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Methylated Bismuth, but Not Bismuth Citrate or Bismuth Glutathione, Induces Cyto- and Genotoxic Effects in Human Cells in Vitro

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Bismuth compounds are widely used in industrial processes and products. In medicine, bismuth salts have been applied in combination with antibiotics for the treatment of *Helicobacter pylori* infections, for the prevention of diarrhea, and in radioimmunotherapy. In the environment, bismuth ions can be biotransformed to the volatile bismuth compound trimethylbismuth (Me_3Bi) by methanobacteria. Preliminary in-house studies have indicated that bismuth ions are methylated in the human colon by intestinal microflora following ingestion of bismuth-containing salts. Information concerning cyto- and genotoxicity of these biomethylated products is limited. In the present study, we investigated the cellular uptake of an organic bismuth compound [monomethylbismuth(III), MeBi(III)] and two other bismuth compounds [bismuth citrate (Bi-Cit) and bismuth glutathione (Bi-GS)] in human hepatocytes, lymphocytes, and erythrocytes using ICP-MS. We also analyzed the cyto- and genotoxic effects of these compounds to investigate their toxic potential. Our results show that the methylbismuth compound was better taken up by the cells than Bi-Cit and Bi-GS. All intracellularly detected bismuth compounds were located in the cytosol of the cells. MeBi(III) was best taken up by erythrocytes (36%), followed by lymphocytes (17%) and hepatocytes (0.04%). Erythrocytes and hepatocytes were more susceptible to MeBi(III) exposure than lymphocytes. Cytotoxic effects of MeBi(III) were detectable in erythrocytes at concentrations >4 μM, in hepatocytes at >130 μM, and in lymphocytes at >430 μM after 24 h of exposure. Cytotoxic effects for Bi-Cit and Bi-GS were much lower or not detectable in the used cell lines up to a tested concentration of 500 μM. Exposure of lymphocytes to MeBi(III) (250 μM for 1 h and 25 μM/50 μM for 24 h) resulted in significantly increased frequencies of chromosomal aberrations (CA) and sister chromatid exchanges (SCE), whereas Bi-Cit and Bi-GS induced neither CA nor SCE. Our study also showed an intracellular production of free radicals caused by MeBi(III) in hepatocytes but not in lymphocytes. These data suggest that biomethylation of bismuth ions by the intestinal microflora of the human colon leads to an increase in the toxicity of the primary bismuth salt.

Introduction

Bismuth is sparsely distributed in the environment and is found as a cometal in lead, copper, and tin ores. It belongs to the group of heavy metals and shows a similar chemical behavior to arsenic and antimony. Unlike arsenic, however, it has been regarded as relatively nontoxic. Bismuth is widely used in metallurgical alloys and in medical applications for treating gastroduodenal ulcers in *Helicobacter pylori* infections, for the prevention and treatment of diarrhea (*1–5*), and for radioimmunotherapy (*6*). Also, it is utilized in the cosmetic industry as a component of metallic pigments (*7*). Although most bismuth salts are sparingly soluble in water at neutral pH, bismuth possesses a significant biomethylation potential. Michalke et al. (*8*) and Feldmann et al. (*9, 10*) reported a high rate of formation of the volatile bismuth compound, trimethylbismuth (Me_3Bi), by anaerobic sewage sludge microflora from low concentrations of inorganic bismuth. In further investigations, Michalke et al. (*11*) observed the biomethylation of bismuth to volatile derivatives in cultures of a common sewage sludge methanobacterium. The authors found that methylcobalamin served as methyl donor in the enzyme-catalyzed methylation of bismuth. Feldmann et al. (*12*) identified Me_3Bi as a major component in landfill and sewage gas. An increased uptake of bismuth by bacterial cells in the presence of lipophilic chelators was demonstrated by Domenico et al. (*13*). More recently, the biomethylation of bismuth by the methanogen *Methanosarcina barkeri* was shown to be dependent on the presence of lipophilic polydimethylated siloxanes (*14*), which are also present in sewage. Recent in-house studies have indicated that bismuth compounds such as bismuth citrate are biotransformed in vivo by intestinal microbes into methylated bismuth derivatives (*15*). The proposed mechanism of bismuth biomethylation is shown in Figure 1 (*16*).

Bismuth accumulation has been shown to occur in various cell types, including kidney cells (*17*), motor neurons (*7, 18*), ganglion cells (*19*), and Leydig cells (*20*). In all of these cases, bismuth was found to be located in lysosomes, which play a vital role in heavy metal metabolism. Intralysosomal bismuth induces lysosomal rupture and decreases numbers of intact

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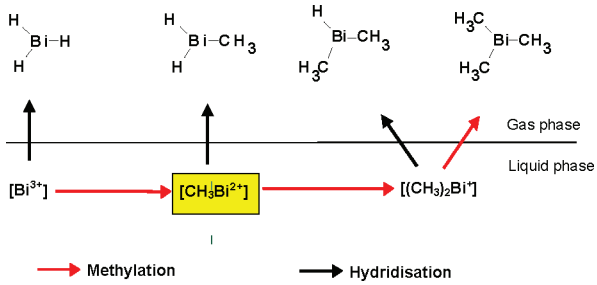

Figure 1. Proposed pathway of bismuth methylation (*16*).

lysosmes (*21*). Bismuth can induce neurotoxic effects in both humans and animals (*22*), but little else is known about the effects of bismuth in the brain. The neurotoxic potential of bismuth was first revealed in an outbreak of encephalopathy among several hundred treated patients in France (*23*). More recent studies have supplied evidence of bismuth uptake in spinal cord motor neurons and accumulation of bismuth in a huge number of neurons in the central nervous system following intraperitoneal injections of bismuth subnitrate (*22*). Stoltenberg et al. (*24*) have shown that bismuth is transported retrogradely in both sensory and motor axons if their ends are exposed to bismuth ions and that it was accumulated in neurons and glia cells in the brain regions.

Poisoning by inorganic bismuth may occur during medical therapy solely by overdosing (*25*) with blood levels of 100 μg/L or more (*4*). Inhalation, ingestion, and skin absorption may cause exodermatitis and may also affect the function of kidney, lung, and liver (*26, 27*). Organometallic bismuth(III) compounds, however, are regarded as toxic. In the 1970s, they became important as biocides (*28*).

Little is known about the cyto- and genotoxicity of bismuth compounds, especially of organobismuth in human cells. Therefore, we initially investigated the cellular uptake of one methylated and two nonmethylated bismuth compounds (monomethylbismuth, bismuth citrate, and bismuth glutathione) by three different human cell types (hepatocytes, lymphocytes, and erythrocytes). We subsequently analyzed the cytotoxic and genotoxic effects of these compounds in human cells to elucidate the toxic potential.

Material and Methods

Cell Cultures. Human hepatocytes (ATCC, HB 8065) (HepG2) were cultured in minimal essential medium (MEM) with Earle's BSS and sodium bicarbonate (CC, PRO, Germany) supplemented with 10% heat-inactivated FCS (Gibco), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), and 100 IU/mL penicillin/streptomycin (CC PRO, Germany) at 37 °C under 5% CO_2 atmosphere. HepG2 is an adherent cell line that grows as a monolayer.

Human lymphocytes were obtained from one voluntary female blood donor and were processed immediately after collection to start the lymphocyte culture. Human lymphocytes were cultivated in McCoy's 5A medium, supplemented with 20% fetal calf serum, 2.5% phytohemeagglutinin, and 10% blood for 20 h at 37 °C. Isolated human erythrocytes were obtained from the Institute of Transfusion Medicine, University Hospital Essen.

Reagents. Colloidal bismuth subcitrate (CBS) was purchased from Yamanouchi Europe (Netherlands), and bismuth glutathione was synthesized at the Institute of Environmental Analysis, University of Duisburg-Essen. Monomethylbismuth was obtained from VeZerf Laborsynthesen GmbH (Idar-Oberstein, Germany). Trypan blue, cytochalasin B, trypsin, and Giemsa solution were purchased from Sigma, and phytohemagglutinine-M was from

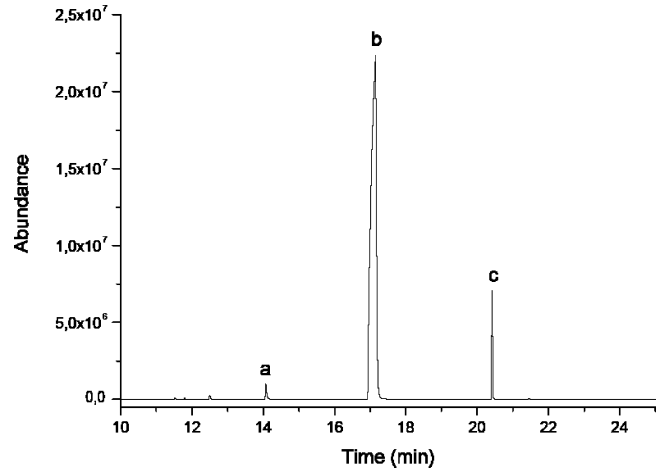

Figure 2. Gas chromatogram (GC-MS) of methyldiethylbismuth ($MeEt_2Bi$) after ethylation of MeBi(III) in PBS and GC separation. Signals: (a) Me_2EtBi, (b) $MeEt_2Bi$, and (c) Et_3Bi.

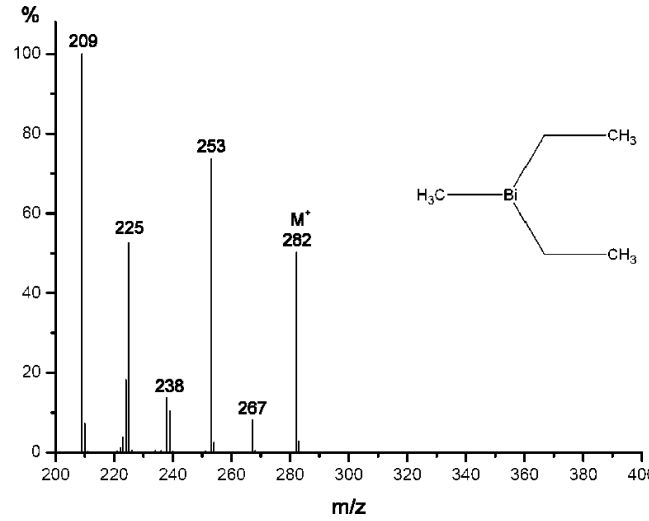

Figure 3. MS spectrum (EI, 70 eV) of methyldiethylbismuth (17.12 min, Figure 2) after ethylation of MeBi(III) and GC separation. Monomethyldiethylbismuth, *m/z* (EI-MS): 282 $[M]^+$, 267 $[M - CH_3]^+$ (M − 15); 253 $[M - C_2H_5]^+$ (M − 29); 238 $[M - CH_3 - C_2H_5]^+$ (M − 44); 225 $[M - C_2H_5 - C_2H_5]^+$ (M − 57); 209 $[M - CH_3 - C_2H_5 - C_2H_5]^+$ (M − 73).

Roche (Germany). Monomethylarsonous acid [MMA(III), $MeAs(OH)_2$] was obtained from Argus Chemicals (Vernio, Italy).

Stability of MeBi(III). MeBi(III) was synthesized by VeZerf Laborsynthesen GmbH (Idar-Oberstein, Germany) as described by Marquardt (*29*). Because of the solubility of MeBi(III) in alcohol (*30*), the charge analysis was done by ^1H NMR in CD_3OD (VeZerf Laborsynthesen GmbH) and corresponds to structure (500 MHz, 1.9 ppm, s, 3H, CH_3). Analysis was confirmed by ^1H NMR analysis at the University of Duisburg-Essen. In aqueous solution, a slight production of methane could be detected using a headspace GC-flame ionization detector (FID) system. ^1H NMR analysis of MeBi(III) in D_2O over a 2 day period showed a stable CH_3-Bi signal with a chemical shift of 2.1 ppm, which is typical for $Bi-CH_3$ group (*31*). The small deviation in the chemical shift (1.9 ppm vs 2.1 ppm) may be explained by the use of different solvents (CD_3OD vs D_2O). To ensure stability of this compound in cell experiments, we prepared a 100 ppm standard in PBS. Derivatization of 100 μL of this MeBi standard with sodiumtetraethylborate produced the derivative methyldiethylbismuth ($MeEt_2Bi$), which could be detected via GC-parallel inductively coupled plasma-mass spectrometry(ICP-MS)/EI-MS as described previously (*32*) (Figures 2 and 3). Integration of peak areas resulted in a concentration of 95% $MeEt_2Bi$.

Table 1. Uptake of Bismuth Compounds by Human Lymphocytes[a]

	detected bismuth concentrations ± SD			
	whole cell extract		cell free (membrane removed) extract	
concn of Bi in exposure medium (μM)	(ng/10^6 cells)	% of bismuth	associated with cell (ng/10^6 cells)	% of bismuth associated with cell
bismuth-citrate				
control	ND		ND	
0.05	ND	ND	ND	2.67 ± 5.04
0.25	ND	ND	ND	1.68 ± 1.25
0.5	ND	ND	ND	1.27 ± 0.47
2.5	ND	0.42 ± 0.16	ND	0.28 ± 0.04
5	0.03 ± 0.03	2.60 ± 2.38	ND	0.10 ± 0.02
25	0.02 ± 0.01	0.46 ± 0.12	0.01 ± 0	0.22 ± 0.02
50	0.12 ± 0.02	1.01 ± 0.18	0.03 ± 0	0.21 ± 0
250	0.58 ± 0.12	1.11 ± 0.08	0.07 ± 0	0.15 ± 0.02
500	0.80 ± 0.62	0.81 ± 0.64	0.24 ± 0.01	0.24 ± 0.02
monomethylbismuth				
control	ND		ND	
0.05	0.01 ± 0.01	1.67 ± 1.43	0.04 ± 0	8.90 ± 0.28
0.25	0.04 ± 0.01	2.09 ± 0.29	0.20 ± 0.23	10.88 ± 12.91
0.5	0.08 ± 0.03	2.25 ± 0.27	0.50 ± 0.59	12.25 ± 13.83
2.5	2.86 ± 1.31	7.83 ± 0.38	1.98 ± 1.04	5.33 ± 0.15
5	3.99 ± 0.76	9.92 ± 1.35	2.78 ± 0.81	6.90 ± 1.64
25	35.26 ± 4.97	15.51 ± 2.19	24.88 ± 0.04	10.95 ± 0.02
50	84.58 ± 0.96	23.23 ± 5.12	64.66 ± 14.68	17.31 ± 0.08
250	461.46 ± 2.06	21.69 ± 0.75	340.76 ± 32.07	15.99 ± 1.03
500	605.70 ± 143.34	11.81 ± 0.30	586.94 ± 143.25	11.43 ± 0.37
bismuth-glutathion				
control	ND		ND	
0.05	ND	ND	ND	0.65 ± 0.99
0.25	ND	ND	ND	0.08 ± 0.09
0.5	ND	ND	ND	ND
2.5	ND	0.19 ± 0.02	ND	0.18 ± 0
5	ND	0.26 ± 0.11	ND	0.23 ± 0.06
25	0.01 ± 0	0.27 ± 0.06	ND	0.19 ± 0.03
50	0.01 ± 0	0.15 ± 0.01	ND	0.18 ± 0.02
250	0.03 ± 0.01	0.15 ± 0.03	0.03 ± 0	0.15 ± 0.01
500	0.02 ± 0	0.04 ± 0	0.01 ± 0	0.02 ± 0
MMA(III) positive control				
control	ND		ND	
0.05	ND	ND	ND	ND
0.25	ND	ND	ND	ND
0.5	ND	ND	ND	ND
2.5	ND	ND	ND	ND
5	0.01 ± 0	25.10 ± 0.38	0.01 ± 0	25.55 ± 0.35
25	0.01 ± 0	14.36 ± 0.74	0.02 ± 0	22.59 ± 1.67
50	0.04 ± 0	21.24 ± 1.50	0.04 ± 0	20.26 ± 0.80
250	0.09 ± 0.02	9.36 ± 2.23	0.07 ± 0	7.13 ± 0.30
500	0.10 ± 0.01	4.94 ± 0.41	0.09 ± 0.01	4.55 ± 0.29

[a] Note: The amount of bismuth (ng/10^6 cells) as well as the percentage of substrate loading (%) were measured in whole cell extracts and in membrane-removed cell extracts by inductively coupled plasma mass spectrometry (ICP-MS). Present values represent means of duplicate incubations and five replicate analyses. ND, not detected (<0.01 ng Bi per 106 cells).

The MeBi standard was analyzed after 0, 1.5, 3, and 24 h to ensure stability over the whole exposure time. No significant decrease in methylbismuth concentration (visible area of the methyldiethylbismuth peak) was detected over this period. All cell experimentation was performed within a 24 h time window.

Cellular Uptake. Human lymphocytes were cultured in McCoy's 5A medium, supplemented with 20% fetal calf serum, 2.5% phytohemeagglutinin, and 10% blood for 20 h at 37 °C. A 10^7 amount of HepG2 cells were cultivated in Earl's minimum essential medium (EMEM) for 24 h. Fresh human erythrocyte concentrate, human lymphocytes, and HepG2 cells were exposed to Bi-Cit to Bi-GS and to MeBi(III) at concentrations ranging from 0.05 to 500 μM for an exposure time of 1 h. MMA(III) (0.05–500 μM) was used as a positive control (exposure time, 1 h). After incubation, cells were washed twice with PBS and subsequently resuspended in 10 mL of fresh culture medium. After cell counting, the cell suspension was centrifuged for 5 min at 190*g* and cells were lysed by immersion in distilled water (10 mL) for 30 min. The suspension was controlled under the microscope for complete cell lysis. From this cell solution, two kinds of samples were prepared as follows: (i) whole cell extract with membranes and proteins present and (ii) cell-free extract, obtained by centrifugation for 20 min at 2000*g* to remove the membranes. Samples were stored at −80 °C until ICP-MS analysis. All experiments were performed at least in duplicate. The total bismuth concentration in whole cell and cell-free extract was determined by ICP-MS (Agilent 7500a, Agilent Technologies, Germany). Quantitation was performed by external calibration with a bismuth standard solution (semimetals plasma standard, Alfa Aesar, Massachusetts) and validated by analyzing CRM SERO B2.

Trypan Blue Cytotoxicity Test. HepG2 cells, human lymphocytes, and erythrocytes were treated with different concentrations of Bi-Cit, Bi-GS, and MeBi(III) (0.5−500 μM) for 1 and 24 h. Cell viability was evaluated immediately after exposure. Treated and untreated HepG2 cells were harvested by trypsination (0.25% trypsin). Cell counting was performed following trypan blue staining; that is, the cell suspension was mixed with an equivalent volume of 0.4% trypan blue solution and subsequently evaluated under the light microscope. The membrane of dead cells was permeable to trypan blue (blue stained cells), whereas living cells remained unstained. Cell viability was expressed as a percentage of surviving cells as compared to the total number of cells. The

Table 2. Uptake of Bismuth Compounds by Human Hepatocytes[a]

	detected bismuth concentrations ± SD			
	whole cell extract		cell free (membrane removed) extract	
concn of Bi in exposure medium (μM)	(ng/10^6 cells)	% of bismuth associated with cell	(ng/10^6 cells)	% of bismuth associated with cell
bismuth-citrate				
control	ND		ND	
0.05	ND	ND	ND	ND
0.25	0.01 ± 0	0.07 ± 0	ND	0.05 ± 0.01
0.5	0.02 ± 0.01	0.09 ± 0	0.04 ± 0.02	0.17 ± 0.04
2.5	0.06 ± 0.01	0.06 ± 0	0.09 ± 0.01	0.09 ± 0
5	0.09 ± 0.02	0.05 ± 0.01	0.07 ± 0.02	0.04 ± 0.01
25	0.93 ± 0.20	0.10 ± 0.01	0.69 ± 0.01	0.07 ± 0
50	1.22 ± 0.02	0.08 ± 0	1.12 ± 0.10	0.07 ± 0.01
250	26.20 ± 2.54	0.12 ± 0.01	21.53 ± 2.23	0.10 ± 0.01
500	9.70 ± 7.29	0.03 ± 0.01	9.07 ± 6.37	0.03 ± 0.01
monomethylbismuth				
control	ND		ND	
0.05	ND	ND	ND	ND
0.25	ND	ND	ND	ND
0.5	ND	ND	ND	ND
2.5	ND	ND	ND	ND
5	ND	ND	ND	ND
25	0.10 ± 0.02	0.02 ± 0	0.06 ± 0.02	0.01 ± 0
50	0.22 ± 0.04	0.02 ± 0	0.18 ± 0.03	0.01 ± 0
250	1.15 ± 0.01	0.01 ± 0	0.87 ± 0.07	0.01 ± 0
500	13.23 ± 2.28	0.06 ± 0.01	9.89 ± 1.53	0.04 ± 0.01
bismuth-glutathion				
control	ND		ND	
0.05	ND	ND	0.01 ± 0.01	0.58 ± 0.58
0.25	ND	ND	0.05 ± 0	0.17 ± 0.06
0.5	0.01 ± 0	0.02 ± 0	0.04 ± 0.02	0.07 ± 0.02
2.5	0.05 ± 0	0.02 ± 0	0.08 ± 0	0.03 ± 0
5	0.15 ± 0.16	0.02 ± 0.02	0.15 ± 0.13	0.02 ± 0.01
25	0.35 ± 0.03	0.01 ± 0	0.34 ± 0.02	0.01 ± 0
50	0.39 ± 0.24	0.01 ± 0	0.33 ± 0.20	0.01 ± 0
250	1.37 ± 0.06	0.01 ± 0	1.18 ± 0.10	ND
500	3.46 ± 1.20	0.01 ± 0	4.19 ± 1.48	0.01 ± 0
MMA(III) positive control				
control	ND		ND	
0.05	ND	ND	ND	ND
0.25	ND	ND	ND	ND
0.5	ND	ND	0.02 ± 0.02	0.61 ± 0.87
2.5	0.15 ± 0.02	2.07 ± 0.25	0.08 ± 0.11	1.02 ± 1.45
5	2.06 ± 1.40	5.31 ± 0.23	1.52 ± 0.90	4.05 ± 0.27
25	7.13 ± 0.80	2.24 ± 3.17	6.39 ± 0.10	4.36 ± 0.07
50	15.99 ± 12.89	0.08 ± 0	11.00 ± 6.49	0.06 ± 0.01
250	toxic	toxic	toxic	toxic
500	toxic	toxic	toxic	toxic

[a] Note: The amount of bismuth (ng/10^6 cells) as well as the percentage of substrate loading (%) were measured in whole cell extracts and in membrane-removed cell extracts by ICP-MS. Present values represent means of duplicate incubations and five replicate analyses. ND, not detected (<0.01 ng Bi per 106 cells).

experiments were performed in duplicate. Significance was tested by using the Student's *t* test.

Chromosomal Aberrations (CA) and Sister Chromatid Exchanges (SCE). Human lymphocytes were exposed to MeBi(III), Bi-Cit, and Bi-GS in a concentration range from 2.5 to 250 μM [MeBi(III) for 1 and 24 h; Bi-Cit and Bi-GS for 1 h]. Following incubation, cells were washed twice and resuspended in 5 mL of McCoy's 5A medium. For the analysis of SCE and for the evaluation of first post-treatment metaphases with CA, cells were prelabeled with 20 μM BrdU (Serva, Heidelberg, Germany). Thereafter, cells were cultivated for an additional 44 h for the examination of CA and 68 h for SCE. Cell growth was stopped by treatment of lymphocytes with 0.08 μg/mL colcemid solution (Ciba, Basel, Switzerland) for a further 4 h. Metaphases were stained with Hoechst-Giemsa solution (Bisbenzimid, Merck, Germany) as described by Hill and Wolff (*33*). At least 100 uniformly stained first post-treatment metaphases were analyzed for CA, and as possible, 50 differentially stained second post-treatment metaphases were evaluated for SCE. All experiments were performed in duplicate. The trivalent monomethylarsenic compound, monomethylarsonous acid [MMA(III), 0.5 μM, 1 and 24 h exposure], was run alongside as a positive control (for details see refs *34, 35*). The χ square test was used for the statistical analysis of CA results, and the ANOVA test was used for evaluating the data from the SCE experiments.

Radical Measurements with H_2DCF-DA. Dichlorodihydrofluorescein diacetate (H_2DCF-DA) can be used to detect cellular production of reactive oxygen species (ROS), in particular hydrogen peroxide (H_2O_2) (*36, 37*). HepG2 cells were exposed to MeBi(III) (100 μM) for 3, 6, 9, and 12 h and human lymphocytes for 1, 6, and 12 h [250 μM MeBi(III)]. Thereafter, cells were incubated with H_2DCF-DA (10 μM) for 20 min. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm in 96 well plates with an Elisa Plate Reader (Tecan Deutschland GmbH, Crailsheim, Germany). An increased fluorescence level is an indication of intracellular ROS formation. The trivalent monomethylarsenic compound MMA(III) (5 μM for HepG2 and 250 μM for human lymphocytes) was used as positive control, and unexposed cells were used as a negative control. The experiments were carried out in triplicate. Significance was tested with Student's *t* test. The results shown in Figure 6 are relative values related to the negative control.

Table 3. Uptake of Bismuth Compounds by Human Erythrocytes[a]

	detected bismuth concentrations ± SD			
	whole cell extract		cell free (membrane removed) extract	
concn of Bi in exposure medium (μM)	(ng/106 cells)	% of bismuth associated with cell	(ng/106 cells)	% of bismuth associated with cell
bismuth-citrate				
control	ND		ND	
0.05	ND	ND	ND	ND
0.25	0.01 ± 0	1.21 ± 0.26	ND	0.25 ± 0.05
0.5	0.07 ± 0.01	6.51 ± 0.86	ND	0.13 ± 0.06
2.5	0.08 ± 0.03	1.61 ± 0.60	0.01 ± 0	0.24 ± 0.01
5	0.08 ± 0	0.78 ± 0.01	0.03 ± 0	0.26 ± 0.01
25	0.36 ± 0	0.72 ± 0.01	0.10 ± 0.03	0.20 ± 0.06
50	0.40 ± 0.04	0.40 ± 0.04	0.12 ± 0.04	0.12 ± 0.04
250	0.43 ± 0.17	0.09 ± 0.03	0.27 ± 0.07	0.05 ± 0.01
500	0.52 ± 0.21	0.05 ± 0.02	0.25 ± 0.05	0.03 ± 0.01
monomethylbismuth				
control	ND		ND	
0.05	ND	ND	ND	ND
0.25	ND	ND	ND	ND
0.5	0.20 ± 0.11	19.65 ± 11.46	0.22 ± 0.12	22.08 ± 12.38
2.5	1.82 ± 0.19	36.36 ± 3.83	1.80 ± 0.11	36.04 ± 2.15
5	3.25 ± 0.09	32.47 ± 0.87	2.86 ± 0.14	28.56 ± 1.43
25	13.42 ± 1.46	26.84 ± 2.93	13.76 ± 0.99	27.53 ± 1.98
50	16.21 ± 1.67	16.21 ± 1.67	16.89 ± 1.22	16.89 ± 1.22
250	48.12 ± 0.19	9.62 ± 0.04	44.34 ± 3.25	8.87 ± 0.65
500	56.13 ± 12.42	5.61 ± 1.24	55.84 ± 3.01	5.58 ± 0.30
bismuth-glutathion				
control	ND		ND	
0.05	ND	ND	ND	ND
0.25	ND	0.05 ± 0.21	0.01 ± 0.01	1.63 ± 1.68
0.5	ND	0.12 ± 0.06	ND	0.25 ± 0.03
2.5	0.01 ± 0	0.19 ± 0.02	0.01 ± 0	0.10 ± 0
5	0.02 ± 0	0.15 ± 0.01	0.01 ± 0	0.10 ± 0.02
25	0.09 ± 0.02	0.19 ± 0.03	0.08 ± 0	0.16 ± 0
50	0.21 ± 0	0.21 ± 0	0.13 ± 0.01	0.13 ± 0.01
250	0.33 ± 0.01	0.07 ± 0	0.18 ± 0	0.04 ± 0
500	0.67 ± 0.04	0.07 ± 0	0.46 ± 0.06	0.05 ± 0.01
MMA(III) positive control				
control	ND		ND	
0.05	ND	0.24 ± 0.15	ND	0.24 ± 0.09
0.25	ND	0.11 ± 0.02	ND	0.13 ± 0.05
0.5	0.01 ± 0	0.11 ± 0	0.01 ± 0	0.13 ± 0.03
2.5	0.02 ± 0	0.08 ± 0	0.02 ± 0	0.07 ± 0.01
5	0.02 ± 0	0.05 ± 0	0.03 ± 0	0.05 ± 0
25	0.04 ± 0	0.01 ± 0	0.04 ± 0.01	0.02 ± 0
50	0.04 ± 0	0.01 ± 0	0.04 ± 0	0.01 ± 0
250	0.07 ± 0.01	ND	0.06 ± 0.01	ND
500	0.08 ± 0	ND	0.08 ± 0	ND

[a] Note: The amount of bismuth (ng/10^6 cells) as well as the percentage of substrate loading (%) were measured in whole cell extracts and in membrane-removed cell extracts by ICP-MS. Present values represent means of duplicate incubations and five replicate analyses. ND, not detected (<0.01 ng Bi per 106 cells).

Results

Our results demonstrate that the uptake of bismuth-glutathione by human lymphocytes, hepatocytes, and erythrocytes is relatively low (<0.3%). In comparison to Bi-GS, bismuth-citrate was taken up to a higher extent by human lymphocytes (up to 2.6%) and by human erythrocytes (up to 6.5%). Uptake of methylbismuth by lymphocytes and erythrocytes was, in comparison, significantly higher; up to 23% was taken up by lymphocytes and up to 36% by erythrocytes. In contrast, the uptake of MeBi(III) by human hepatocytes was significantly lower than that of Bi-Cit (Tables 1−3).

Exposure of cells to bismuth compounds led to an accumulation of the metal in the cells in a dose-dependent manner. The bismuth species were not associated with the cell membrane of human lymphocytes, hepatocytes, and erythrocytes; they were located in the cytosol (no significant differences between whole cell extract and cell-free (membrane removed) extract (Tables 1−3). In contrast to MeBi(III) uptake by lymphocytes (increasing up to the highest applied concentration, Table 1), the relative MeBi(III) uptake by erythrocytes decreases at concentrations >5 μM (Table 3). An increased active efflux of this Bicompound at higher concentrations might be the reason. The same effect can be observed for Bi-Cit uptake by erythrocytes (Table 3).

In the cytotoxicity test, human hepatocytes were more susceptible to MeBi(III) treatment than erythrocytes and lymphocytes after 1 h of exposure (Figure 4a). After an extended exposure time of 24 h, erythrocytes were most sensitive to treatment with the organic bismuth compound followed by hepatocytes and lymphocytes. In hepatocytes, the cytotoxic effect was significant only after MeBi(III) treatment for 1 h at concentrations >350 μM and after 24 h of exposure at concentrations >130 μM. In erythrocytes, the cytotoxicity of all three bismuth compounds was <5% up to a tested concentration of 500 μM after 1 h of exposure. After 24 h of exposure, MeBi(III) was highly toxic at concentrations above 3.8 μM (>50% cell death), and Bi-Cit showed cytotoxic effects at

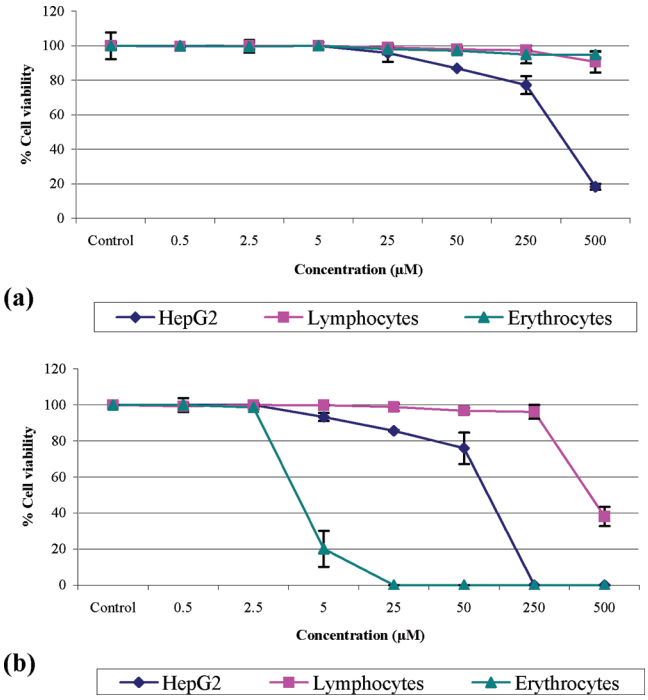

Figure 4. Cell viability of human hepatocytes, erythrocytes, and lymphocytes after exposure to MeBi(III) at different concentrations (0.5–500 μM) for 1 (a) and 24 h (b). All experiments were carried out in duplicate.

concentrations above 113 μM (>48% cell death). In lymphocytes, MeBi(III) was cytotoxic only at relatively high concentrations and long exposure times (>430 μM, 24 h) (Figure 4b). Bi-Cit and Bi-GS did not show any toxic effect in hepatocytes and lymphocytes (cell viability always >95%).

Incubation of lymphocytes for 1 h with MeBi(III) at a concentration of 250 μM induced a significant number of chromosomal aberrations (14.8%, $p < 0.01$, Table 4). Also, a significant increase in SCE (7%, $p < 0.05$) occurred after exposure of lymphocytes to MeBi(III) at the same concentration (250 μM). To validate the observed genotoxic effects, a longer exposure time (24 h) was chosen for additional experiments with MeBi(III). The results also revealed a higher amount of aberrations and SCE [9% CA at a concentration of 25 μM MeBi(III) and 15% CA at a concentration of 50 μM MeBi(III), Table 4]. Higher concentrations than 250 μM MeBi(III) and longer exposure times than 24 h were not tested because of increased cytotoxic effects. Bi-Cit and Bi-GS did not induce CA or SCE up to the highest tested concentration of 250 μM. MMA(III) (0.5 μM, 1 h) was used as a positive control and induced 9% CA in lymphocytes ($p < 0.05$, Table 4). After exposure of cells to MeBi(III), mainly chromosomal type aberrations such as single and double strand breaks occurred in lymphocytes (Figure 5a). Chromatid exchanges (Figure 5b), dicentrics (Figure 5c), and rings appeared rarely.

Intracellular radical formation was evident in hepatocytes exposed to 100 μM MeBi(III) after an exposure time of 3−12 h ($p < 0.05$), whereas radical formation in human lymphocytes exposed to 250 μM MeBi(III) was not significantly elevated (Figure 6).

Discussion

Until now, cyto- or genotoxic effects of bismuth compounds in human cells have been sparsely described in detail. In the literature, there are only some data available regarding measurement of bismuth uptake and accumulation, for example, in rat Leydig cells (*38, 39*), intragastric uptake of bismuth into motor neurons in mice (*7*), uptake by lysosomes, macrophages, and dendritic cells of mice (*40*), as well as uptake into the nervous system and organs in mice (*18*). Therefore, we investigated cellular uptake, cyto- as well as genotoxicity, of some bismuth compounds in human blood cells and in hepatocytes. Our results show clearly that the uptake of bismuth compounds is dependent on bismuth species and cell type. The absolute uptake (ng/10^6 cells) of the metal into all cell types occurred in a dose-dependent manner. However, the relative uptake [% of Bi found intracellularly in relation to the extracellular applied Bi concentration (*34, 35, 41, 42*)] of MeBi(III) and Bi-Cit by lymphocytes and erythrocytes is different. Whereas an accumulation of MeBi(III) and Bi-Cit can be observed with increasing concentrations (up to 250 μM) in lymphocytes, an active extrusion occurs in erythrocytes already at 2.5 μM intracellular MeBi(III) concentration and at 0.5 μM intracellular Bi-Cit. The mechanisms for metal ion homeostasis as described by Rosen (*43*) involve a balance between uptake and efflux systems and would therefore seem to be different in these blood cell types.

The uptake capability of hepatocytes for bicompounds is in general low (<0.1%) as compared to lymphocytes and erythrocytes. Different membrane permeabilities and cell type-specific properties might be responsible for the observed differences.

The methylated bicompound MeBi(III) was best membrane permeable in lymphocyte and erythrocyte cultures. It is known that methylated compounds are more lipophilic than nonmethylated species. Therefore, the uptake of Bi-Cit and Bi-GS might be less effective than that of MeBi(III), which seems to better pass the cytoplasmic membrane. Some toxic metals (e.g., monomethylmercury) are able to cross cell barriers (e.g., membrane barriers or blood/brain barrier) by forming a cysteine complex, which can be taken up by large amino acid transporters (*44*). It might be that a similar mechanism is working for organobismuth compounds.

The reason for the observed enhanced toxicity of organometallic compounds over the inorganic derivatives can be explained with the existence of hydrophobic or lipophilic groups on the same species also having a hydrophilic dipole (*45*). This allows transport in aqueous body fluids and also solubility and transport through fatty tissue and cell walls by diffusion. As Lewis acids similar to inorganic metal species, there is good bonding to Lewis base coordination sites within the organism (e.g., thiol groups) (*45*).

Rao et al. (*46*) and Sun et al. (*47*) noted that trivalent bismuth nitrate and colloidal bismuth subcitrate display protein-specific binding. The authors investigated the distribution of bismuth in the body. Bismuth is distributed via blood to the spleen, liver, brain, heart, skeletal muscle, and, in particular, the kidney, resulting in the manifestation of bismuth toxicity in vivo (*25*). The apparent lower intracellular concentration of bismuth in hepatocytes—as shown in the present study—may be explained by an inhibition of uptake or by the presence of an enhanced efflux mechanism in these cells as described also for arsenic compounds in bacteria, yeast, and mammalian cells (*48, 49*).

As a result of the better uptake of MeBi(III) (as compared to Bi-GS and Bi-Cit), the methylated bismuth compound showed toxic effects in all three cell types, when exposed for 24 h. The results demonstrate that even at micromolar concentrations, MeBi(III) exerts cytotoxic action on hepatocytes and blood cells.

Hepatotoxicity after 72 h of treatment of mice with colloidal bismuth citrate was detected by a significant increase in serum liver enzymes (*50*). It is uncertain if bismuth compounds can

Table 4. Occurrence of Chromosomal Aberrations and Sister Chromatid Exchanges in Human Lymphocytes after Exposure of Cells to MeBi(III) (Concentrations, 2.5–250 μM) for 1 and 24 h and Bi-Cit (2.5–250 μM) and Bi-GS (2.5–250 μM) for 1 h[a]

bismuth compound doses (μM)	no. of metaphases analyzed for aberrations	no. of aberrations			% aberrations	mean of sister chromatid exchanges (SCE) per metaphase
		chromatid exchanges	breaks	dicentrics and rings		
MeBi(III) (1 h)						
0	200		5		2.50	4.77
2.5	270		7		2.59	5.23
5	250		12	1	5.20	5.94
25	130		3	2	3.85	5.38
50	120		5		4.17	5.58
250	115	2	15		14.78	7.07
MeBi(III) (24 h)						
0	100		3		3.00 ± 0.71	3.34 ± 0.47
2.5	105		5		4.76 ± ;0.71	4.25 ± 0.78
5	100		5		5.00 ± 0.71	4.60 ± 0.57
25	120		9	2	9.17 ± 0.71*	7.25 ± 0.35*
50	135		17	2	14.07 ± 2.12**	6.92 ± 0.31*
250					toxic	toxic
bismuth citrate (1 h)						
0	100		3		3.00 ± 0.71	3.16 ± 0.78
2.5	100		1		1.00 ± 0.71	3.23 ± 0.58
5	100		2		2.00 ± 0.00	3.37 ± 0.05
25	100		3		3.00 ± 0.71	3.00 ± 0.00
50	100		2		2.00 ± 1.40	3.20 ± 0.21
250	100		2	1	3.00 ± 0.71	4.02 ± 0.84
bismuth glutathione (1 h)						
0	100		2		2.00 ± 1.41	3.50 ± 0.28
2.5	100		2		2.00 ± 0.00	3.00 ± 0.35
5	100		2		2.00 ± 1.41	3.05 ± 0.49
25	100		2		2.00 ± 1.41	3.30 ± 0.04
50	100		2		2.00 ± 0.00	3.30 ± 0.57
250	120		2	1	2.50 ± 0.71	3.85 ± 0.21
positive control [MMA(III)] (1 h)						
0	100		2		2.00 ± 1.41	
0.5	100		8	1	9.00 ± 0.71*	
positive control [MMA(III)] (24 h)						
0	100		3		3.00 ± 0.71	
0.5	130	1	28	1	23.08 ± 4.24**	

[a] MMA(III) (0.5 μM) was used as a positive control. Experiments were repeated three times. Significance testing: *$p < 0.05$ and **$p < 0.01$.

be biomethylated in hepatocytes as it is known to occur for inorganic arsenicals (*51*). Human lymphocytes are less susceptible to the methylated bismuth compound and MeBi(III) affects these cells only after 24 h of exposure at a high concentration of >430 μM. It seems that lymphocytes are able to accumulate higher amounts of bismuth compounds than hepatocytes and erythrocytes before showing cytotoxic effects. Arata et al. (*52*) also described cytotoxic effects with another organic bismuth compound, triphenylbismuth, at micromolar concentrations in rat thymocytes. In contrast to our results, Hutson (*53*) demonstrated in viability testing a significant effect in macrophages after treatment with bismuth-citrate at 6.25 μM after 24 h of exposure. Magnusson et al. (*54*) also found a time- and dose-dependent decrease of viability between 12 and 24 h of incubation with bismuth-citrate (50 μM) in a macrophage cell line. Macrophages may however be more susceptible to bismuth-citrate exposure than our test cells. We only observed cytotoxicity with bismuth-citrate in erythrocytes at concentration of >113 μM after 24 h of incubation. These different results further emphasize that bismuth toxicity depends on the cell type.

MeBi(III) is able to induce genomic damage in human lymphocytes. Chromosomal aberrations are recognized as very sensitive end points for detection of genotoxic effects induced by chemicals. Significant genotoxic effects in bone marrow cells of mice were detected by Gurnani et al. (*55*) after treatment of mice with bismuth trioxides. The authors found an increased frequency of aberrations in dependence on concentration and incubation time. In their investigations, chromosomal aberrations were mainly gaps and single strand breaks. In the present study, a significant number of chromosomal aberrations and sister chromatid exchanges were detected after MeBi(III) exposure. The concentration at which aberrations occurred was not in the cytotoxic range and showed a cell viability of >97%. Chromosomal type aberrations such as single and double strand breaks were mainly found in our study; dicentric chromosomes and chromatid type aberrations such as chromatid exchanges were detected rarely. Chromosome gaps were not assessed as aberrations in our analysis.

The mechanism of genotoxicity of organobismuth compounds is not known. We have tested the hypothesis that free radicals are involved in MeBi(III)-induced genotoxic damage, but formation of ROS is only evident in hepatocytes but not in lymphocytes. Another mechanism seems to be responsible for the observed effects. It is known that bismuth is a powerful metallothionein inducer (*54*). Metallothionein (MT) is a cysteine-rich metal-binding protein, which decreases cytotoxicity and induces "hypoxia-like" stress under nonhypoxic conditions. Its function is transport, metabolism, and detoxification of metals as well as inactivation of radicals. Metallothionein is not produced in tumor cells (*56*). This explains why we found ROS formation in HepG2 cells (hepatoma cells) but not in lymphocytes.

Sun et al. (*56*) concluded from their investigations that Bi^{3+} binds strongly to MT and can readily displace Zn^{2+} and Cd^{2+}. It was shown by several authors that metals are able to interact

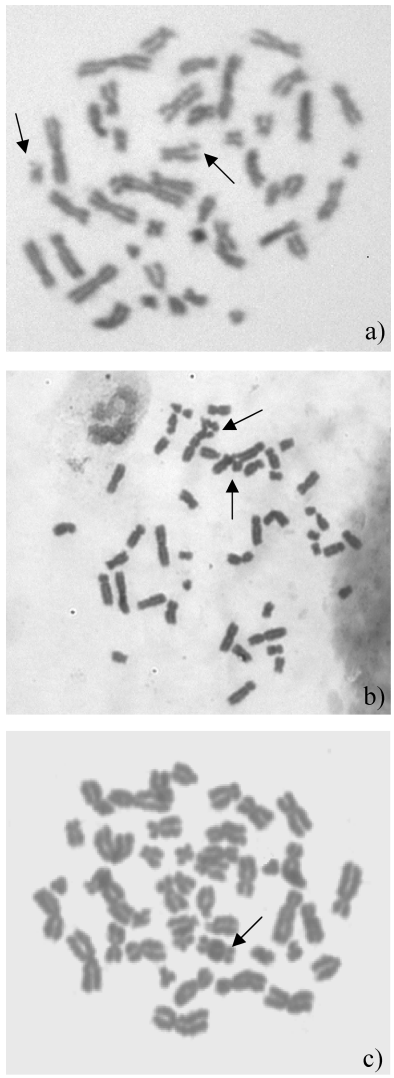

Figure 5. Metaphase chromosomes of human lymphocytes with (a) two single strand breaks (arows), (b) two chromatid aberrations (arrows), and (c) one dicentric chromosome (arrow) after treatment of cells with MeBi(III), 250 μM, for 1 h.

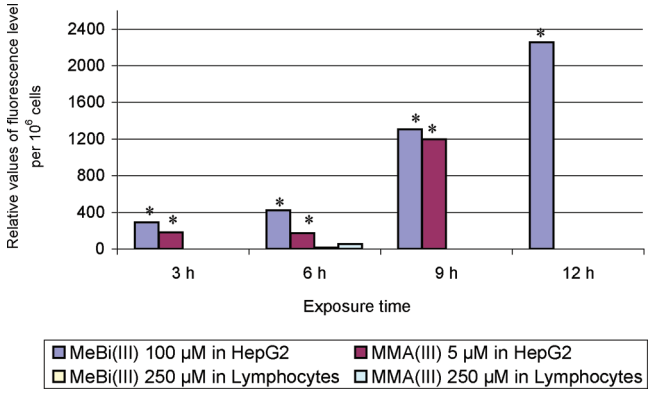

Figure 6. Time-dependent formation of intracellular radicals (ROS) in human hepatocytes and lymphocytes after MeBi(III) exposure. MMA(III) was used as a positive control. The experiments were carried out in triplicate; SD < 12%. Data are presented as relative values related to the negative control (unexposed cells). Significance testing: *p < 0.05.

with so-called zinc finger proteins (*57, 58*). The zinc finger proteins comprise a family of proteins where zinc is complexed through four invariant cysteine and/or histidine residues forming a zinc finger domain, which is involved mostly in DNA binding but also in protein–protein interactions (*59*). Even though most zinc finger structures have been described as DNA-binding motifs in transcription factors, they have also been identified in several DNA repair enzymes. Zinc finger domains are potential targets for metal toxicity, according to one or more among several molecular mechanisms proposed, direct (metal ion exchange, mixed complex formation) or indirect (e.g., oxidative) (*60–62*). Also, a direct interaction of methylbismuth with DNA seems possible, similar to interactions of platinum with nucleic acids (*63*).

With respect to our investigations, we hypothesize that MeBi(III) may inhibit the DNA repair mechanism by displacement of Zn^{2+} from the zinc finger protein of DNA repair enzymes leading to increased DNA damage. Formation of ROS is probably not involved in MeBi(III)-induced genomic damage.

In summary, our results show that the methylated bismuth compound MeBi(III) is more membrane permeable and more cytotoxic than the bismuth compounds Bi-GS and Bi-Cit. Also, MeBi(III) induced chromosome damage in human lymphocytes at noncytotoxic concentrations. Bi-GS and Bi-Cit did not show any genotoxic effects. We conclude from our study that the uptake of bismuth compounds into cells and the possibility to methylate these compounds in vivo lead to species that are more toxic than the primary bismuth salt and are able to induce DNA damage and cell death.

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