Cod: 4.1011

ANTIBIOTIC POTENTIAL AND CYTOTOXICITY OF HALOGENATED CARBAZOLES PRESENT IN GERMAN SOILS

M. Mandelshtam[1], K.I. Mohr[2], J. Wink[2], F. Mertes[3], K.W. Schramm[3]

[1]*Ostfalia, Department of Supply Engineering, Salzdahlumer Straße 46/48, 38302 Wolfenbüttel, Germany*

[2]*Helmholtz Centre for Infection Research (HZI), Research Group Microbial Strain Collection (MISG), Inhoffenstrasse 7, 38124 Braunschweig, Germany*

[3]*Helmholtz Zentrum München – German Research Center for Environmental Health (GmbH), Molecular EXposomics, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany*

Introduction

In recent years carbazoles have been found in environmental samples, especially the group of halogenated carbazoles (bromo- and chlorocarbazoles) was found in soils and sediments (1, 2). Until know there is little known about their origin, occurrence and environmental impact, despite their similarity to persistent organic pollutants (POPs), the polycyclic heteroaromatic carbazoles are not assigned to environmental pollutants. The halogenated carbazoles exhibit dioxin-like toxicities and are furthermore resistant to degradation (3); therefore halogenated carbazoles might significantly affect the overall toxicity of environmental samples where currently only PCDD/Fs and PCBs are considered. Possible reasons of environmental occurrence of halogenated carbazoles are natural formation mediated by haloperoxidases as well as anthropogenic sources. In case of natural formation their role as signaling compounds is not understood. One hypothesis is that they act as allelopathic, resp. antibiotic compound, which is investigated here.

Materials and methods

Minimal inhibition concentration in microorganisms

To test the bioactivity of different mono- and dihalogenated carbazoles, the minimal inhibition concentration (MIC) of 3-chlorocarbazole (3-Cl Carb), 3,6-dichlorocarbazole (3,6-Cl Carb), 3-bromocarbazole (3-Br Carb), and 3,6-dibromocarbazole (3,6-Br Carb), all solved in MeOH, was determined for different microorganisms. Therefore Gram positive bacteria (Bacillus subtilis DSM 10, Micrococcus luteus DSM 20030, Staphylococcus aureus DSM 346, Mycobacterium smegmatis ATCC 700048), Gram negative bacteria (Chromobacterium violaceum DSM 30191, Escherichia coli DSM 1116), yeasts (Candida albicans DSM 1665, Rhodotorula glutinis DSM 10134) and a filamentous fungi (Mucor hiemalis DSM 2656) served as test organisms; all microorganisms were ordered from German collection of Microorganisms (DSM) and American Type Culture Collection (ATCC), respectively. Stocks of bacterial test germs were diluted in commercial Mueller-Hinton Bouillon (Roth). M. smegmatis was cultivated in Middlebrook medium (Sigma-Aldrich) and yeasts and filamentous fungi in MYC medium (1.0% glucose, 1.0% phytone peptone, 50 mM HEPES [11.9 g/l], pH 7.0). In a dual approach initial concentrations of 66.6 and 6.6 µg/ml of each carbazole were tested in a total range between 66.6 – 0.05 µg/ml in the serial dilution test. A total of 2µl and 20 µl carbazoles, respectively, were pipetted on the first row (A) of the 96 well plate. Negative control wells were left blank. Using a multichannel pipet, 150 µL of a mixture of the test strain and the appropriate culture medium was aliquoted in all the rows. To the first row, an additional 150 µL of the test strain-medium mixture was added and mixed by repeated pipetting, before transferring the same amount to the second row. A 1:1 serial dilution was done in the subsequent rows, and 150 µl discarded after the last row (H). The test organisms were cultivated under stirring at 160 rpm at 30 °C or 37 °C for P. aeruginosa for 24−48 h. The lowest concentration of the drug preventing visible growth of the pathogen was taken as the MIC. Oxytetracycline, gentamycin, and kanamycin for bacteria and nystatin for fungi served as positive control; MeOH as negative control.

Cytotoxicity in vitro assay

The cytotoxicity of mono- and dihalogenated carbazoles was tested in vitro with the rat hepatoma cell line H4IIE together with the reference substance 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The assay was performed in 96-well plates with 100 µl volume by seeding 1×104 cells per well in DMEM (w 3,7 g/l NaHCO3, w 4.5 g/l D-Glucose, w/o L-glutamin, w/o phenol red) supplemented with 10 % FBS, 3.5 mM L-glutamin and 25 mM HEPES. The cells were exposed to increasing concentrations of the halogenated carbazoles at 0.0, 0.1, 1.0, 3.9, 7.8, 10.0, 15.6, 31.2, 62.5, 125.0, 250.0 µM respectively

together with 2,3,7,8-TCDD at 0.0, 0.9, 1.9, 3.7, 6.2, 12.4, 18.6, 37.3 pM all in quadruplicates. After 24 and 72 h of exposure in incubation conditions of 37 °C and 7.5% CO2, measurement of total protein content in wells was performed with the BCA protein assay kit (Novagen). After removal of medium 100 µL BCA protein reagent was added into all wells and incubated for 90 min at room temperature. Absorbance was recorded at 540 nm by a Tecan Fluorostar.

Results and discussion

Minimal inhibition concentration in microorganisms

The monohalogenated carbazoles showed very few activities to almost no activities (3-bromocarbazole) against the tested organisms. M. luteus and S. aureus were slightly inhibited by 3-chlorocarbazole (MIC values: 16.6 and 67.0 µg/ml). The dihalogenated 3,6-dichlorocarbazole was active against S. aureus (6.7 µg/ml) and also showed a minimal inhibition concentration of 4.2 µg/ml against the filamentous fungi M. hiemalis. The most active substance 3,6-dibromocarbazole inhibits growth of three Gram positive strains (B. subtilis: 2.1 µg/ml; M. luteus: 4.2 µg/ml; S. aureus: 8.3 µg/ml), one Gram negative strain (C. violaceum 3.3 µg/ml) and all tested fungi (8.3-33.3 µg/ml). None of the tested carbazoles was active against Mycobacterium smegmatis. With one exception (3,6-dibromocarbazole against B. subtilis) the reference antibiotics showed the same or improved activity against the tested organisms in comparison to carbazoles. Results for all tested carbazoles in test organisms are summarized in table 1.

Cytotoxicity testing in cells

All four mono- and dihalogenated carbazoles were tested for their cytotoxicity in the rat hepatoma cell line H4IIE at time points of 24 and 72 h based on overall protein content in wells with the reference substance 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). After 24 h only 3,6-chlorocarbazole reduced cell growth slightly at concentrations ≥ 31.3 µM, whereas the remaining carbazoles exhibited no influence on overall cell growth for the concentration range (0.01 to 1000 µM) tested. After 72 h of incubation, 3,6-bromocarbazole and 3,6-chlorocarbazole significantly reduced cell growth at higher concentrations (≥ 62.5 µM). Cell growth for the other carbazoles was not inhibited after 72h. Results for cytotoxicity testing are depicted in figures 1 and 2.

Acknowledgements

We thank Wera Collisi, Claudia Corsten and Silke Bernhöft for excellent technical support.

References

1. J. Mumbo et al., Persistence and dioxin-like toxicity of carbazole and chlorocarbazoles in soil. Environ Sci Pollut Res Int 22, 1344-1356 (2015).
2. L. Trobs, B. Henkelmann, D. Lenoir, A. Reischl, K. W. Schramm, Degradative fate of 3-chlorocarbazole and 3,6-dichlorocarbazole in soil. Environ Sci Pollut Res Int 18, 547-555 (2011).
3. N. Riddell et al., Characterization and Biological Potency of Mono- to Tetra-Halogenated Carbazoles. Environ Sci Technol 49, 10658-10666 (2015).

Table 1: MIC values of carbazoles: 3-chlorocarbazole (3-Cl Carb), 3,6-dichlorocarbazole (3,6-Cl Carb), 3-bromocarbazole (3-Br Carb), and 3,6-dibromocarbazole (3,6-Br Carb) and reference antibiotics against different test organisms. /: no inhibition.

MIC µg/ml	*B. subtilis*	*M. luteus*	*S. aureus*	*M. smegmatis*	*C. violaceum*	*E. coli*	*P. aeruginosa*	*M. hiemalis*	*R. glutinis*	*C. albicans*
3-Cl Carb	/	16.6	67.0	/	/	/	/	/	/	/
3,6-Cl Carb	/	/	6.7	/	/	/	/	4.2	/	/
3-Br Carb	/	/	-67.0	/	/	/	/	/	/	/
3,6-Br Carb	2.1	-4.2	8.3	/	3.3	/	/	16.6	8.3	33.3
Oxytetracyclin	8.3	0.4	6.7		0.4	1.7				
Nystatin								16.6	8.3	8.3
Gentamycin							≤ 0.5			
Kanamycin				10.5						

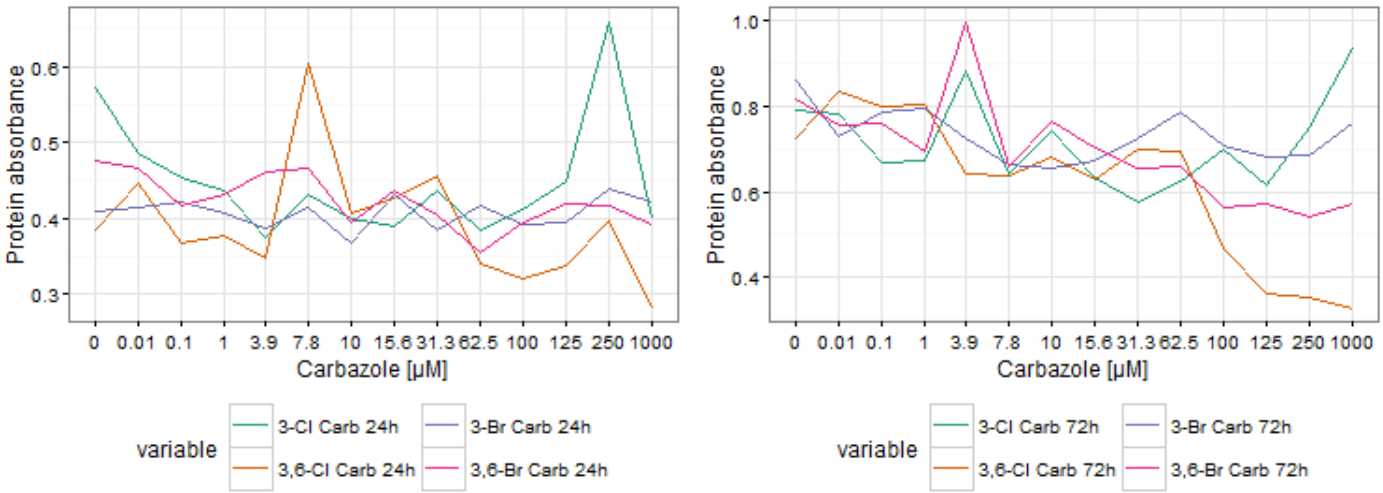

Figure 1: Cell growth from H4IIE cells deduced from protein content in wells for 3-chlorocarbazole (3-Cl Carb), 3,6-dichlorocarbazole (3,6-Cl Carb), 3-bromocarbazole (3-Br Carb), and 3,6-dibromocarbazole (3,6-Br Carb) at time points of 24 and 72 h.

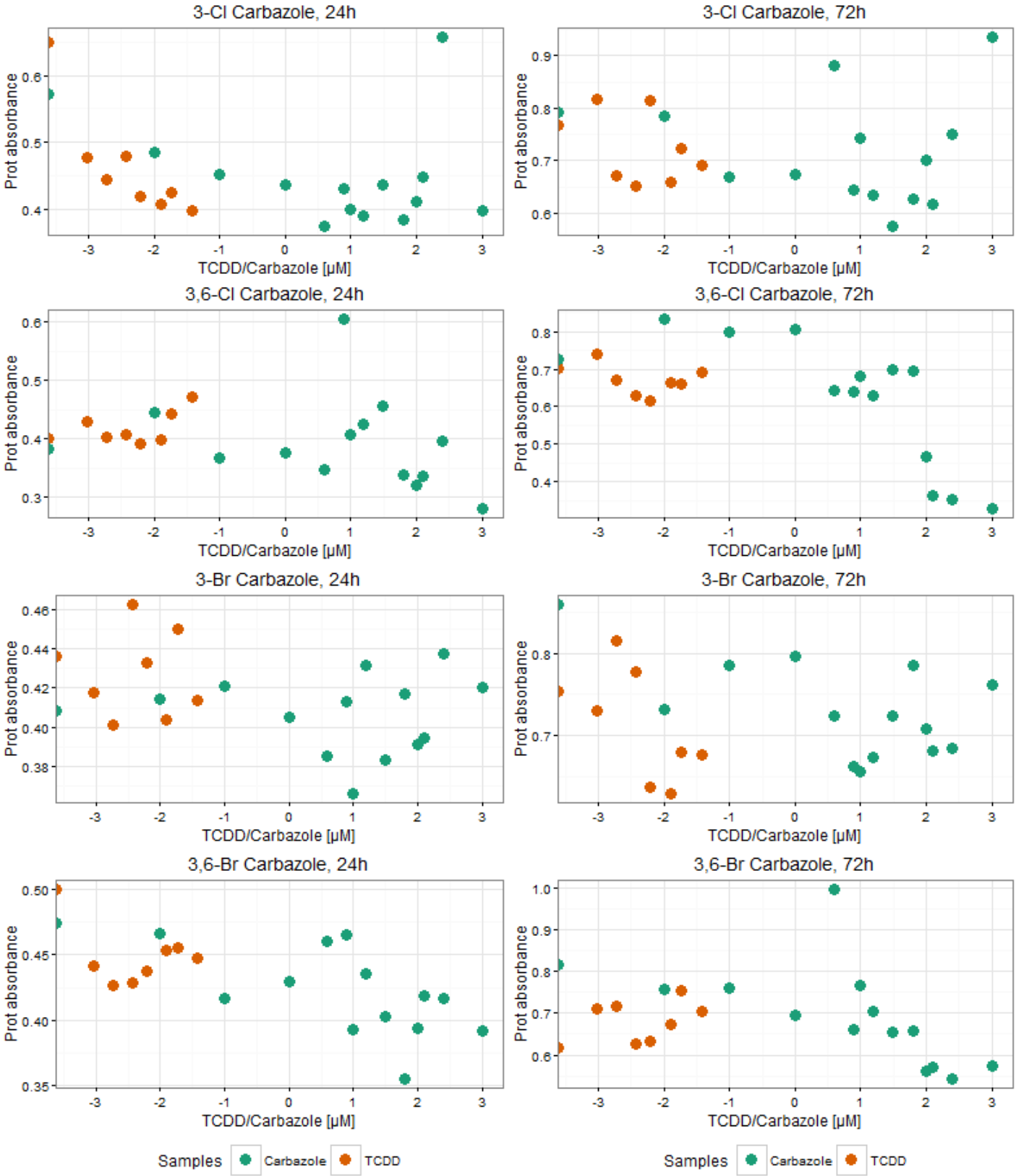

Figure 2: Comparison of cell growth deduced from protein content in wells for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-chlorocarbazole (3-Cl Carb), 3,6-dichlorocarbazole (3,6-Cl Carb), 3-bromocarbazole (3-Br Carb), and 3,6-dibromocarbazole (3,6-Br Carb) at time points of 24 and 72 h.