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3-Selenocyanate-indoles as new agents for the treatment of superficial and mucocutaneous infections†

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The development of resistance to the current antifungal agents is an alarming problem. Therefore, the search for new molecules capable of combating fungi infections is imperative. This study presents the *in vitro* activities of a library of seven 3-selenocyanate-indoles against *Candida* spp. and dermatophytes of the genera *Trichophyton* and *Microsporum*. The antifungal susceptibility of compounds **4a** and **4b** presented geometric mean values of 4.1 and 6.0 μg mL^{-1} against *Candida* spp. and 1.2 and 2.2 μg mL^{-1} against dermatophytes following the CLSI guidelines. The 3-selenocyanate-indole **4a** showed a fungicidal effect against the whole fungal panel. The toxicological results revealed that the selenocyanates **4a** and **4b** did not show mutagenicity or cause changes in the human leukocyte cells and were classified as non-irritant by the *ex vivo* HET-CAM test. The mechanism of action of the 3-selenocyanate-indoles has not been clearly elucidated. However, a genotoxic potential in higher concentrations, observed by the comet assay, leads us to believe that these molecules have their mechanism of action related to the nucleus of the fungal cells.

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1. Introduction

Dermatophyte fungi are pathogens capable of invading keratinized tissues and cause cutaneous infections that are difficult to eradicate. The therapeutic options include ketoconazole, griseofulvin, allylamines, and triazoles. However, resistance to these drugs is frequently observed.[1–3] Similarly, there are few options available for the treatment of systemic and mucocutaneous infections caused by *Candida* spp. The indiscriminate use of these drugs has led to resistant and multidrug-resistant infections.[4,5] Currently, *C. albicans* is the major *Candida* species in hospital infections. However, non-*albicans Candida* infections caused by *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* are becoming quite common. Hence, the development of new antifungals is imperative.[6,7]

Privileged scaffolds are structures that can interact with different molecular cell-targets due to their appropriate molecular size and shape. Substituents can be designed into these structures, allowing the preparation of libraries of molecules with great diversity.[8] Indoles are a well-known example of privileged scaffolds. Several commercial drugs, which interact with a myriad of molecular targets, are indole-derivatives, including indomethacin, ergotamine, frovatriptan, ondansetron, tadalafil, delavirdine, zafirlukast, and sumatriptan.[8]

In 1993, 2-((phenylsulfinyl)methyl)-3-(phenylthio)-1*H*-indole was identified as a potent HIV reverse transcriptase inhibitor.[9] Since then, several libraries of 3-thio-indole derivatives have been synthesized as new anti-retroviral agents[10,11] and as strong poxvirus inhibitors.[12] Also, arylthioindoles, such as 5-bromo-3-((3,4,5-trimethoxyphenyl)thio)-1*H*-indole (**1**) (Fig. 1), have been proposed as anti-tubulin inhibitors to be used as new antitumor drugs.[13,14]

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† Electronic supplementary information (ESI) available: Synthesis of 3-selenocyanate-indoles **4**, tables of the mechanism of action, antibacterial susceptibility test, and HET-CAM assay. See DOI: 10.1039/c8nj04935a

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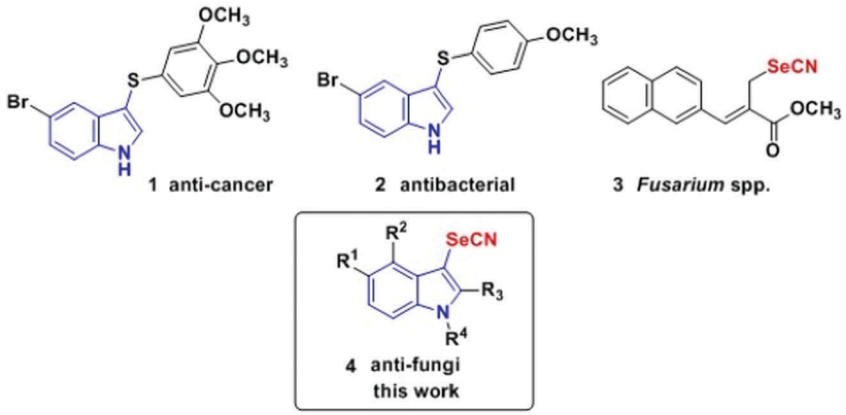


Fig. 1 Pharmacological properties of 3-chalcogenyl-indoles **1**, **2**, and **4** as well as the allylic selenocyanate **3**.

Commercially available drugs that are first prescribed to treat cancer, including 5-fluorouracil, gallium compounds, and mitomycin C, are being repurposed as antibacterials.[15] With these points in mind, we demonstrate that a library of 3-calcogenyl-indoles **3** (Fig. 1) presents good activities against *Staphylococcus aureus* isolates.[16] Similarly, anticancer drugs are being repurposed as new antifungals. For instance, the geldanamycin derivative 17-AAG, which is an Hsp90 inhibitor, dramatically improves the fluconazole activity in a *Galleria mellonella* model of systemic candidiasis.[17]

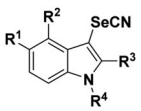
Organoselenium compounds have several chemical and pharmacological applications[18–22] due to their unique mechanisms of action including broad antimicrobial activity.[23] For instance, diphenyl diselenide and its analogues showed growth-inhibition and fungistatic activity against filamentous fungi and pathogenic *Candida* spp.[24] 2,2′-Dithienyl diselenide demonstrated fungistatic activity toward *C. albicans*.[25] Ebselen analogues presented activity against *C. albicans* and filamentous fungi.[26–28] Selenocyanates have a vast range of biological applications and can be prepared using different methodologies.[29] Efficient preparation of 3-selenocyanate-indoles has been reported in the literature by the use of diverse selenocyanate species.[30–34]

Recently, a library of allylic-selenocyanates, such as **3**, was prepared by us as new agents to combat *Fusarium* spp. involved in human infections.[35] Based on these previous studies and with our ongoing program seeking new molecules with antimicrobial properties[16,35–37] that can be developed as new leads for future *in vivo* studies, we decided to prepare a collection of seven 3-selenocyanate-indoles **4** (Fig. 1) and screen them against *Candida* spp. and dermatophytes. Therefore, this work discloses the results of these screening tests and toxicological studies (cytotoxicity, genotoxicity, mutagenicity, and allergenicity) to the best hit identified. The results are shown in the following sections.

2. Results and discussion

Seven 3-selenocyanate-indoles, **4a–g** (Table 1), were synthesized in one chemical step by an electrophilic aromatic substitution of the respective indole with electrophilic selenocyanate species. Triselenium dicyanide (TSD), which can be easily prepared *in situ* from malononitrile and selenium dioxide in dimethylsulfoxide, was chosen as the electrophilic selenium reagent.[32] So, the indoles reacted with TSD in dimethylsulfoxide at room temperature to yield the target indole selenocyanides in excellent yields (78–99%, ESI†). In a total, five new compounds, **4c–g**, were prepared.

Table 1 Screening (μg mL^{-1}) of selenocyanate-indoles **4a–g** against *Candida* spp.



	R^1	R^2	R^3	R^4	*C. albicans* ATCC 18804	*C. tropicalis* ATCC750	*C. glabrata* RL37	*C. krusei* CK01	*C. parapsilosis* RL13
4a	H	H	H	H	6.2	6.2	3.1	6.2	3.1
4b	Br	H	H	H	1.5	1.5	3.1	3.1	6.2
4c	I	H	H	H	50	>50	12.5	25	25
4d	CO_2CH_3	H	H	H	50	>50	50	>50	25
4e	H	CN	H	H	>50	>50	>50	>50	25
4f	H	H	Ph	H	>50	>50	50	>50	50
4g	H	H	Ph	CH_3	50	12.5	3.1	12.5	6.2
FCZ					1	4	8	8	0.5
AFB					0.5	0.2	0.1	0.03	0.06

Fluconazole (FCZ) and amphotericin B (AFB).

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The 3-selenocyanate-indoles **4a–g** were screened against a panel of *Candida* spp.: *C. albicans* (ATCC18804), *C. glabrata* (RL37), *C. krusei* (CK01), *C. tropicalis* (CT750), and *C. parapsilosis* (RL13) (Table 1). The selenocyanate derivatives **4a–g** presented antifungal activity with MICs $\leq$ 50 μg mL^{-1} for *C. parapsilosis* (RL13). In addition, all compounds were also active against *C. glabrata* (RL37), except for **4e**, considering the breakpoint chosen (50 μg mL^{-1}). Compounds **4a** (MICs 3.12–6.25 μg mL^{-1}) and **4b** (MICs 1.56–6.25 μg mL^{-1}) were active against the whole panel of yeasts and chosen as the lead compounds. Their MICs are in the same range as that of the commercial drug fluconazole (MICs 0.5–8 μg mL^{-1}) (Table 1). Substitution at the 5- (**4c** and **4d**), 4- (**4e**), and 3-positions (**4f**), and the nitrogen of the indole-ring were deleterious to activities against all fungi tested. The only exceptions are observed to be **4g** against *C. glabrata* and *C. parapsilosis*, whose MICs are in the same range as those of **4a** and **4b** to these microorganisms (Table 1).

Next, 3-selenocyanate-indoles **4a** and **4b** were screened against a panel of 25 *Candida* spp. and 15 filamentous fungi. These compounds demonstrated a broad spectrum of action toward yeast (MICs 1.5–12.5 μg mL^{-1}) and filamentous fungi of the genera *Microsporum* and *Trichophyton* (0.1–12.5 μg mL^{-1}) (Table 2).

It has been identified that a halogen atom at the 5-position of the indole ring of 3-arylthioindoles results in a reduction of the free energy associated with the binding of the compounds to tubulin, which decreases their cytotoxicity.[13] It is known that several compounds presenting anti-cancer activity also demonstrate antimicrobial capacity.[15,17,38–40] Preliminary results of our group demonstrated that 3-chalcogenyl-indoles are active toward Gram-positive bacteria (Fig. 1). The lead compound in these screening tests presents a 5-bromoindole moiety.[16] Similarly, this work shows the 3-selenocyanate-indoles **4a** and **4b** as lead compounds for further broad-spectrum antifungal drug development toward *Candida* spp. and dermatophytes of the genera *Trichophyton* and *Microsporum*. These fungi are typical dermatomycosis agents. Noteworthy, compound **4a** also has a 5-bromoindole moiety as observed in the previous studies with different targets. The presence of iodine (**4c**) or other electron withdrawing groups such as CO_2CH_3 (**4d**) at the fifth position of the indole ring was deleterious to the activities (Table 1).

Table 2 Minimum inhibitory concentrations (MIC range/geometric mean, μg mL^{-1}) of **4a**, **4b**, fluconazole (FCZ), and terbinafine (TBF)

Yeasts (n = 25)	4a	4b	FCZ[b]
C. tropicalis (n = 5)	3.1–6.2	1.5–12.5	0.5–16
C. krusei (n = 5)	1.5–12.5	3.1–12.5	0.25–32
C. parapsilosis (n = 5)	3.1	6.2–12.5	0.5–2
C. glabrata (n = 5)	3.1–6.2	3.1–12.5	0.25–32
C. albicans (n = 5)	1.5–6.2	1.5–6.2	0.5–32
Geometric mean[a]	4.1	6.0	2.5
Dermatophytes (n = 15)	**4a**	**4b**	**TBF**
M. gypseum (n = 5)	0.1–3.1	0.7–12.5	0.001–0.1
T. mentagrophytes (n = 5)	1.5–6.2	0.7–12.5	0.008–0.1
T. rubrum (n = 5)	0.4–0.8	1.5–3.1	0.001–0.06
Geometric mean[a]	1.2	2.2	0.02

n = Number of isolates. [a] Geometric mean interspecies. [b] MICs read after 24 h considering the reduction of fungal growth ($\cong$50%).

The lead compounds **4a** and **4b** were screened against a panel of 25 *Candida* strains and 15 filamentous fungi. These compounds presented a broad spectrum fungicidal profile against both genera tested and geometric means of 4.1 and 6.0 μg mL^{-1} toward *Candida* spp. and 1.2 and 2.2 μg mL^{-1} against *Trichophyton* and *Microsporum* (Table 2). These results are similar to the ones obtained using FCZ (2.5 μg mL^{-1}), which is the prescribed drug in the treatment of candidiasis.[41] Meanwhile, the selenocyanates **4a** and **4b** were 50–100 times less active than TBF (0.02 μg mL^{-1}) against dermatophytes. However, since resistance to these microorganisms is continuously observed,[42–44] **4a** and **4b** might be an alternative for the reference drug (TBF), especially considering that they do not share the same mechanism of action as that of TBF.

The MIC of anidulafungin against yeasts and MEC for filamentous fungi increased significantly in the presence of sorbitol after 7 and 8 days, respectively, as an indication of its fungal cell wall activity. This was not observed for 3-selenocyanate-indoles **4a** and **4b**, since their MICs did not change (Table S1, ESI†). Similarly, ergosterol (100, 150, 200, and 250 μg mL^{-1}) was added into the culture medium to verify if the compounds have action on the fungal cell membrane. Amphotericin B was used as the control. The MICs also did not vary for the selenocyanate-indoles **4a** and **4b** (Table S2, ESI†).

The ergosterol and sorbitol assays indicated that the broad-spectrum antifungal mechanism of action of compounds **4a** and **4b** are not related to interactions with the membrane and fungal cell wall chemicals. Hence, scanning electron microscopy (SEM) allowed us to visualize the effects of the 3-selenocyanate-indole **4a** on the morphology and amount of *C. albicans* cells after *in vitro* treatment. A considerable reduction in the number of blastoconidia, hyphae, and pseudohyphae was evidenced by SEM images after exposure to **4a** (Fig. 4). The fungicidal activity of this compound may be related to this effect. The formation of hyphae and pseudohyphae by yeast is considered an important mechanism of pathogenicity, which facilitates tissue invasion, colonization, and infection of mucous membranes.[45,46] Therefore, this information might be clinically relevant.

C. albicans (ATCC 18804) was treated with **4a** (sub inhibitory concentration: 3.1 μg mL^{-1}). After 48 h of incubation, the number of fungal cells and pseudohyphae observed by SEM reduced (Fig. 2B) in comparison with that observed for the untreated control (Fig. 2A).

The fungal cells were exposed for 4, 12, 24, and 48 h (*C. albicans*) and 8, 12, 24, and 48 h (*T. rubrum*) to the selenocyanate-indoles **4a**

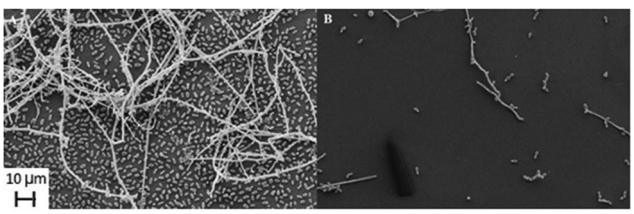

Fig. 2 SEM of *C. albicans* strain (ATCC 18804); (A): untreated control. (B) Treatment with **4a** (sub inhibitory concentration, 3.1 μg mL^{-1}).

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(Fig. 3A and C) and **4b** (Fig. 3B and D) at concentrations MIC/2, MIC, MICx2, and MICx4 in a time-kill assay. Compound **4a** presented a fungicidal profile (a reduction of $>99.9\%$ in $\log_{10}$ relative to the initial inoculum) against *C. albicans* (ATCC 18804) at MICx2 and MICx4 after 4 h of treatment. Meanwhile, at MIC/2 and MIC, microbial inhibition was observed after 12 h of experiment followed by cell proliferation equivalent to the positive control (Fig. 3A). A fungicidal effect was also observed toward *T. rubrum* at all concentrations over the initial 8 h of experiment (Fig. 3C). This complete inhibition of their growth in the early hours demonstrates an excellent fungicidal effect and could be further related to a possible high level of clinical efficacy. The time-kill assay demonstrated that **4b** exhibits a fungistatic effect (a reduction of $<99.9\%$ in $\log_{10}$ in relation to the initial inoculum) against *C. albicans* (ATCC 18804) at MICx2 and MICx4 after 12 and 24 h of treatment, respectively. However, a significant reduction in the activity after 12 to 24 h of treatment followed by cell proliferation can be observed (Fig. 3B). This effect is noted with azole antifungals. These drugs do not completely eliminate fungal cells but inhibit their growth. The fungistatic mechanism usually works in conjunction with the immune system of the host, killing pathogenic or opportunistic microorganisms. However, this mechanism of action might be a major concern in immunocompromised, patients leading to a persistent infection.[47] Meanwhile, the selenocyanate-indole **4b** presented a fungicidal profile against *T. rubrum* (45) at MIC and MICx2 after 24 h and at MICx4 after 8 h of treatment. Cell proliferation is observed at MIC and MICx2 after 8 h of treatment, which would suggest the need for a new therapeutic dose. A dose-dependent effect was noted in the first few hours of the experiment with *T. rubrum*, which required a new therapeutic dose for complete microbial inhibition, as noticed at MIC and MICx2 concentrations after 24 h of treatment (Fig. 3D).

Selenocyanate-indoles **4a** and **4b** (32 μg mL^{-1}) were not cytotoxic to human leukocyte cells, since no statistical differences were observed compared to phosphate-buffered saline (PBS, negative control). Bleomycin (BLE, positive control) reduced the cell viability to approximately 20% (Fig. 4A). Similarly, significant micronucleus frequency, as well as apoptotic and necrotic processes for compounds **4a** and **4b** (32 μg mL^{-1}), was not noticed, inferring a non-mutagenic potential (Fig. 4B). However, the comet assay showed that **4a** and **4b** (32 μg mL^{-1}) caused significant DNA damage (Fig. 4C).

As disclosed by Pukalskienė *et al.*,[48] the divergence in comet and micronucleus results can be explained by the fact that proliferating leukocytes were used for the micronucleus assay. Leukocyte cells have higher repair capacity than cells in the comet's G0 phase (resting cells). Therefore, the DNA damage observed in the comet assay can be repaired during the micronucleus experiment.[49] Since the nuclei of the fungal and mammalian cells have similarities, it is believed that the targets of the compounds are the nucleic acids. Further experiments might be conducted to confirm this hypothesis.

Most imidazole antifungals present cell toxicity or bioavailability problems and are formulated for topical use.[50] Ketoconazole is cytotoxic and presents a possible mechanism of mitochondrial dysfunction in liver cells, compromising mitochondrial DNA synthesis, resulting in induced apoptosis.[51] Thus, even drugs demonstrating cellular toxicity for systemic use are prescribed for topical administration. In addition, in the present study, the toxicity of the selenocyanate-indoles **4a** and **4b** was evaluated at 32 μg mL^{-1} and the MICs (geometric mean) determined were in the range of 1.2 to 6.0 μg mL^{-1}. Thus, DNA damage is being observed at higher concentrations of the compounds compared to the MICs.

The selenocyanate-indoles **4a** and **4b** (32 μg mL^{-1}) are found to be non-irritant (IS = 2.19 and 3.01, respectively) through the HET-CAM assay (Fig. S15, ESI†). The chorioallantoic membrane is highly vascularized and responds to injuries caused by processes, such as inflammation, similar to that observed in the conjunctival tissue of rabbit eyes.[52] This is an indication

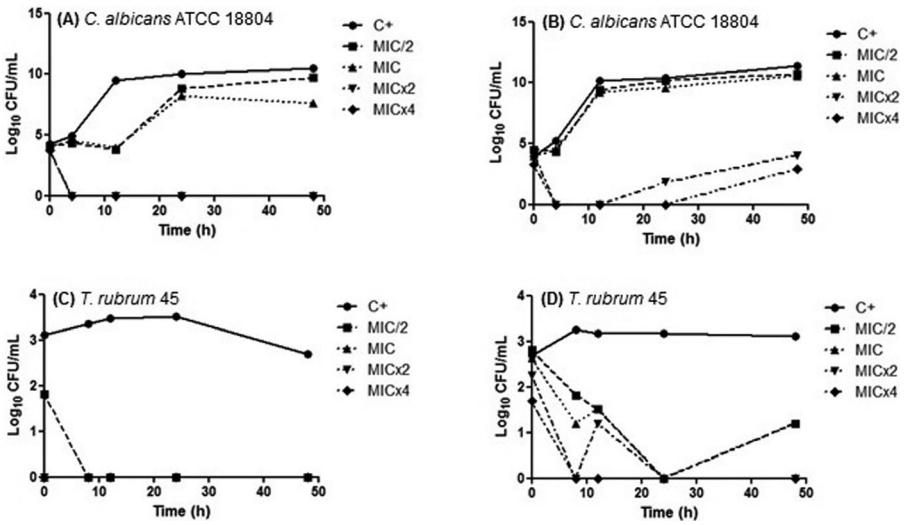

Fig. 3 Time-kill assay of selenocyanates **4a** (A and C) and **4b** (B and D) against *C. albicans* (ATCC 18804) strain and *T. rubrum* (45) isolate at: MIC/2(■), MIC(▲), MICx2(▼), MICx4(♦), and control (●). **4a** MIC: 6.25 μg mL^{-1} (*C. albicans* ATCC 18804), and 0.78 μg mL^{-1} (*T. rubrum* TRU 45); **4b** MIC: 1.56 μg mL^{-1} (*C. albicans* ATCC 18804), and 1.56 μg mL^{-1} (*T. rubrum* TRU 45).

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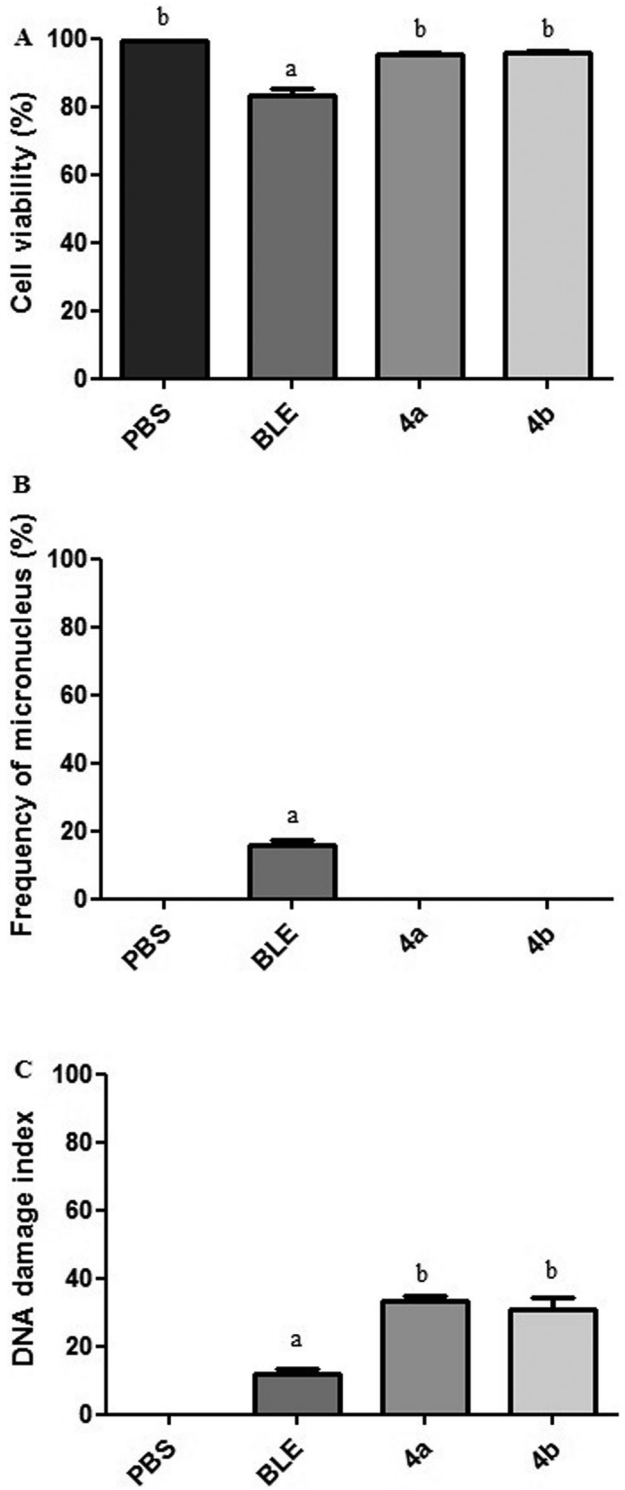

Fig. 4 Toxicological analysis to evaluate the cell viability, mutagenicity, and genotoxicity of selenocyanate-indoles **4a** and **4b** (32 μg mL^{-1}) toward human leukocytes. Phosphate-buffered saline (PBS, negative control) and bleomycin (BLE, positive control).

that formulations for topical administration might be developed without further mucous irritation.

Synergistic effects as a consequence of co-infection of *Candida* spp. with *Staphylococcus* have been demonstrated by *in vitro*[53] and *ex vivo*[54] studies. It is assumed that Gram-positive bacteria adhere or bind to the hyphae of *Candida* spp. For instance, it has been shown that *C. albicans* can transport *S. aureus* into tissues disseminating the infection in an oral co-colonization model. Therefore, co-infection of *Candida* spp. and *S. aureus* results in a more serious infection than that caused by each microorganism individually.[54] 3-Selenocyanate-indoles **4a** and **4b** were also screened against *S. aureus* (ATCC 25923) and the MICs determined were 8 μg mL^{-1} (see the ESI†), indicating that the compounds might have antimicrobial capacities, avoiding this kind of co-infection.

3. Experimental section

3.1. Fungal strains

In total, 40 clinical and ATCC (American Type Culture Collection, Manassas, VA, USA) strains were used in this study, including *C. tropicalis* (ATCC 750, 17P, 72P, RL104, and ATCC 950), *C. krusei* (CK01, CK02, CK03, RL102, and DEN43), *C. parapsilosis* (RL11, RL13, RL33, RL100, and CP0′7), *C. glabrata* (RL03, RL22, RL37, RL105, and CG09), *C. albicans* (CA01, CA02, DEB05, DEB09, and ATCC 18804), *M. gypseum* (MGY 01, MGY 1, MGY 2, MGY 3, and MGY 50), *T. mentagrophytes* (TME 1, TME 2, TME 3, TME 40, and TME 46), and *T. rubrum* (TRU 2, TRU 3, TRU 45, TRU 47, and TRU 51), belonging to the mycology collection of the research group in Applied Mycology, School of Pharmacy (Universidade Federal do Rio Grande do Sul, Brazil).

3.2. Synthesis process

3-Selenocyanate-indoles **4** were synthesized in good yields by the reaction of the respective indole with TSD, which is prepared *in situ* from selenium dioxide and malononitrile in DMSO (ESI†).

3.3. Antifungal susceptibility testing

The minimum inhibitory concentrations (MICs) of the selenocyanate-indoles **4** were determined by the broth microdilution method according to the M27-A3 protocol for *Candida* spp. and M38-A2 for filamentous fungi (*Microsporum* spp. and *Trichophyton* spp.).[55,56] Serial microdilutions were performed in RPMI 1640 medium, and the experiments were carried out in duplicate. MICs are defined as the lowest concentration of compounds at which the microorganisms tested did not demonstrate visible growth after 48 (*Candida* spp.) or 96 h (for *Microsporum* spp. and *Trichophyton* spp.). Fluconazole and terbinafine were used as the reference antifungals against *Candida* spp. and dermatophytes fungi, respectively. The sterility control (negative control: a drug-free medium) and positive control for fungal cell viability were used in parallel (ESI†).

3.4. Mechanism of action

3.4.1. Ergosterol binding assay and sorbitol protection assay. In order to evaluate the ability of selenocyanate-indoles **4** to form a complex with the sterol of fungal membranes, the ergosterol assay was performed.[57] The exogenous (qualitative) ergosterol determination technique was executed with and

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without the addition of ergosterol (Sigma-Aldrich) at concentrations of 100, 150, 200, and 250 μg mL^{-1} against *C. albicans* (ATCC 18804), *C. glabrata* (RL37), *C. krusei* (CK01), *C. tropicalis* (ATCC 750), *C. parapsilosis* (RL13), *T. rubrum* (TRU45), *T. mentagrophytes* (TME46), and *M. gypseum* (MGY50). Ergosterol was dissolved in dimethylformamide (Sigma-Aldrich) and the solution was added to RPMI-1640 medium (containing L-glutamine, without sodium bicarbonate, buffered to pH 7.0, Sigma-Aldrich). The microplates were incubated at 35 °C for 48–72 h. The MICs were determined visually by the absence or presence of fungal growth. Amphotericin B was used as a control. The assay was performed in duplicate.

The antimicrobial effect on the integrity of the fungal cell wall was determined by a sorbitol protection assay.[57] The MICs of the compounds were determined with and without addition of sorbitol (0.8 M, Sigma-Aldrich) against *C. albicans* (ATCC 18804), *C. glabrata* (RL37), *C. krusei* (CK01), *C. tropicalis* (ATCC 750), *C. parapsilosis* (RL13), *T. rubrum* (TRU45), *T. mentagrophytes* (TME46), and *M. gypseum* (MGY50). Sorbitol was dissolved in the RPMI-1640 culture medium (containing L-glutamine, without sodium bicarbonate, buffered at pH 7.0, Sigma-Aldrich). The microplates were incubated at 35 °C and the MICs determined were visually by the absence or presence of fungal growth from the day 2 to the day 7 of incubation for the yeasts and from the day 4 to the day 8 of incubation for the filamentous fungi. Anidulafungin was used as a control. The assay was performed in duplicate.

3.4.2. Scanning electron microscopy (SEM). Only one fungal strain – *C. albicans* (ATCC 18804) was selected to perform scanning electron microscopy (SEM). Selenocyanate-indole **4a** was tested at a concentration of 3.1 μg mL^{-1}. After 48 h of incubation, wells containing the coverslips were washed three times with phosphate buffer saline (PBS). After washing, the adhered cells were added with 500 μL of glutaraldehyde (2.5%, type 1, Sigma Aldrich), diluted with sodium cacodylate (0.1 M, pH 7.2, Sigma Aldrich), and kept for 1 h at room temperature. Then, the wells were washed three times with sodium cacodylate (0.1 M, pH 7.2) containing sucrose (0.2 M) and $MgCl_2$ (2 mM). The adhered cells were dehydrated by immersion in gradients of acetone solutions. Then, the samples were subjected to critical point drying (EM CPD 300, Leica) immediately after dehydration, mounted on metallic stubs, sputter-coated with a 15–20 nm gold-palladium layer, and visualized using a scanning electron microscope (Carl Zeiss EVO® MA10, Oberkochen, Germany) operating at 10 kV.[58]

3.5. Time-kill assay

A time-kill assay was carried out with one representative strain of each genus studied (*C. albicans* ATCC 18804 and *T. rubrum* 45), and the procedures were conducted as previously described by Klepser *et al.*[59] and Ghannoum *et al.*,[60] for *Candida* spp. and dermatophytes, respectively. The activity of the selenocyanate-indoles **4a** and **4b** was evaluated against the isolates at 0, 4, 12, 24, and 48 h (*C. albicans*) and 0, 8, 12, 24, and 48 h (*T. rubrum*) at concentrations of MIC/2, MIC, MICx2, and MICx4. Time-kill curves were constructed by plotting the $\log_{10}$ CFU mL^{-1} mean *versus* the time of exposure of the fungal cells to the selenocyanate-indoles **4**, including the standard deviation. A fungicidal effect is characterized by a decrease ≥99.9% in $\log_{10}$ of the CFU mL^{-1} number compared to the initial inoculum.

3.6. Toxicity (cell viability, genotoxicity, and mutagenicity assays)

The cell culture was prepared using 1 mL of venous blood collected by venipuncture of a young adult volunteer over 18 years of age without using medication. Lymphocytes (Protocol approved by the Ethics Committee of the Universidade Federal do Pampa under number 27045614.0.0000.5323. The procedure was performed with the consent of the volunteer and signing of a term of acceptance of his participation in the research.) obtained by centrifugation gradient were immediately transferred to the culture medium containing 9 mL of RPMI 1640 medium, supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin. Cell culture flasks were stored at 37 °C for 72 h. The negative control was prepared using 500 μL of PBS buffer 7.4, and the positive control with 3 μg mL^{-1} of bleomycin. Selenocyanate-indoles **4a** and **4b** were evaluated at 32 μg mL^{-1}. The cell viability was determined based on the loss of leukocyte membrane integrity measured by the trypan blue method. Each group was analyzed in triplicate. About 100 cells were analyzed per slide for each group in a Neubauer chamber at 400× magnification.[61] The micronucleus test was performed under a microscope[62] (an increase of 1000×) and 300 cells were counted and classified as a number of mononuclear cells with the presence of micronucleus. Finally, the comet assay followed the guidelines for the use of the technique.[63–65] In total, 100 nucleoids per slide (in triplicate for each group) were selected and analyzed. The nucleoids were classified according to the length of the tail for subsequent calculation of the DNA damage index. For classification, the nucleoids received scores from 0 (no migration) to 4 (maximum migration).

3.7. Hen's egg chorioallantoic membrane (HET-CAM) test

Fresh and fertile white eggs (Lohmann selected Leghorn, LSL) were kept under optimized incubation conditions (38–39 °C, 55–60% humidity, 10 days). On the day 10, the eggshell, around the airspace, was removed using a rotary tool (Dremel, WI). Subsequently, 0.3 mL of each selenocyanate-indoles (32 μg mL^{-1}, 0.5% DMSO solution) and controls (negative control: 0.9% NaCl; positive control: 0.1 M NaOH) were added to the CAM of the eggs. Then, the eggs were observed continuously for 5 min for the appearance of haemorrhage, lysis, and coagulation, which was documented. In addition, the severity of each reaction after 1 and 5 min was recorded. From this, the irritation score (IS) was calculated using eqn (1) (see the ESI†) The assay was performed in triplicate. Haemorrhage, lysis, and coagulation time are expressed in seconds considering the first appearance of blood haemorrhage, vessel lysis, and protein coagulation, respectively.[66]

3.8. Statistical analysis

Analysis of variance (ANOVA) followed by the *post hoc* Bonferroni test was performed in the statistical analysis of the toxicity test results. Results with $P < 0.05$ were considered significant. Data were analyzed using the GraphPad Prism software program (GraphPad Software, San Diego, CA, version 5.02 for Windows).

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4. Conclusions

Since the 3-selenocyanate-indoles **4a** and **4b** are non-irritant to the mucous membrane, these compounds could be employed for the development of formulations to treat chronic wound infections caused by *Candida* spp. and dermatophytes of the genera *Trichophyton* and *Microsporum*. In the same way, they could be used for the treatment of co-infections caused by *Candida* and *S. aureus*, where higher concentrations of compounds could be used to tackle infections without concerns regarding genotoxicity. Around 2% of the population in developing countries will develop chronic skin wounds, and the costs of treating these infections is close to US$ 25 billion per year just in the United States.[15] Therefore, formulations containing 3-selenocyanate-indoles might be further applied to preclinical studies in models of superficial and mucocutaneous infections.

Conflicts of interest

The authors declare no conflict of interest.

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