Food Safety and Toxicology

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Antiaflatoxigenic thiosemicarbazones as crop-protective agents: a cytotoxic and genotoxic study

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ABSTRACT

Aflatoxins are secondary fungal metabolites that can contaminate feed and food. They are cause of growing concern worldwide, because they are potent carcinogenic agents. Thiosemicarbazones are molecules that possess interesting antiaflatoxigenic properties but, in order to use them as crop protective agents, their cytotoxic and genotoxic profiles must first be assessed. In this paper, a group of thiosemicarbazones and a copper complex are reported as compounds able to contrast aflatoxins biosynthesis, fungal growth, and sclerotia biogenesis in *Aspergillus flavus*. The two most interesting thiosemicarbazones found are non-cytotoxic on several cell lines (CRL1790, Hs27, HFL1, and U937), and therefore they were submitted to additional analysis of mutagenicity and genotoxicity on bacteria, plants and human cells. No mutagenic activity was observed in bacteria, while a genotoxic activity was revealed by the Alkaline Comet Assay on U937 cells and by the test of chromosomal aberrations in *Allium cepa*.

INTRODUCTION

Mycotoxins, and especially aflatoxins (AFs), are considered the most widespread cause of food contamination and spoilage worldwide.[1,2] These secondary fungal metabolites possess a profile of severe toxicity and carcinogenicity, and can cause teratogenic, carcinogenic, neurotoxic and immunosuppressive effects.[3] The most common producers of mycotoxins are fungi belonging to the *Aspergillus, Penicillium, Fusarium* and *Alternaria* genera, which grow especially in hot and humid climates. They can contaminate a large variety of important agricultural crops, like corn, peanuts, wheat and rice, causing important economic losses and representing a risk for animal and human health.[4]

Up to now, the most common way to fight fungal diffusion and consequent crop contamination by mycotoxins has been the use of pesticides, but this has the consequence of generating noxious long term residues in food and in the environment.[5] Moreover, the problem of resistant pest strains is an emerging global concern. All these considerations push towards the development of novel plant-protection products, able contemporaneously to minimize a prolonged use of synthetic fungicides and to block the production of AFs. We have recently found a series of molecules, belonging to the thiosemicarbazone class, with very promising antifungal and anti-aflatoxigenic profiles.[6,7,8,9] Our investigations highlighted that modifications on the backbone of the thiosemicarbazone scaffold provide a means to tune their activity against the two major genera of mycotoxigenic fungi that contaminate cereals, i.e. *Fusarium* and *Aspergillus*, and that metal chelation could improve their activity. By using these thiosemicarbazones and their copper complexes, in fact, it is possible to obtain relevant anti-aflatoxigenicity, in concert with moderate fungistatic activity. This behavior has the advantage of avoiding problems related to undesired modifications of the composition of the microbiota in the environment.

The analysis of the previous results indicates that the presence of the C=S group is essential to observe antiaflatoxigenic activity (the corresponding semicarbazones are inactive).[8] In addition the antiaflatoxigenic activity depends deeply on the substituents on the phenyl ring and it is increased by an increase in the lipophilicity of the thiosemicarbazone.[9] This last observation could be obviously associated to the ability of the molecule to cross the cell membrane.[10,11] As a prosecution of this research, we here present the study on the phenyl and cyclohexyl thiosemicarbazones **(1) - (4)** (**Figure 1**).

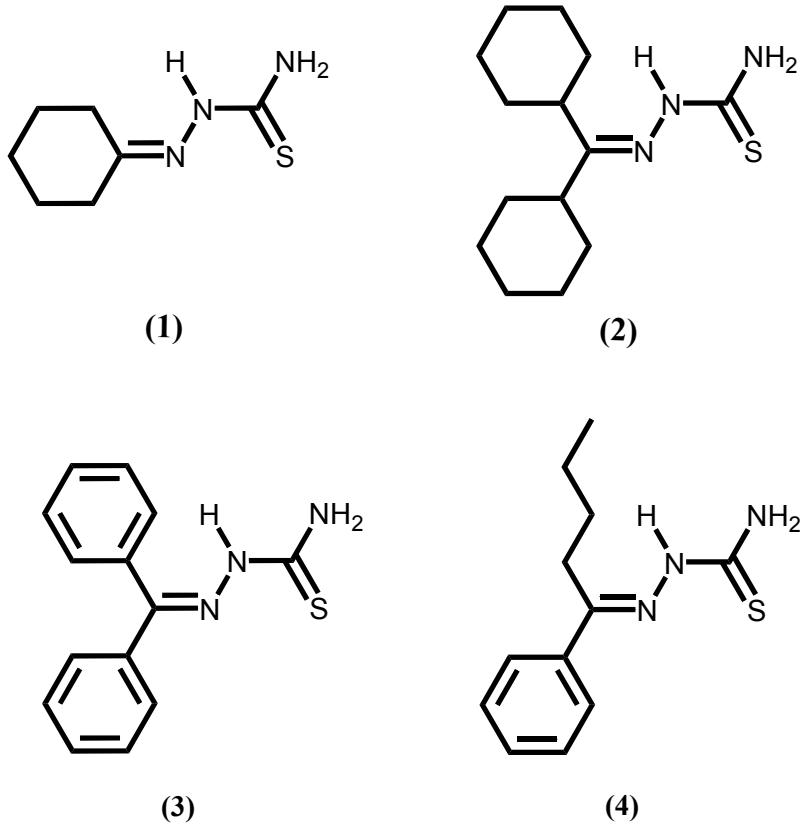

Figure 1. Schematic representation of thiosemicarbazones **(1) - (4)**

These compounds have been tested for their ability to inhibit sclerotia biogenesis and AFs biosynthesis in *A. flavus*. We also synthesized compound **(5)**, the copper complex of **(1),** because

previous results[12] suggested that metal chelation can lead to improved AFs inhibition profiles and that in particular copper compounds are good candidates to obtain inhibition of mold and bacteria growth and of aflatoxin production.

In view of potential agronomical applications, it is important to assess the genotoxic potential risks for the environment and the human health of the crop-protective agents. We have therefore investigated the cyto- and geno-toxic effects of the most active compounds **(3)** and **(4)** on healthy human cells, and in particular on human cell lines coming from tissues that can come into contact with chemicals (gastrointestinal tract, pulmonary epithelium and epidermis). Finally, the toxic and genotoxic effects of (**3)** and (**4)** were investigated on bacteria and plant cells.

MATERIALS AND METHODS

The following commercial products were used: chemicals (Sigma-Aldrich Srl, Milano, Italy); Ham's Nutrient Mixture F-12 and Fetal bovine serum (FBS) (EuroClone s.p.a., Milano, Italy); HFL1 (ATCC, CCL-153), CRL 1790 (ATCC, CCD 841 CoN), and Hs27 (ATCC, CRL1634) (American Type Culture Collection, ATCC); U937 cells (American Tissue Culture Collection, Rockville, MD); CellTiter96® AQ_{ueous}One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA); Dulbecco's Modified Eagle's medium (DMEM) and RPMI-1640 (Lonza Group Ltd, Basel, Switzerland).

Chemistry. The purity of the compounds was ≥ 95% as determined by elemental analysis. Technical details about NMR, ATR-IR, ESI-MS, and ICP analyses were reported elsewhere.[9]

The synthesis of **(1) - (4)** (**Figure 1**) was performed by using a slightly modified literature procedure.[9]

Cyclohexanone thiosemicarbazone **(1)**. Thiosemicarbazide (3.30 mmol) was dissolved in refluxing ethanol. Cyclohexanone (1.2 eq., 3.96 mmol) and few drops of glacial acetic acid were added to the solution and the mixture was reacted for 24 hours. The solvent was partially evaporated and a precipitate was isolated by filtration. The solid was recrystallized from ethanol, to give a yellow solid. Yield = 31%. M.p.: 170-171 °C. IR (ATR, cm^{-1}): 3374 ν(NH_2); 3205, 3143 ν(NH); 2940 ν(CH); 1582 ν(C=N); 1073, 1035, 831 ν(C=S). MS-EI: m/z= 171.1 (100, M^+). ^{1}H-NMR (DMSO-d_6, 25°C, ppm), δ: 10.12 (s, 1H, NH), 7.93(s, 1H, NH_2), 7.50 (s, 1H, NH_2), 2.40 (t, 2H, $H_{cyclohexyl}$), 2.23 (t, 2H, $H_{cyclohexyl}$), 1.63-1.56 (m, 6H, $H_{cyclohexyl}$).

Dicyclohexylketone thiosemicarbazone **(2)**. Thiosemicarbazide (3.30 mmol) was dissolved in refluxing ethanol. Dicyclohexylketone (1.2 eq., 3.96 mmol) and some drops of glacial acetic acid were added and the mixture was reacted for 50 hours. Solvent was removed by vacuum and the solid residue was purified by chromatographic column (hexane:ethyl acetate 7:3) to give a yellow solid. Yield = 15%. M.p.: 169-170 °C. IR (ATR, cm^{-1}): 3430 ν(NH_2); 3276, 3230, 3140 ν(NH); 2921 ν(CH); 1693 ν(C=N); 1081, 843 ν(C=S). MS-EI: m/z= 255.3 (80, M^+). ^{1}H-NMR (DMSO-d_6, 25°C, ppm), δ: 10.21 (s, 1H, NH), 8.03 (s, 1H, NH_2), 7.41 (s, 1H, NH_2), 3.03 (t, 1H, HC-C=N), 2.25 (t, 1H, HC-C=N), 1.69-1.15 (20H, $CH_{cyclohexyl}$).

Benzophenone thiosemicarbazone **(3)**: Thiosemicarbazide (3.3 mmol) was dissolved in refluxing ethanol. Benzophenone (1.2 eq., 3.96 mmol) and some drops of glacial acetic acid were added, then the mixture was reacted for 36 hours. After concentration by vacuum, the precipitate was isolated by filtration and recrystallized from ethanol to give a pale orange powder. Yield = 15%. M.p.: 172-173 °C. IR (ATR, cm^{-1}): 3430 ν(NH_2); 3276, 3236, 3141 ν(NH); 2922 ν(CH); 1693 ν(C=N); 1081, 844 ν(C=S). MS-EI: m/z= 235.3 (80, M^+). ^{1}H-NMR (DMSO-d_6, 25°C, ppm), δ: 8.66 (s, 1H, NH), 8.40 (d, 2H, NH_2), 7.76-7.65 (m, 6H, CH_{arom}), 7.42-7.34 (6H, CH_{arom}).

Valerophenone thiosemicarbazone **(4)**: Thiosemicarbazide (3.3 mmol) was dissolved in refluxing ethanol. Valerophenone (butylphenylketone) (1.2 eq., 3.96 mmol) and some drops of glacial acetic acid were added to the solution, that was reacted for 40 hours. Solvent was removed by vacuum; the solid was then purified by chromatography (CH_2Cl_2:CH_3OH 9:1). Yield = 23%. IR (ATR, cm^{-1}): 3378 ν(NH_2); 3226, 3149 ν(NH); 2957, 2929 ν(CH); 1588 ν(C=N); 1066, 847 ν(C=S). MS-EI: m/z= 267.5 (100, M^+). ^{1}H-NMR (DMSO-d_6, 25°C, ppm), δ: 10.34 (s, 1H, NH), 8.24 (s, 1H, NH), 8.90-7.88 (3H, NH+CH_{arom}), 7.38-7.39 (3H, CH_{arom}), 2.87 (t, 2H, H_2C-C=N), 1.37 (t, 4H, $CH_{cyclohexyl}$), 0.88 (t, 3H, $CH_{cyclohexyl}$).

$Cu_2(HL)(L)_2$ (**5**). HL: *Cyclohexanone thiosemicarbazone*. The solution obtained by mixing under N_2 100 mg (2 eq.) of **(1)** in 10 ml of degassed methanol, and 1 eq. of $CuCl_2 \cdot 2H_2O$ in 5 mL of the same solvent was stirred at room temperature. After 4 hours, it was cooled and the precipitate was filtered off and washed with cold diethyl ether. Green powder. Yield=34%. IR (ATR, cm^{-1}): 3436 ν(NH_2); 3248, 3165 ν(NH); 2932, 2856 ν(CH); 1604 ν(C=N); 1034 ν(C=S).^{1}H-NMR (DMSO-d_6, 25°C) δ: 10.52 (s, 1H, NH); 7.64, 8.47 (2s, br, 1H+1H, NH_2); 2.60 (s, br, $CH_{cyclohexyl}$); 1.58-1.69 (m, br, $CH_{cyclohexyl}$). ESI-MS (CH_3OH): m/z=701, $[Cu_2(HL)_2L+H]^+$, 5%; 405, $[Cu(HL)L+H]^+$, 100%; 234, $[Cu(HL)]^+$, 50%. ICP: Cu found 20.3 ± 0.7%, calcd. for $Cu_2(L)_2(HL)$: 19.8 %.

***Aspergillus flavus* strains.** Two strains were used: **t**he toxigenic strain CR10 and the atoxigenic strain TOϕ isolated from corn kernels in the Po Valley.[5]

Effect on aflatoxin accumulation. Aflatoxin accumulation in a coconut-milk derived medium (CCM) was valued by the high throughput procedure described in our previous works.[9,13,14]

Effect on fungal growth and on sclerotia biogenesis. Once assessed the AF accumulation rate, mycelia from single wells were recovered at the 6th day of incubation, slightly dried on hands paper, and weighted. Biomass measures were then converted in percentage inhibition respect to controls (DMSO-treated cultures).

A 5 µl aliquot of aflatoxigenic strain CR10 spore suspension (approximately 10^6 conidia/ml) was point-inoculated in the center of Petri dishes (Ø = 5 cm) poured with Czapek Dox Agar (CZA) medium added with 100 µM thiosemicarbazones. Control plates were added with 1 % (v/v) DMSO. Plates were replicated in triplicate and incubated at 30˚C in darkness; after two weeks of incubation, sclerotia were manually scraped from the colonies surface and washed with a 70% ethanol solution to completely remove conidia, then dried for three days at 60 °C. Dry weight was assessed. Thiosemicarbazones inhibition rate on sclerotia production was expressed as percentage respect to the control. Plates were inoculated in triplicate.

One-way analysis of variance (Past 3.x software) was used for data analyses. Tukey's test was applied to the data relative to mycelial growth, aflatoxin accumulation and sclerotia production; differences were regarded as significant if $p< 0.05$.

Cytotoxicity. The biological activity of the molecules with antifungal potential was assessed on normal cells: human fibroblast cell line Hs27 (ATCC, CRL1634), human lung epithelial cell line HFL1 (ATCC, CCL-153), and human colon epithelial cell line Crl1790 (ATCC, CCD 841 CoN). These cell lines were chosen to investigate the exposition risks, because they are attributable to human districts that could be involved in xenobiotic ways of interaction with the human body.[7] We also performed toxicological assays on human histiocytic lymphoma cell line U937 (ATCC, CRL-3253) cultured in RPMI-1640. Hs27 and CRL1790 were cultured in DMEM. HFL1 were cultured in Ham's Nutrient Mixture F-12. All media were supplemented with 10% (v/v) FBS, 1%

penicillin (100 U/ml)/streptomycin (100 µg/ml) and 1% L-glutamine (2 mM). Flasks and plates were maintained at 37°C and 5% CO_2 in a humidified atmosphere. Culture medium was refreshed every two or three days during sub-culturing. Hs27, CRL1790 and HFL1 cells were used between passage numbers 5 and 20. MTS assay (CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay) was performed to identify the antiproliferative effect of the compounds. Briefly, 100 µL of a suspension of cells in exponential growth (5 $\times 10^4$/mL in complete medium without phenol red supplemented with 5% FBS) were added into 96-well plates 24h before treatment. Plates were incubated at 37 °C in a humidified 5% CO_2 incubator. After this recover period, increasing concentrations of compounds (0.5-1.0-5.0-10.0-50.0-100.0 µM) were added to the medium and cells were left exposed for 24-48-72h. Negative control was represented by 100 µM DMSO. After treatment period, 20 µl of MTS reagent was added to each well. At the end of exposure time, the absorbance at 485 nm was measured by a microwell plate reader (TECAN SpectraFluor Plus, Männedorf, Switzerland).

The cytotoxicity response parameters GI_{50} were extrapolated from concentration-response curves.[15]

Genotoxicity on U937 cells. To verify primary DNA damage the alkaline version of Comet assay was carried out on U937 cells as detailed elsewhere.[16] U937 cells were seeded 24h before treatment at a concentration of 1 x 10^5 cell/mL in 1 mL of complete medium. Cells were treated with increased concentrations (25.0-50.0-75.0-100.0 µM) of the compounds for 1h and 24h. DMSO (100 µM) and ethylmethanesulfonate (EMS) (2 mM) were used as negative and positive controls, respectively. After treatment period at 37 °C, the percentage of live cells was assessed by Trypan blue exclusion method. Only the treatments with a viability higher than 70% have been processed in the Comet assay.[9,16] Percentage of DNA in the tail region of the comet (TI, tail

intensity) provided representative data on genotoxic effects. For each sample, coded and evaluated blind, 100 cells were analyzed.

The "IBM SPSS Statistics 24" software was used for data analyses as already described.[9]

Mutagenicity assessment on bacteria. To assess the presence of mutagenic activity, the standard plate incorporation method of Ames test was performed[17] with *S. typhimurium* TA98 and TA100 strains, with and without metabolic activation (S9 mix). Molecules were dissolved in DMSO and assayed at increasing doses (0.1, 1, 10, 50, 100 μM/plate, corresponding to a range of doses from 0.025 to 25.5 μg/plate and 0.023 to 23.5 μg/plate for molecules **3** and **4**, respectively); negative and positive controls were also introduced.[17] After 48 h, the revertant colonies grown on the plates are counted and the mean of three replicates are computed with their relative standard deviation (net revertants). The results were expressed as mutagenicity ratio (RM) dividing the revertants/plate by spontaneous mutation rate (number of revertants in negative controls). They were considered positive if two consecutive dose levels or the highest non-toxic dose level produced a response at least twice that of the control and at least two of these consecutive doses showed a dose-response relationship.[18,19]

Mutagenicity assessment on plants. *Allium cepa toxicity test:* 12 equal-sized young onion bulbs were exposed for 96 hours in the dark to different concentrations of molecules dissolved in DMSO changing the sample solution every day. Root length was used to calculate the EC_{50} value of each compound and to identify the concentration to undergo the *Allium cepa* genotoxicity assay being the highest dose correspondent to the EC_{50} value identified (the concentration that gives a 50% reduction in root growth). Root macroscopic parameters (turgescence, consistency, change in color, root tip shape) were used as toxicity indexes.[20]

Allium cepa genotoxicity tests. To detect chromosome aberrations and micronuclei[21, 22] six equal-sized young bulbs per sample were exposed to sample solutions for 24 hours, then the roots were fixed in acetic acid and ethanol (1:3) for 24 hours, and lastly stored in 70% ethanol for the chromosome aberrations (CA) test.[22] In the micronuclei (MN) test the bulbs, after exposure, were replaced in saline solution (Rank's solution) for 44 hours of recovery time, to cover two rounds of mitosis so that damage induced in chromosomes during mitosis to be visible as micronuclei in interphase cells. Then the roots were fixed in acetic acid and ethanol (1:3) for 24 hours and lastly stored in 70% ethanol.[21] The negative control was DMSO in Rank solution (the dose of DMSO corresponding to volume of samples) and positive control was maleic hydrazide (10 mg/l, 6h exposure). Five roots of each sample were used for microscopic analysis: 1000 cells/slide (5000 cells/sample) were scored for mitotic index (as a measure of cell division and hence of sample toxicity), 200 in mitosis cells/slide (1000 cells/sample) for chromosomal aberrations and 2000 in interphase cells/slide (10000 cells/sample) were scored for micronuclei frequency.

Statistical analysis was performed using chi-square test for mitotic index and chromosomal aberrations, the analysis of variance and Dunnett's t-test were performed for MN. All the experiments were in duplicate (two independent assays).

RESULTS AND DISCUSSION

Chemistry. Compounds **(1)-(4)** (**Figure 1**) were synthesized by condensation between a ketone and thiosemicarbazide accordingly to a modified literature procedure[9] and they were characterized by spectroscopic tools and mass spectrometry (Supporting Information). In their IR spectra it is possible to observe the NH and NH_2 stretching vibration bands (3430 - 3140 cm^{-1}), and ν(C=N) at about 1580 cm^{-1}, while the C=S stretching absorptions are at about 1035-1080

and 830-950 cm^{-1}. In the ^{1}H-NMR spectra recorded in d_6-DMSO, the resonances of the hydrazone NH and of NH_2 groups (10.3-8.6 and 7.4-8.4 ppm, approximately) are clearly visible. Even if these molecules could give rise to thione-thiol equilibrium, there are not evidences of the presence of the thiolic form in solution.

In our previous investigations,[7-9] we considered the possibility that copper coordination could improve the biodisponibility of the metal ion, favoring adsorption into lipid membranes and thus promoting a better anti-aflatoxigenic activity. It is well known that copper salts have been widely applied in agriculture against plant pathogens. In order to further explore this aspect, we synthesized the copper complex **(5)**, by reacting **(1)** with $CuCl_2{\cdot}2H_2O$ in methanol. As we have already observed,[9] also in this case the metal center undergoes a reduction to Cu(I). In **(5)** the metal is present as diamagnetic d^{10} Cu(I) ion and it is in fact possible to record its ^{1}H-NMR spectrum in DMSO-d_6 (Supporting Information). It is known from the literature that thiosemicarbazones are subjected to intramolecular oxidative cyclization when some bases,[23] oxidants[24] or redox-active metals[25] are present. However, IR and ^{1}H NMR spectra of **(5)** do not register the presence of cyclized ligand. In the IR spectra of **(5)** the strong bands attributable to the symmetric and asymmetric stretching modes of NH and NH_2 are clearly visible between 3500 and 3000 cm^{-1}. The C=N stretching band shifts from 1582 cm^{-1} (the parent ligand) to 1604 in the complex, suggesting the coordination of the metal ion to the iminic nitrogen. Also the stretching band relative to the C=S group is shifted upon coordination, from 1073 to 1034 cm^{-1}. In the literature, it is reported that by reacting copper(II) chloride with (+)-camphor and (–)-carvone thiosemicarbazones,[26] polynuclear copper(I) complexes are obtained; in **(5)** data analysis suggests a 2:3 metal to ligand stoichiometry. In conclusion, if the monoprotic ligand (**1**) is indicated as HL, for complex **(5)** it is possible to propose the formula $Cu_2(HL)(L)_2$ (Supporting

246 information). The $Cu_2(HL)(L)_2$ stoichiometry is also supported by the ESI-MS mass (Supporting
247 Information) and ICP analysis.

248 **Fungal growth, aflatoxin inhibition and sclerotia development. Figures 2** and **3** shows the
249 data concerning the effect of the treatments with **(1)-(5)** on AFs biosynthesis, biomass
250 accumulation and sclerotia development.

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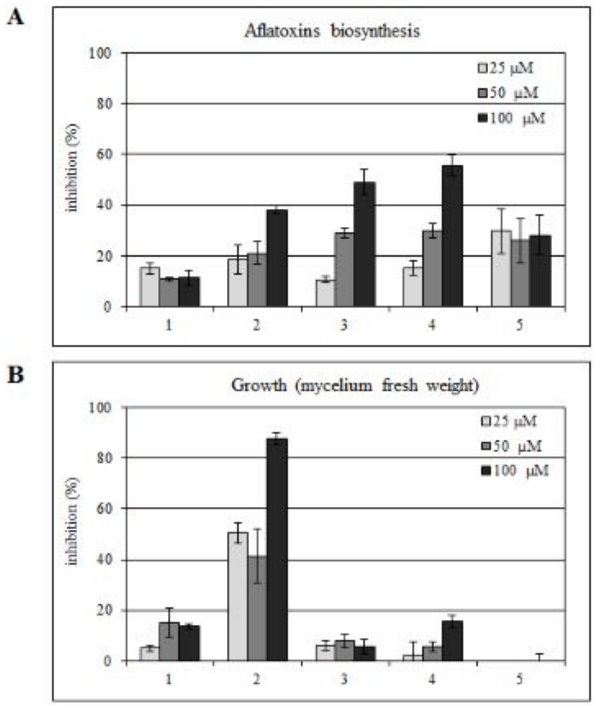

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253 **Figure 2:** Antifungal and anti-aflatoxigenic activities of compounds **(1)-(5)** at 25, 50 and 100
254 µM concentration. Results referred to aflatoxin accumulation (**A**) and mycelium fresh weight (**B**)
255 in CCM medium; they are expressed as mean percentage inhibition in comparison with controls
256 (0.25%, 0.5% and 1% DMSO respectively). Error bars indicate the standard deviations of four
257 replications (p-value < 0.05).

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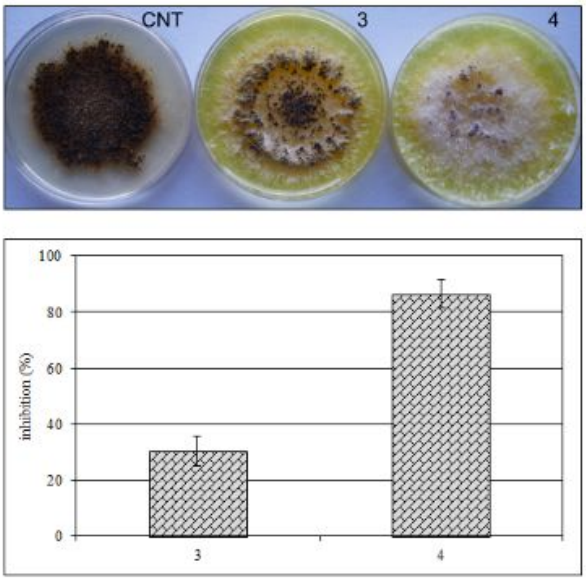

Figure 3. Effects of **(3)** and **(4)** on sclerotia biogenesis. Assessments were performed in Czapek Dox Agar solid medium amended with 100 μM thiosemicarbazones or 1% DMSO as control (CNT). Error bars indicate the standard deviations of three replications (p value <0.05).

Compound **(1)** inhibited only slightly AFs accumulation, whereas **(2)**, **(3)** and **(4)** determined an increasing AFs inhibition, ranging from 40% to 55% at 100 μM (**Figure 2A**). **(5)**, the copper complex of **(1)**, inhibited AFs accumulation slightly more than the ligand alone, but it did not show a dose dependent inhibition and its anti-mycotoxin effect was rather scarce. The biomass production evaluation revealed that **(2)** induced a notable reduction of fresh weight (over 80% of inhibition at the thiosemicarbazone highest concentration, **Figure 2B**). Poor antifungal activity in conjunction with inhibition of aflatoxin production is highly desirable, since this can assure the preservation of the environmental microbiota while ensuring the protection from noxious secondary metabolites. Therefore **(3)** and **(4)** were chosen for further analyses due to their ability to halve AFs accumulation without significantly affecting biomass production.

Since AFs biosynthesis is known to share several regulatory steps with other developmental processes belonging to the secondary metabolisms such as sclerotia biogenesis,[27] the effect on the production of these structures was assessed. Sclerotia formation was induced by culturing the aflatoxigenic and sclerotigenic *A. flavus* CR10 strain in CZA solid medium amended with 100 μM of **(3)** and **(4)** and 1% DMSO as control. The presence of the thiosemicarbazones in the culture medium reduced the formation of sclerotia, while, as expected, no mycelium growth inhibition was observed. With respect to the control, the exposure to **(3)** and **(4)** limited the sclerotia biogenesis to 30% and 86% respectively (**Figure 3**). These results are in line with our previous observations regarding other thiosemicarbazones, effective in containing AFs production, but with a slight effect on fungal growth, that severely impair sclerotia development in *A. flavus.*[7]

Cytotoxicity. The cytotoxicity of the most interesting compounds (**3**) and (**4**) was screened over a panel of human cell lines. Three normal healthy cell lines were chosen to represent possible different routes of exposure to chemicals: epidermal contact (skin fibroblast, Hs27), inhalation (lung epithelial cells, HFL1) and ingestion (colon epithelial cells, CRL1790). On the other hand, the tumor cell line U937 is a model commonly used to identify cytotoxicity and genotoxic activity of drugs.[28] Growth inhibition (GI) was determined by MTS assay; data are obtained as a mean of four independent experiments (**Table 1)**.

Table 1. GI_{50} value (μM), concentration of compound that causes a 50% reduction of cell proliferation, obtained after 24h treatment in human healthy cell lines CRL1790 (colon epithelial

cells), Hs27 (skin fibroblast), HFL1 (lung epithelial cells), and U937 (histiocytic lymphoma cells) cancer cell line.

	CRL1790	Hs27	HFL1	U937
(3)	>100.0	>100.0	>100.0	73.0
(4)	>100.0	79.5	>100.0	58.0

Compound **(3)** showed no antiproliferative activity against colon, skin and lung cell lines and presented a mild cytotoxic effect only on cancer cells (**Table 1**). A mild cytotoxic effect against skin fibroblast and a more pronounced cytotoxicity on U937 cells (**Table 1**) were observed with **(4)**.

Since **(3)** and **(4)** present a good profile in term of cytotoxicity on normal cell lines, they were considered for further analysis.

Genotoxicological assessment. The Alkaline Comet Assay was performed on U937 cells to identify the genotoxic potential of **(3)** and **(4)**. The test is able to measure DNA damage and is usually carried out at pH>13 to detect, in addition to single and double strand breaks, alkali-labile sites such as adducts, apurinic and apyrimidinic sites, oxidation of the nitrogenous bases, etc. The percentage of DNA in the tail of the comet (TI%) provided a representative data on genotoxic effects of the molecules. Cells were treated with 25.0-50.0-75.0-100.0 μM of the molecules for 1 and 24h. Against U937 cells, compound **(3)** induced DNA strand breaks in a dose-dependent manner in both the treatment times (1 and 24h) (**Figure 4A**). Compound **(4)** after 1h of treatment induced DNA damage at the highest concentrations (75.0 and 100.0 μM),

while after 24h it was genotoxic already at 25.0 µM (**Figure 4B**); it caused, as expected, a reduction in cell viability and showed a toxic effect at the highest concentrations.

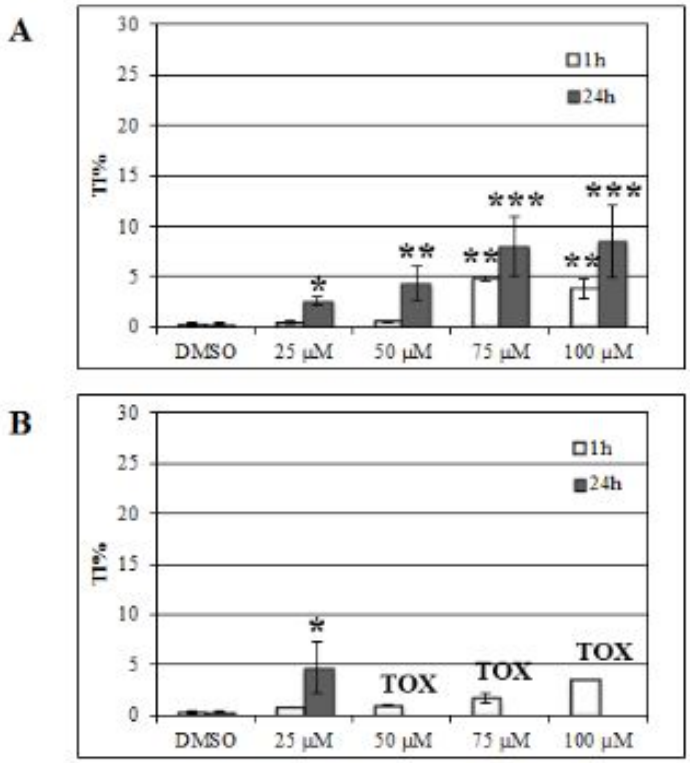

Figure 4. Genotoxic activity of compounds (**3**) (**A**) and (**4**) (**B**) by using Alkaline Comet Assay on U937 cells treated for 1 h and 24 h. The significance of the difference between the control (DMSO) and the exposure group is indicated by: *p < 0.05; **p < 0.01; ***p < 0.001 (ANOVA, Bonferroni's post-hoc test). TOX: viability <70%

The results of the Ames test showed that **(3)** and **(4)** had no mutagenic activity both in *Salmonella typhimurium* strains TA98 and in TA100, with and without metabolic activation (**Table 2**).

Table 2. Results of the Ames test expressed as revertants/plate and as mutagenicity ratio (MR).

Samples	Doses (μM/plate)	TA 98		TA98+S9		TA 100		TA100+S9	
		mean ± SD	MR	mean ± SD	MR	mean ± SD	MR	mean ± SD	MR
(3)	0.1	14.5 ± 0.7	1.0	22.3 ± 4.11	1.0	94.5 ± 19.1	1.2	108.0 ± 0.0	1,2
	1	18.5 ± 2.1	1.2	30.0 ± 1.41	1.3	92 ± 7.1	1.1	99.5 ± 10.6	1,1
	10	11.5 ± 0.7	0.8	20.5 ± 0.71	0.9	96.5 ± 20.5	1.2	83.5 ± 6.4	0,9
	50	19.0 ± 2.8	1.2	22.0 ± 0.0	0.9	100.5 ± 7.8	1.2	86.5 ± 14.8	0,9
	100	16.5 ± 0.7	1.1	20.5 ± 6.36	0.9	95.5 ± 7.8	1.2	79.0 ± 17.0	0,9
(4)	0.1	12.0 ± 4.2	0.8	15.5 ± 6.36	0.7	75.5 ± 12.0	0.9	86.0 ± 17.0	0,9
	1	17.5 ± 12.0	1.1	26.0 ± 0.71	1.1	77.5 ± 12.0	1.0	86.5 ± 3.5	0,9
	10	15.5 ± 4.9	1.0	16.0 ± 2.12	0.7	74 ± 5.7	0.9	101.5 ± 16.3	1,1
	50	14.5 ± 0.7	1.0	22.0 ± 6.36	0.9	82 ± 1.4	1.0	92.5 ± 10.7	1,0
	100	17.5 ± 3.5	1.1	22.0 ± 3.54	0.9	75 ± 9.9	0.9	75.5 ± 3.5	0,8
Negative controls		15.25 ± 4.11		23.3 ± 10.6		81.5 ± 4.65		91.3 ± 11.32	

Negative control: DMSO, 100 μM/plate. Positive control –S9: TA98 10 μg/plate 2-nitrofluorene; TA100 10 μg/plate sodium azide; positive control +S9: TA98, TA100 20 μg/plate 2-aminofluorene. Positive control results: TA 98±S9 > 1,000 revertants/plate; TA100±S9 > 1,000 revertants/plate.

Unfortunately, in preliminary tests, solutions of **(4)** exposed to the roots of *Allium cepa* showed a high toxicity already to the lowest doses, with a decrease in the number of cell division (too low mitotic index); for this reason, it was not possible to carry out a genotoxicity test. On the contrary, for **(3)** it was evaluated, by a root toxicity test, the dose corresponding to the EC_{50}, which was used as the maximum dose for the further genotoxicity evaluations. The molecule had EC_{50} = 50 μM, with a slight toxic activity at the highest tested dose (100 μM). The test of

chromosomal aberrations in *Allium cepa* showed a clear genotoxic activity for **(3)** already at the lowest dose tested, with a statistically significant increase of chromosomal aberrations compared to the negative control, without a reduction in the number of cell divisions and mitotic index (**Table 3**).

Table 3. *Allium cepa* chromosomal aberrations test on compound **(3)**: mitotic index and total chromosomal aberrations in metaphase root cells.

		1st experiment			2nd experiment		
	Doses (μM)	Mitotic index (%)	Cromosomal Aberration AC (%)	p values*	Mitotic index (%)	Cromosomal Aberration AC (%)	p values*
	10	12.6	3.5	< 0.01	13.3	3.7	< 0.01
	25	11.6	4.5	< 0.01	11.2	4.6	< 0.01
	50	10.3	4.7	< 0.01	10.0	5.1	< 0.01
	100	12.3	7.4	< 0.01	11.8	7.4	< 0.01
C-		10.5	1.2		9.9	1.9	
C+		7.2	8.8		8.7	10.1	

Negative control, C-: 100 μM DMSO; positive control, C+: maleic hydrazide (10^{-2}M)
* Statistically significant according to χ^2 test

In the linear trend analysis, the sample gave a positive response, showing a clear dose-response relationship which is statistically significant ($p<0.05$) (**Figure 5**).

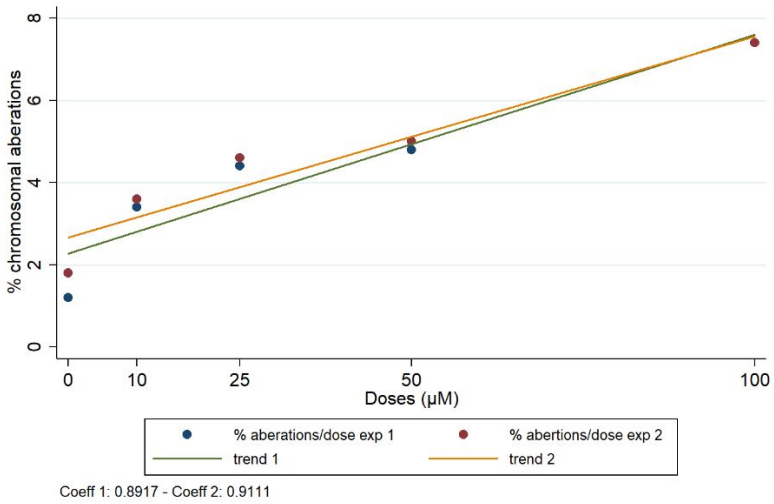

Figure 5: Linear trends of chromosomal aberrations in *Allium cepa* root cells tested with compound **(3)**

The frequency of micronuclei in interphase cells of *Allium cepa* is reported in **Table 4**: there is no increase of micronuclei compared with negative control for compound **(3)**.

Table 4. *Allium cepa* micronuclei test on compound **(3)**: mitotic index and total chromosomal aberrations in metaphase root cells.

		1st experiment		2nd experiment	
	Doses (μM)	Mitotic index (%)	MCN (mean±DS)	Mitotic index (%)	MCN (mean±DS)
	10 μM	11.5	0.03 ± 0.03	11.8	0.03 ± 0.07
	25 μM	11.4	0.03 ± 0.03	11.5	0.04 ± 0.04
	50 μM	9.6	0.08 ± 0.03	10.4	0.07 ± 0.07
C-		12.5	0.04 ± 0.07	12.6	0.02 ± 0.04

C+	7.6	16.8 ± 8.4	7.2	17.2 ± 12.0

C-: negative control, DMSO; C+: positive control, maleic hydrazide (10^{-2}M). MCN, micronuclei frequency

In conclusion, we were focused on finding specific inhibitors of AFs biosynthesis, rather than inhibitors of fungal growth, and benzophenone thiosemicarbazone **(3)** and valerophenone thiosemicarbazone **(4)** seem interesting in this sense. Compound **(3)** and **(4)** are promising also because they showed no antiproliferative activity against colon, skin and lung cell lines and were characterized by the absence of mutagenic activity on bacteria. However, the Alkaline Comet Assay performed on U937 cells points out that **(3)** and **(4)** produce DNA damages. **(4)** is toxic for the root of *Allium cepa*, and the test of chromosomal aberrations still in *Allium cepa* showed a clear genotoxic activity for **(3)**, already at the lowest doses. *Allium cepa* micronuclei test was negative instead: compound **(3)** caused directly DNA damage and chromosomal alterations, but did not cause disturbance in the mitotic cycle and damage in mitotic spindle.

Compounds **(3)** and **(4)** revealed to be promising hit compounds and the next efforts will be devoted to investigate the action mechanisms of the antiaflatoxigenic thiosemicarbazones and, at the same time, control their genotoxic character.

ASSOCIATED CONTENT

384 **Supporting Information**. The Supporting Information is available free of charge on the

385 ACS Publications website at DOI: xxx.

386 Figures: IR and ^{1}H NMR spectra of compounds **(1)**, **(2)**, **(3)**, and **(4)** are reported,

387 together with IR, ^{1}H NMR and mass spectra of **(5).**

388

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Jennifer Bartoli, Serena Montalbano, and Giorgio Spadola are considered co-first authors. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. DR, JB, MC, FB, GP: molecular design and chemical synthesis; FMR, FD, GS: fungal growth, sclerotia development and aflatoxin inhibition evaluation. AB, SM, OS: cyto- and geno-toxicological assessment on human cells. DF, CZ: genotoxicological assessment on bacteria, and plants.

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Notes

The authors declare no competing financial interest

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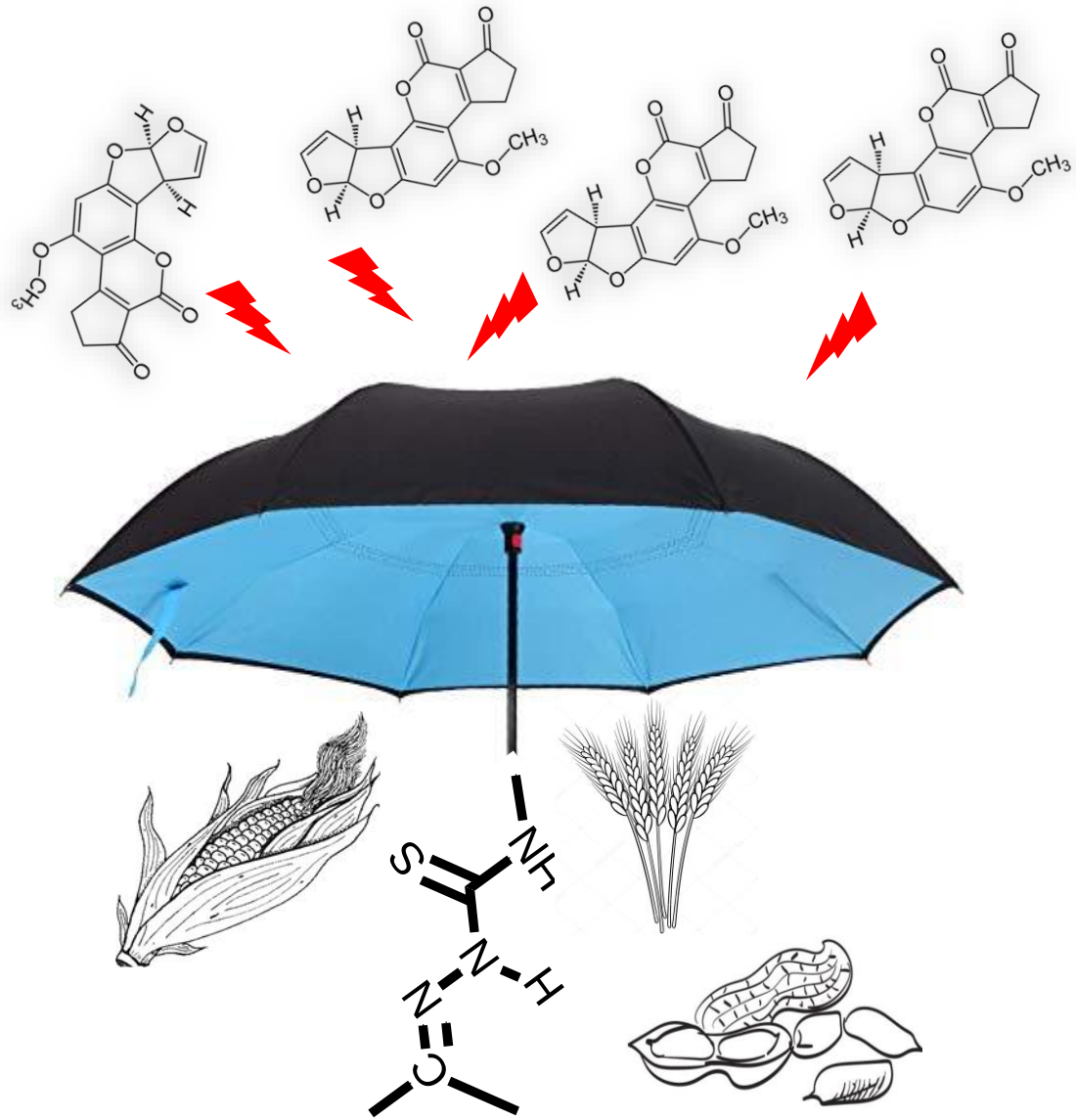

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