and tested, but the first ones discovered, doxorubicin and daunorubicin, continue to be the drugs of choice. Despite their extensive use in chemotherapy, little is known about the DNA repair mechanisms involved in the removal of lesions caused by anthracyclines. The anthracycline cosmomycin D is the main product isolated from *Streptomyces olindensis*, characterized by a peculiar pattern of glycosylation with two trisaccharide rings attached to the A ring of the tetrahydrotetracene.
Methods We assessed the induction of apoptosis (Sub-G_1) by cosmomycin D in nucleotide excision repair-deficient fibroblasts (XP-A and XP-C) as well as the levels of DNA damage (alkaline comet assay).
Results Treatment of XP-A and XP-C cells with cosmomycin D resulted in apoptosis in a time-dependent manner, with highest apoptosis levels observed 96 h after treatment. The effects of cosmomycin D were equivalent to those obtained with doxorubicin. The broad caspase inhibitor Z-VAD-FMK strongly inhibited apoptosis in these cells, and DNA damage induced by cosmomycin D was confirmed by alkaline comet assay.
Conclusions Cosmomycin D induced time-dependent apoptosis in nucleotide excision repair-deficient fibroblasts. Despite similar apoptosis levels, cosmomycin D caused considerably lower levels of DNA damage compared to doxorubicin. This may be related to differences in structure between cosmomycin D and doxorubicin.

Keywords DNA repair · Cosmomycin D · Apoptosis · Anthracyclines

Abbreviations

CPD	Cyclobutane pyrimidine dimers
GGR	Global genome repair
TCR	Transcription-coupled repair
TTD	Trichothiodystrophy
XP	Xeroderma pigmentosum

Introduction

Anthracyclines have been widely used over the past decades as chemotherapeutics in the treatment of cancer, being one of the most effective classes of anti-tumor drugs. The first anthracycline described was β-rodomycin, isolated from *Streptomyces purpurascens* by Brockmann and Bauer in 1950 [1]. Since then, about 2,000 new anthracyclines have been isolated, although only a few molecules, mainly doxorubicin and daunorubicin, have been employed in cancer treatment. The anthracycline family of compounds are

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tetracyclic aromatic polyketides with a common 7,8,9,10-tetrahydrotetracene-5,12-quinone structure [2]. They are normally glycosylated with a wide array of different sugars, normally aminosugars. Other differences between members of the family are often found in the aglycone moiety such as substitutions in the quinone-hydroquinone rings. As most antineoplasic agents, these compounds were later associated either to the development of tumor resistance or to toxicity to healthy tissues, mainly cardiotoxicity [3]. In the search for less toxic anthracyclines or those with new biological activities, various derivatives have been designed, although until now, none of them have really proved to be less so to patients and doxorubicin or daunorubicin are still the most widely used.

The biological activity of anthracyclines is associated with several different mechanisms such as inhibition of topoisomerase II due to its intercalation in DNA and stabilization of a ternary complex DNA-anthracycline-topoisomerase, DNA damage due to the formation of reactive oxygen species, and intercalation-induced distortion in the double helix [2, 4] All these mechanisms can ultimately lead the cell to apoptosis. The deoxysugars attached to these polyketides are determinant to their biological activity, since they interact with the DNA minor groove near the intercalation site. They also play a key role in forming and stabilizing the ternary complex [5]. Furthermore, differences in the structure and number of sugar groups have been reported as attenuating anthracyclines cardiotoxicity [6, 7].

Cosmomycin D (Cos D) is the main product isolated from *Streptomyces olindensis*. It is an interesting anthracycline molecule since it has an uncommon structure with two trisaccharide chains, each one formed by a terminal L-rhodinose linked to a 2-deoxy-L-fucose and a L-rhodosamine, the latter ultimately attached to the C-7 and C-10 of the A anthracycline ring (Fig. 1). The DNA-binding properties of cosmomycin D were recently described as being as stable as those of daunorubicin [3, 8]. Nevertheless, further characterization of cosmomycin D properties is still necessary. Here, we studied the effects of cosmomycin D on cells deficient in nucleotide excision repair (NER). NER is one of the most versatile DNA repair pathways, primarily removing UV-induced photolesions (cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine pyrimidone photoproducts ((6-4) PPs)), but also acting on other bulky lesions that cause major distortions in the double helix [9, 10]. This repair pathway is a coordinated mechanism that involves more than 30 proteins, including XP (XPA through XPG) and CS proteins (CSA and CSB) [10–12]. Mutations in XP proteins are found in xeroderma pigmentosum (XP), a rare disease characterized by photosensitivity and a high incidence of skin cancer, besides being sometimes associated with premature aging and neurological symptoms [12]. CS proteins are mutated in Cockayne's syndrome, which is characterized by growth and mental retardation, retinal abnormalities and severe photosensitivity [13]. Mutations in XP genes can also result in trichothiodystrophy (TTD), a disease characterized by brittle hair and nails due to a deficiency in sulfur-rich protein synthesis [14].

NER works in two sub-pathways, either by repairing the genome as a whole (global-genome repair—GGR) or by specifically acting on the transcribed strand of active genes

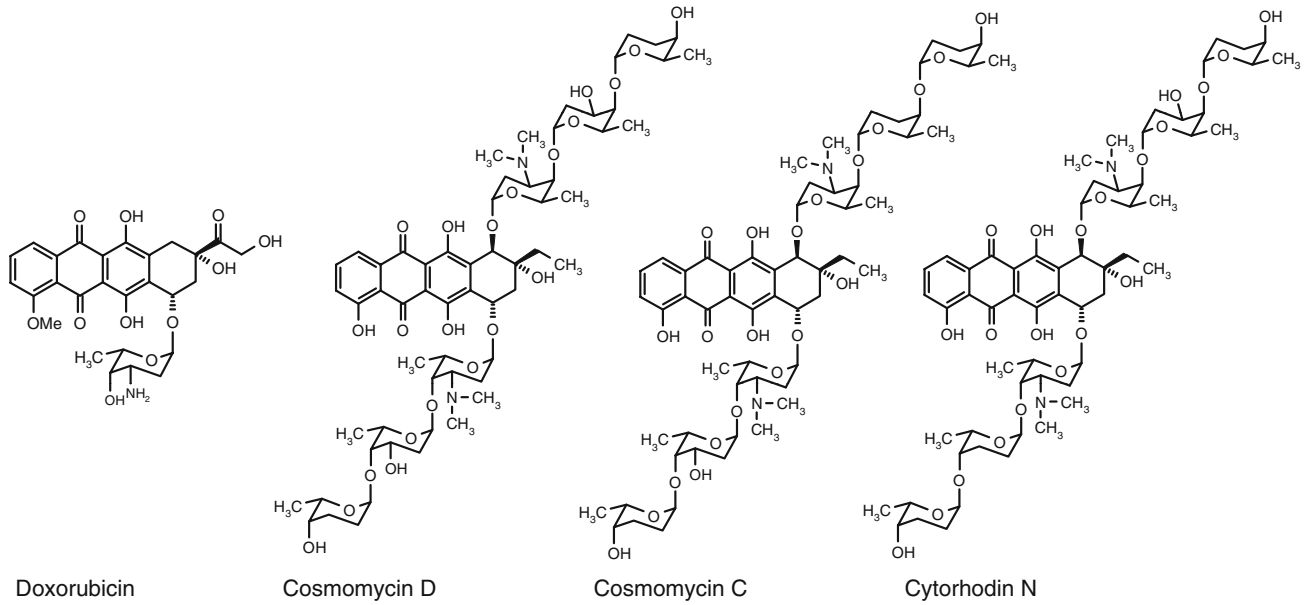

Fig. 1 Chemical structures of doxorubicin and cosmomycin D produced by *S. olindensis*

(transcription-coupled repair—TCR) [10]. Cells from XP patients are deficient in both TCR and GGR, with the exception of XPC and XPE complementation groups which are exclusively impaired for GGR. On the other hand, CS cells demonstrate exclusively TCR impairment [13]. As one of the consequences of unrepaired DNA damage, cells may trigger apoptosis, thereby avoiding the perpetuation of mutations. Apoptosis is a controlled mode of cell death, characterized by cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation in a characteristic internucleosomal pattern [15, 16].

In spite of the wide use of anthracyclines in clinical treatments, little is known regarding the DNA repair pathways involved in the removal of lesions caused by these drugs. Most reports link mismatch repair (MMR) deficiency to the resistance of tumors or cancer cell lines to anthracyclines [17, 18]. Recombination repair has also been implicated in the removal of anthracyclines-induced DNA lesions [19]. However, there are also some indications that the NER proteins XPB, XPD and XPG may be involved in the repair of these lesions [19, 20]. In order to define the possible role of NER, and specifically XPA and XPC proteins, in the repair of anthracycline-induced DNA lesions, we investigated apoptosis induced by the anthracycline cosmomycin D in XP-A (impaired for TCR and GGR) and XP-C (deficient only in GGR) deficient cells. Apoptosis levels were higher in NER-deficient compared to NER-proficient cells, with XP-C deficient cells being slightly more sensitive. The levels of apoptosis in cosmomycin D-treated cells were equivalent to those observed in doxorubicin-treated ones. DNA damage induction (single-strand breaks and alkaline sensitive sites) in cells treated with cosmomycin D was confirmed by the comet assay. These results reinforce the idea of a possible role of NER in the removal of anthracycline-induced lesions.

Materials and methods

Cell lines and culture

The SV40 transformed fibroblasts employed in this work were either DNA repair-proficient (MRC5) or NER-deficient from XP patients: XP12RO (XP-A cells) and XP4PA (XP-C cells). Normal cell line MRC5 was used as a control. These cells were kindly provided by Dr. Alain Sarasin (IRC, Villejuif, France). Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, USA), supplemented with 10% fetal calf serum (FCS, Cultilab, Campinas, SP, Brazil), 100 U/mL of penicillin G sodium, 100 μg/mL of streptomycin and 0.25 mg/mL of amphotericin B, at 37°C and in a humidified, 5% CO_2 atmosphere.

Purification of cosmomycin D

Cos D was purified from *S. olindensis* DAUFPE 5622, by HPLC, as previously described [21]. For purification, 5 mL culture samples were centrifuged and supernatants were applied to cartridges with 50 mg of C-18 as the solid phase (Sep-Pak Vac—Waters, Milford-MA, USA). The cartridge was washed twice with distilled water and the adsorbed compounds were eluted with 1 mL of methanol (Merck, Darmstadt, Germany) and dried under vacuum. The extracts were then resuspended at the desired concentrations with methanol (Merck).

Cell treatments

Cells were treated with 0.1–0.5 μg/mL of Cos D or doxorubicin (Sigma Chemical Co, St. Louis, MO, USA) in complete medium and then incubated for 24–96 h at 37°C. Cells were then harvested and assayed for apoptosis by flow cytometry. For caspase inhibition, cells were pre-treated for 2 h with 50 μM of the broad caspase inhibitor Z-VAD-FMK (Calbiochem, San Diego, CA, USA) followed by treatment with 0.1 μg/mL of CosD.

Comet assays

Cells were treated for 3 h with cosmomycin D or doxorubicin (Sigma). Alkaline comet assays were carried out as described [22]. Briefly, 20 μL of cell suspension (~10,000 cells) was mixed with 90 μL low melting point agarose, spread onto a normal agarose pre-coated microscope slide and left for 5 min at 4°C to allow for solidification. Cells were lysed for 2 h in high salt and detergent (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris with 1% Triton X-100 and freshly added 10% DMSO). They were then exposed to alkali (300 mM NaOH/1 mM Na_2EDTA, pH > 13, 30 min, 4°C) to allow for DNA unwinding and cleavage of alkali-labile sites. For electrophoresis, an electric field of 78 V/cm was applied for 25 min at 4°C. Afterward, the slides were neutralized, stained with ethidium bromide (Sigma) and analyzed by fluorescence microscope (Axiovert 200, Zeiss, Germany). One hundred cells were visually scored according to tail length and the amount of DNA present in the tail. Each comet was given an arbitrary value of 0–4 (0, undamaged; 4, maximally damaged). Damage scores were thus assigned to each sample and could range from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4).

Apoptosis detection

Apoptosis was measured by quantifying hypodiploid nuclei (sub-G_1 events) after cell cycle analysis of propidium

iodide (Sigma) staining [23]. Briefly, cells were harvested by tripsinization, along with the floating cells, resuspended with 70% ethanol/PBS and stored at 4°C. Cells were then stained with 50 μg/mL propidium iodide for 1 h in the presence of 40 μg/mL of DNAse-free RNAse A (Sigma). Measurements were carried out in a FACScalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). Results were analyzed with CellQuest Software (Becton-Dickinson).

Results

Induction of apoptosis by cosmomycin D

Cosmomycin D has been previously shown to induce apoptosis in HL60 cells [24]. The purpose of this work was to analyze the induction of apoptosis in NER-deficient cells, in order to evaluate a possible role of this repair pathway on the removal of DNA lesions caused by cosmomycin D. XP12RO cells, deficient in XPA protein, and XP4PA cells, deficient in XPC protein, were treated with 0.1 μg/mL of HPLC-purified cosmomycin D for different times. Apoptosis levels, measured by the percentage of cells in sub-G_1 are shown in Fig. 2a. The results for cells treated with doxorubicin are also shown for comparison. Cells deficient in NER were clearly more sensitive to apoptosis induction by both cosmomycin D and doxorubicin. Figure 2b shows there was no dose-dependent response when the same cells were treated with 0.1–0.5 μg/mL of cosmomycin D for 96 h. Induction of apoptosis was higher with 0.1 μg/mL, decreasing at higher concentrations, possibly due to an increase in the percentage of necrotic cells (not shown). These results may suggest a possible role for NER in the removal of lesions caused by these anthracyclines.

Apoptosis induction was greatly reduced, in all cells tested, by the broad caspase inhibitor Z-VAD-FMK (Fig. 2c) when cells where pre-treated with 50 μM of this inhibitor as shown by the decrease in the percentage of cells in Sub-G_1, thereby confirming the role of caspases in the process of cosmomycin D-induced cell death.

DNA damage detection by the comet assay

DNA damage was evaluated in cells treated with cosmomycin D concentrations ranging from 0.1 to 0.5 μg/mL, by using the alkaline version of the comet assay. Under these conditions, DNA single- and double-strand breaks, alkali-labile sites and incomplete excision repair events are detected. Results are shown in Fig. 2d. There was a general increase in the levels of DNA damage with increasing cosmomycin D concentrations. When used in the same concentration, DNA damage levels induced by doxorubicin were higher compared to cosmomycin D. Since apoptosis levels were comparable in both cases, these results may indicate slightly different mechanisms of action for cosmomycin D and doxorubicin.

Discussion

Despite their wide use in cancer treatment, the mechanisms of removal of DNA lesions induced by anthracyclines are not well understood. NER is widely known to remove bulky DNA lesions induced by UV light and several chemicals including cisplatin [10, 11]. NER has also been shown to play a role in the removal of interstrand crosslinks. Chinese hamster ovarian (CHO) cells with mutations in NER proteins are more sensitive to crosslinking agents than normal cells [25]. Nevertheless, XPF-deficient cells are much more sensitive than XPA, XPB or XPD. Moreover, crosslinked DNA generated in vitro and tested with cell extracts from CHO cells with mutated NER proteins [26] showed that removal of the oligomer containing the crosslink depends on XPF endonuclease.

Here, we confirmed previous results from other research groups indicating a role of NER proteins in the removal of anthracyclines-induced DNA lesions [19, 20]. XP12RO and XP4PA cells, deficient for XPA and XPC proteins, respectively, were more sensitive to apoptosis induction when compared to NER-proficient MRC5 cells. When analyzing the effects of different DNA adduct-forming anthracyclines, Spencer et al. [19] showed some interesting data. Similar cytotoxicity was observed in CHO cells deficient in XPB, XPD and XPG, treated with doxorubicin. However, different results were observed when anthracyclines barminomycin or doxoform were used. In this case, decreased cytotoxicity and lower apoptosis levels were observed for the same NER-deficient cells. However, the authors had not tested XPA and XPC cells.

It is interesting to note that in the present work, DNA damage levels were slightly higher for XP12RO when compared to XP4PA cells, despite the fact the latter showed higher levels of cosmomycin D induced apoptosis. This may be the result of operative TCR repair in XP4PA, absent in XP12RO cells, which quickly removes lesions from the transcribed strand of active genes. However, this does not explain why XP4PA cells underwent higher levels of apoptosis. The difference in behavior comparing these two cells is even more intriguing if we consider apoptosis induction by UV radiation, since XPA-deficient cells are known to be more susceptible to UV radiation than XPC-deficient ones [23]. These results may be influenced by cosmomycin D structure, mainly the types and numbers of deoxysugars, and also by the considerably higher molecular mass (1,189 kDa) when compared to doxorubicin

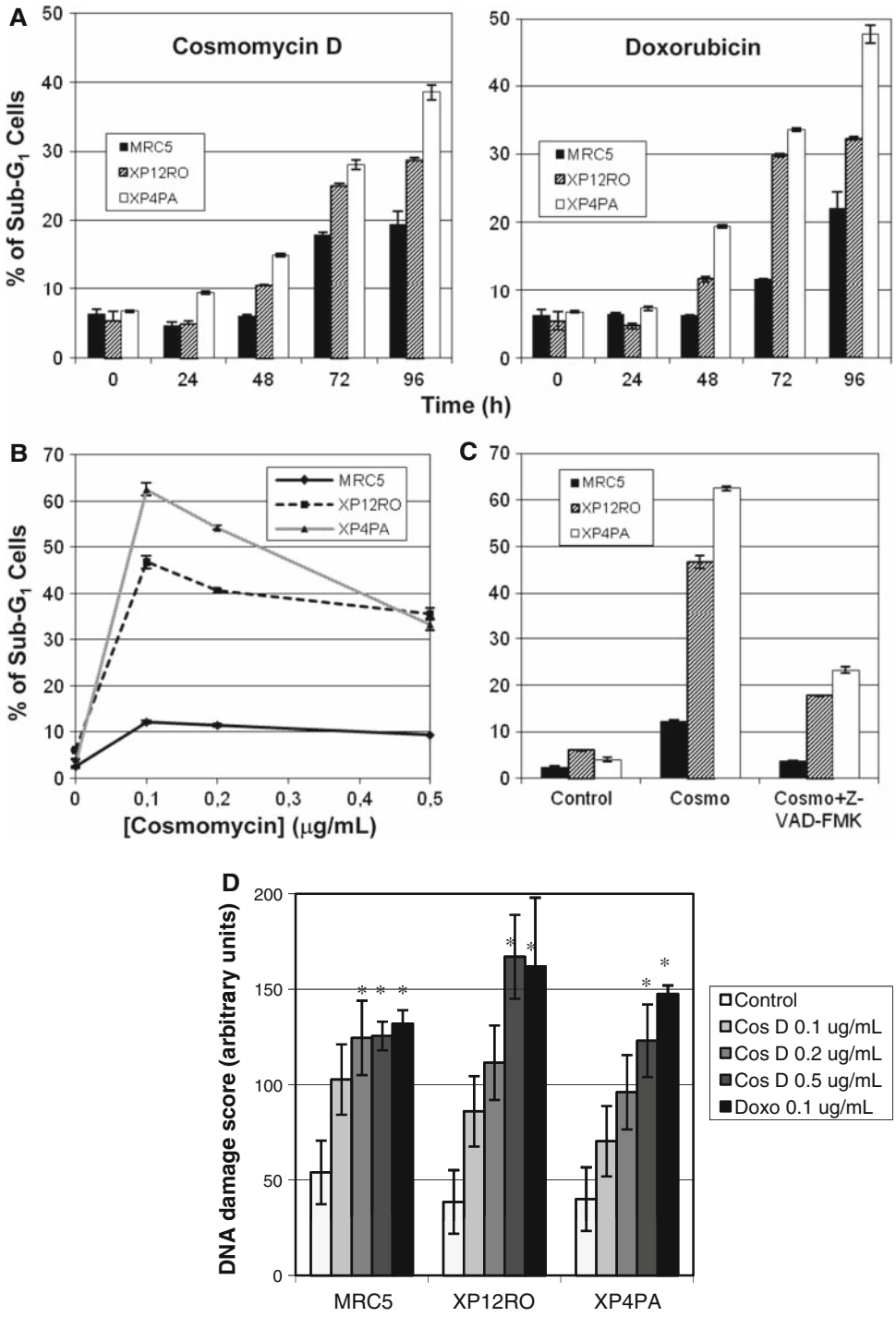

Fig. 2 Induction of apoptosis and DNA damage by cosmomycin D. **a** Time course analysis. MRC5 (normal), XP12RO (XPA) or XP4PA (XPC) cells were treated with 0.1 μg/mL of cosmomycin D or doxorubicin for 24–96 h. **b** Apoptosis induction by different cosmomycin D doses. Cells were treated with 0.1–0.5 μg/mL of cosmomycin D for 96 h. **c** Inhibition of apoptosis by the caspase inhibitor Z-VAD-FMK. Cells were pretreated with 50 μM of the broad caspase inhibitor Z-VAD-FMK for 2 h and then with 0.1 μg/mL of cosmomycin D for 96 h. Subsequently, cells were harvested, stained with propidium iodide and apoptosis levels were assessed by quantification of the number of cells with Sub-G_1 DNA by FACS. **d** Cosmomycin D-induced DNA damage measured by the comet assay. Cells were treated with 0.1–0.5 μg/mL of cosmomycin D or 0.1 μg/mL of doxorubicin for 3 h, transferred to glass slides, subjected to electrophoresis and then stained with ethidium bromide. Subsequently, cells were analyzed by fluorescence microscopy and scored according to tail length. *Error bars* represent the mean of 2–3 determinations ±SEM. *$P < 0.05$ compared to control

(543 kDa). Recently, Despras et al. [27] found that HeLa cells in which XPC was knocked down by RNA interference, showed increased sensitivity to the chemotherapeutic drug etoposide, which also affects topoisomerase II activity and stabilizes covalent enzyme-cleaved DNA complexes [28]. The authors found that XPC silencing reduced DNA Double-Strand Break Repair, while no effect was observed when XPA was knocked down. These results, as well as those presented here, reinforce the idea of a possible role for the XPC protein on the repair or regulation of repair of anthracycline-caused lesions. Arlett et al. [29] recently described an XP-C patient who showed increased clinical and cellular sensitivity to ionizing radiation. However, the complementation of cells derived from this patient with the XPC gene did not result in correction of radiosensitivity despite the correction of UV sensitivity.

So far, a clear role of XPA and XPC proteins in the removal of anthracyclines-induced DNA lesions remains

elusive. Nevertheless, this work confirms the susceptibility of NER-deficient cells to anthracyclines, comparing its effect on cells which are deficient for proteins involved in GGR and TCR (XPA) or only in GGR (XPC). Moreover, this is the first work to describe the susceptibility of NER-deficient cells to the anthracycline cosmomycin D. Further studies are still necessary to determine the precise role of XPA and XPC proteins on the removal of cosmomycin D and doxorubicin-induced DNA lesions.

Acknowledgments This work is supported by Fundação de Amparo à Pesquisa do Estado de São Paulo—FAPESP (São Paulo, Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (Brasília, Brazil). H. Carvalho acknowledges his fellowship from FAPESP.

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