Transport of aflatoxin M_1 in human intestinal Caco-2/TC7 cells

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Aflatoxin M_1 (AFM_1) is a hydroxylated metabolite of aflatoxin B_1 (AFB_1). After it is formed, it is secreted in the milk of mammals. Despite the potential risk of human exposure to AFM_1, data reported in literature on the metabolism, toxicity, and bioavailability of this molecule are limited and out of date. The aim of the present research was to study the absorption profile of AFM_1 and possible damage to tight junctions (TJ) of the intestinal Caco-2/TC7 clone grown on microporous filter supports. These inserts allowed for the separation of the apical and basolateral compartments which correspond to the *in vivo* lumen and the interstitial space/vascular systems of intestinal mucosa respectively. In this study, the Caco-2/TC7 cells were treated with different AFM_1 concentrations (10–10,000 ng/kg) for short (40 min) and long periods of time (48 h). The AFM_1 influx/efflux transport and effects on TJ were evaluated by measuring trans-epithelial electrical resistance and observing TJ protein (Zonula occludens-1 and occludin) localization. The results showed that: (i) when introduced to the apical and basolateral compartments, AFM_1 was poorly absorbed by the Caco-2/TC7 cells but its transport across the cell monolayer occurred very quickly (P_{app} value of 105.10 ± 7.98 cm/s $\times 10^{-6}$). (ii) The integrity of TJ was not permanently compromised after exposure to the mycotoxin. Viability impairment or barrier damage did not occur either. The present results contribute to the evaluation of human risk exposure to AFM_1, although the AFM_1 transport mechanism need to be clarified.

Keywords: aflatoxin M_1, Caco-2/TC7 cells, transport, tight junctions, intestinal barrier

INTRODUCTION

Aflatoxins, a group of mycotoxins produced primarily by *Aspergillus flavus* and *parasiticus*, are common contaminants found in a wide variety of agricultural commodities such as corn, sorghum, barley, rye, wheat, peanuts, soy, rice, cottonseed, and feed (Hussein and Brasel, 2001).

Aflatoxin B_1 (AFB_1), the most potent hepatocarcinogen known in mammals (Creppy, 2002), is biotransformed into aflatoxin M_1 (AFM_1) at the hepatic level by microsomal cytochrome P450 (Van Egmond, 1989) and can be secreted in the milk of mammals (Holzapfel et al., 1966; Applebaum et al., 1982; Van Egmond, 1989; Wood, 1991; Neal et al., 1998). AFM_1 has 2–10% of the carcinogenic potency of the parent molecule (Creppy, 2002) and is classified as a probable human carcinogen, categorized in group 2B by the [International Agency for Research on Cancer , IARC].

Human exposure to AFM_1 is partly from consumption of contaminated milk and dairy products and partly from endogenous production through AFB_1 metabolism in the liver (Neal et al., 1998). Milk has the greatest demonstrated potential for introducing directly AFM_1 in human diet (Rahimi et al., 2010). AFM_1 intake from milk is calculated to be 6.8 ng/person/day in the European diet, 3.5 ng/person/day in the Latin American diet, 12 ng/person/day in the Far Eastern diet, 0.7 ng/person/day in the Middle Eastern diet and 0.1 ng/person/day in the African diet (Creppy, 2002). The potential presence of AFM_1 in milk and its

by-products represents a worldwide concern as these products are primarily consumed by infants and children who are more susceptible to the adverse effects of mycotoxins (Boudra et al., 2007). In order to protect consumers, many countries have regulated the level of AFM_1 in milk. The Commission of the European Community has prescribed a maximum tolerance limit of 50 ng/kg in milk and 25 ng/kg in milk-based food for infants [Commission of the European Communities (CEC), 2004, 2006, 2010], with the intention of decreasing this limit to 10 ng/kg. The US Food and Drug Administration (FDA) has however established an action level of 500 ng/kg in whole, low fat, and skim milk (FDA, 2005).

Despite the potential risk of human exposure to AFM_1, data reported in literature regarding the metabolism, toxicity, and absorption of this molecule, particularly in humans, are limited and out of date. In general, AFM_1 and AFB_1 cause almost identical effects of acute toxicity and carcinogenicity in different mammalian systems (Sinnhuber et al., 1970; Pong and Nogan, 1971; Shibahara et al., 1995). However, AFM_1 seems to be the weaker hepatic carcinogen compared to AFB_1 (Bailey et al., 1994) and little evidence is available with regard to AFM_1 embryotoxicity (Vismara et al., 2006).

A dose-dependent absorption of AFM_1 in differentiated Caco-2 cells and significant lactate dehydrogenase release, particularly evident in undifferentiated cells, was reported previously (Caloni et al., 2006).

The purpose of this study was to investigate AFM$_1$ transport and possible damage to tight junctions (TJ) of Caco-2/TC7 cells, a clone derived from late passage of the human parental colorectal adenocarcinoma Caco-2 cell line. This clone was seen to express higher metabolic competence, such as hydrolase sucrose isomaltase and UDP-glucuronyltransferases (Turco et al., 2011), and more regular morphology than parental cells and showed more of a similarity to the *in vivo* intestinal cells considering certain defined parameters (Zucco et al., 2005). The experiments were carried out on microporous filter supports which separated the apical (Ap) compartment (corresponding to the *in vivo* intestinal lumen) from the basolateral (Bl) compartment (which *in vivo* faces the interstitial space and the vascular systems) and allowed for separate evaluation of the absorptive influx (Ap to Bl) and exsorptive components (Bl to Ap).

MATERIALS AND METHODS
CHEMICALS
The 0.5-ng/μl AFM$_1$ solution in methanol was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water (H$_2$O), acetonitrile (ACN), and methanol (MeOH) for HPLC analysis were obtained from J.T. Baker® (Deventer, The Netherlands) and 2-propanol (IPA) from Merck (Darmstadt, Germany). Dimethylsulfoxide (DMSO) was purchased from Carlo Erba (Milan, Italy). Hanks Balanced Salt Solution (HBSS), *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), and 2-(*N*-morpholino) ethane sulfonic acid (MES) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagles' Medium (DMEM) high glucose, Fetal Calf Serum (FCS), glutamine, Non-Essential Amino Acids (NEAA), penicillin/streptomycin were all purchased from GIBCO BRL (Gaithersburg, MD, USA). All other chemicals were of analytical grade.

CELL CULTURE CONDITIONS
Caco-2/TC7 clone, derived from late passage of Caco-2 wild type cells (provided by Dr. Ming Hu, Washington State University, Pullman) was routinely grown in an atmosphere of 5% carbon dioxide at 37°C in DMEM high glucose standard medium (Caloni et al., 2006). The cells were seeded at a density of 1.5×10^5 cells/filter on 1 μm pore size 12-well plate polycarbonate inserts (Millicell®, Millipore Corporation). The cells were used between passage 60 and 65 and maintained in a standard culture medium (regularly changed three times a week) during the whole differentiation phase. The experiments were performed after 21 days of culture when the differentiation process was completed.

ABSORPTION EVALUATION
Experiment 1
Experiment 1 was performed to evaluate AFM$_1$ *in vitro* intestinal absorption profile after exposure for 48 h. Caco-2/TC7 cells were treated for 48 h with different concentrations of AFM$_1$ (1,000, 5,000, 10,000 ng/kg corresponding to 3.2, 16, 32 nM) in both Ap and Bl sides. AFM$_1$ was dissolved in DMSO; the same final concentration of the solvent (2% maximum) was used in the corresponding control cells. At the end of the incubation time, Ap and Bl media, and the cellular layer were collected separately and processed for HPLC analysis.

Experiment 2
Experiment 2 was performed to evaluate AFM$_1$ *in vitro* intestinal absorption kinetic profile at different times of exposure up to 40 min. Caco-2/TC7 cells were treated for 40 min with different concentrations of AFM$_1$ (10, 100, 1,000 ng/kg corresponding to 0.032, 0.32, 3.2 nM) in Phosphate buffer (HBSS) in pH gradient (HBSS-Mes, pH = 6–6.5, in Ap compartment (donor) and HBSS-Hepes, pH = 7.2–7.4, in Bl compartment). At different time points (10, 20, 30, and 40 min after exposure), samples of buffer were taken from the receiver compartment and replaced by an equal volume of fresh buffer. At the end of the experiments, buffers from the donor compartments as well as cellular lysates were collected to allow mass balance calculation. All samples were processed for HPLC analysis.

BARRIER INTEGRITY ASSAY (TRANS-EPITHELIAL ELECTRICAL RESISTANCE EVALUATION)
In Experiment 1 and Experiment 2 barrier impairment after exposure to AFM$_1$ was assessed by measuring the trans-epithelial electrical resistance (TEER) which quantifies ion movement across the cellular barrier. TEER values were recorded in the culture medium at 37°C with chopstick electrodes (Millicell®-ERS, Millipore) and were expressed as $\Omega \times cm^2$ according to the following equation:

$$\text{TEER} = \left(\Omega \text{ cell monolayer} - \Omega \text{ filter cell - free}\right) \times \text{filter area}.$$

For each filter, three separate measures were collected.

FLUORESCENT STAINING OF CELLULAR STRUCTURES
Experiment 3
Experiment 3 was carried out to evaluate the AFM$_1$ effects on TJ proteins. The expression of Zonula occludens-1 (ZO-1) and occludin, two TJ proteins located in different cellular compartments, was examined. In addition, considering that apoptosis might contribute to loss of intestinal barrier integrity (Sun et al., 1998; Abreu et al., 2000; Gitter et al., 2000; Chin et al., 2002), nuclear staining was performed as a marker of apoptosis.

In detail, Caco-2/TC7 cells were seeded on filters as described previously and treated with AFM$_1$ concentrations of 1,000 ng/kg (3.2 nM) and 10,000 ng/kg (32 nM) for 60 min. After two washes with PBS, monolayers were fixed with a solution of paraformaldehyde (4%) and sucrose (0.12 M) and permeabilized with TRITON×-100 (0.2%). For ZO-1 and occludin staining, cells were incubated overnight at 4°C with anti-ZO-1 (1:100 in PBS) and anti-Occludin (1:50 in PBS) and then labeled with the secondary fluorescent conjugated antibodies. For nuclear staining, after two washes with deionized water, 250 μl of Hoechst solution was added to the Ap compartment and incubated at 37°C for 30 min. Cells were observed using an inverted fluorescent microscope (LEICA DM IRB, Nussloch, Germany).

EXTRACTION PROCEDURE FOR HPLC ANALYSIS
After exposure to AFM$_1$ the cells were processed and the samples were analyzed for AFM$_1$ presence by using HPLC. All procedures were conducted in absence of artificial light. In short, 3 ml of H$_2$O was added to each medium sample (1 ml) and then extracted by the Immunoaffinity Column (Afla M$_1$ TM, Vicam, USA) as described

by Sharman et al. (1989). The Immunoaffinity Column was first conditioned with 10 ml of PBS, subsequently treated with the sample, then washed with 10 ml H$_2$O and finally dried. The AFM$_1$ was slowly eluted from the column with methanol (2 ml) into a glass vial, dried under nitrogen, and dissolved in 200 µl of ACN:H$_2$O (25:75). Pellet analysis was carried out after adding 100 µl of ACN with subsequent sonication for 15 min followed by centrifugation for 10 min 500 × g. Supernatants were analyzed without extraction (Caloni et al., 2006).

HPLC ANALYSIS

Samples were processed as described previously and analyzed by HPLC (Series 200, Perkin-Elmer, USA) using a Waters Spherisorb 5 µm ODS 2 250 mm × 4.6 mm (Supelco, Inc., Sigma-Aldrich, St. Louis, MO, USA), MeOH-NaH$_2$PO$_4$ 0.1 M (33:67, v:v) as a mobile phase (flow rate of 1 ml/min) and a fluorescence detector (LC 240 Perkin-Elmer, USA) set at an excitation wavelength of 365 nm and emission wavelength of 420 nm (Sharman et al., 1989). AFM$_1$ chromatographic conditions were described by Pietri et al. (1997).

MEDIAN APPARENT PERMEABILITY COEFFICIENT (P_{app}) VALUE AND UPTAKE RATIO CALCULATION

The apparent permeability coefficient (P_{app}) value for both directions (from Ap to Bl and from Bl to Ap) was calculated using the following general equation (Prieto et al., 2010; Turco et al., 2011):

$$C_R(t) = [M/(V_D + V_R)] \\ + \left(C_{r,0} - [M/(V_D + V_R)]\right) e^{-P_{app} A(1/V_D + 1/V_R)t}$$

where V_R is the volume in the receiver compartment and V_D is the volume of the donor compartment. M is the amount of toxin in the system, A is the area of the filter, $C_{R,0}$ is the toxin concentration in the receiver compartment at the beginning of the interval and t is the time from the start of the interval.

Uptake ratio (absorption), i.e., the ratio between Ap → Bl and Bl → Ap P_{app} values and efflux ratio (secretion), i.e., the ratio between Bl → Ap and Ap → Bl P_{app} values, were also calculated.

STATISTICAL ANALYSIS

Two separate experiments, performed in triplicate, were carried out for each assay. Results were expressed as mean ± standard deviations (SD). Statistical evaluation was performed by two tailed Student's *t*-test. The level of significance was established at $P < 0.05$.

RESULTS
EFFECTS ON TJ
Trans-epithelial electrical resistance

Trans-epithelial electrical resistance values in Experiment 1 were recorded before the treatment and after exposure for 6 and 24 h to different concentrations of AFM$_1$ (from 1,000 to 10,000 ng/kg). Both the Ap and Bl sides were subjected to treatment. The mean TEER value of untreated cells was 256 ± 6 Ω × cm^2.

As shown in **Figure 1**, a slight (15–20%) but significant ($P < 0.01$) TEER decrease was reported starting from the sixth hour of treatment. The reduction was not dose-dependent. This
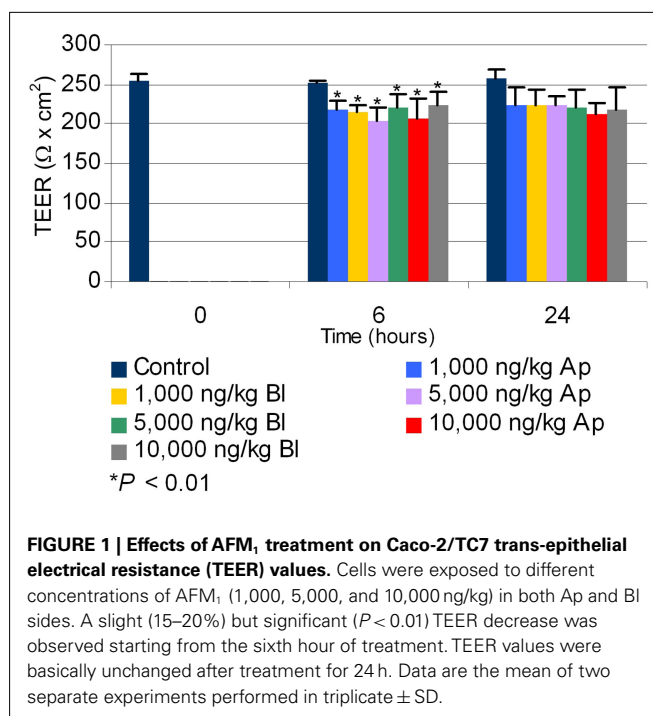

FIGURE 1 | Effects of AFM$_1$ treatment on Caco-2/TC7 trans-epithelial electrical resistance (TEER) values. Cells were exposed to different concentrations of AFM$_1$ (1,000, 5,000, and 10,000 ng/kg) in both Ap and Bl sides. A slight (15–20%) but significant ($P < 0.01$) TEER decrease was observed starting from the sixth hour of treatment. TEER values were basically unchanged after treatment for 24 h. Data are the mean of two separate experiments performed in triplicate ± SD.

decrease could indicate an alteration in paracellular permeability in presence of AFM$_1$.

Trans-epithelial electrical resistance values were basically unchanged after 48 h of AFM$_1$ treatment (data not shown). Before and after the 40-min absorption studies (Experiment 2), TEER values of all inserts were determined in order to verify monolayer integrity. No significant variations were reported at any of the concentrations tested; moreover, the mean TEER value was always within the range of the acceptance criteria defined for this cell line (i.e., >200 Ω cm^2).

Fluorescent staining of cellular structures

After a 1-h treatment with AFM$_1$ concentrations of 1,000 and 10,000 ng/kg, no loss of ZO-1 was observed. Occludin staining continuity was reported, indicating integrity of TJ (**Figures 2A–I**). Caco-2/TC7 monolayers exhibited uniform fluorescent nuclear staining (**Figures 2J–L**) characteristic of viable cells indicating no apoptotic changes induced by AFM$_1$ at concentration*s* of 1,000 and 10,000 ng/kg.

RESULTS OF HPLC DETERMINATION

The detection limit for AFM$_1$ in medium and cells in both experiments was 5 ng/kg and the volume injected was 50 µl. AFM$_1$ extraction recoveries from Ap and Bl media samples for each transport study were calculated on 20 replicates, obtaining a range of 91.2–98.5%. Recoveries from Caco-2/TC7 cells extractions were about 100%.

AFM$_1$ ABSORPTION PROFILE

Absorption of AFM$_1$ was evaluated on the insert culture system. Caco-2/TC7 cells were exposed to different concentrations of AFM$_1$ (1,000–10,000 ng/kg) in both Ap and Bl compartment*s*
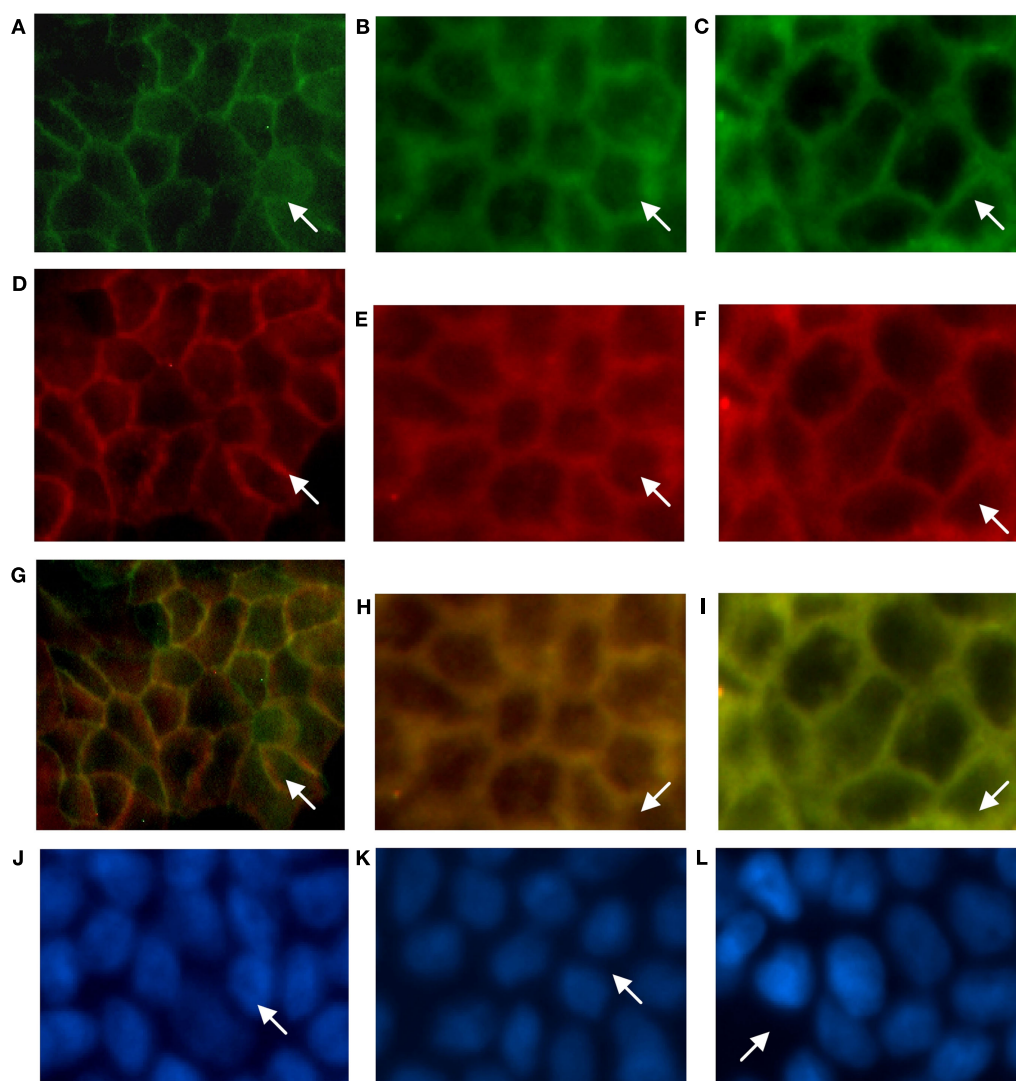
FIGURE 2 | Effects of AFM₁ treatment on Caco-2/TC7 TJ complex components ZO-1 and occludin and nuclear structure. No differences between control and treated monolayer was observed (arrows). **(A–C)** ZO-1 labeling, **(A)** control monolayer, **(B)** monolayer treated with 1,000 ng/kg AFM₁ for 1 h, **(C)** monolayer treated with 10,000 ng/kg AFM₁ for 1 h, **(D–F)** Occludin labeling, **(D)** control monolayer, **(E)** monolayer treated with 1,000 ng/kg AFM₁ for 1 h, **(F)** monolayer treated with 10,000 ng/kg AFM₁ for 1 h, **(G–I)** Merging of ZO-1, and occludin labeling, **(G)** control monolayer, **(H)** monolayer treated with 1,000 ng/kg AFM₁ for 1 h, **(I)** monolayer treated with 10,000 ng/kg AFM₁ for 1 h, **(J–L)** Nuclear labeling, **(J)** control monolayer, **(K)** monolayer treated with 1,000 ng/kg AFM₁ for 1 h, **(L)** monolayer treated with 10,000 ng/kg AFM₁ for 1 h.

and distribution between compartments was determined after 48 h by HPLC analysis. After Ap exposure more than 70% of the mycotoxin was found in the donor compartment while, after Bl exposure a uniform distribution between donor and acceptor compartment*s* was reported. In both cases, the same low concentration of mycotoxin was detected in the cells, indicating that no significant absorption occurred into this cell line (**Table 1**). Moreover, the trend was independent of the dose in all the experimental conditions utilized.

Forty-minutes transport studies were performed with AFM₁ concentrations ranging from 10 to 1,000 ng/kg in both Ap and Bl compartment*s* and distribution in the compartments was evaluated after 10, 20, 30, and 40 min of exposure (**Table 2**).

A P_{app} value of 105.10 ± 7.98 cm/s $\times 10^{-6}$ was obtained for both passage directions (from Ap to Bl and from Bl to Ap). AFM₁ uptake (absorption) and efflux (secretion) ratios were <2.

DISCUSSION

The intestinal tract represents the first barrier to ingested chemicals or food contaminants and the evaluation of its integrity is crucial in assessing risk subsequent to food contaminant exposure.

The disruption of the intestinal barrier allows increased penetration of normally excluded luminal substances that could promote intestinal disorders (Pinton et al., 2009).

Although epidemiological evidence is still required, it is believed that food-associated exposure to certain mycotoxins

Table 1 | AFM$_1$ detection in donor compartment, acceptor compartment, and cellular fractions after exposure for 48 h.

AFM$_1$ ng/kg	Apical exposure (mean ± SD)			Basolateral exposure (mean ± SD)		
	Donor medium	Acceptor medium	Cells	Donor medium	Acceptor medium	Cells
1,000	731.7 ± 91.8	163.6 ± 9.1	53.1 ± 9.3	339.2 ± 77.8	451.6 ± 48.0	59.3 ± 7.5
5,000	4324.4 ± 297	149.0 ± 34.4	59.2 ± 8.3	2178.7 ± 239.2	2330.9 ± 111.8	60.2 ± 9.0
10,000	7341.7 ± 450	1914.5 ± 391.5	53.1 ± 9.2	3984.2 ± 497.8	4611.8 ± 449.1	60.5 ± 7.6

Results are the mean of two separate experiments performed in triplicate ± SD.

Table 2 | AFM$_1$ detection in donor compartment, acceptor compartment, and cellular fractions after exposure for 40 min.

AFM$_1$ ng/kg	Time (min)	Apical exposure (mean ± SD)			Basolateral exposure (mean ± SD)		
		Donor medium	Acceptor medium	Cells	Donor medium	Acceptor medium	Cells
10	10	NA	0.63 ± 0.21	NA	NA	0.57 ± 0.06	NA
	20	NA	1.35 ± 0.21	NA	NA	0.93 ± 0.25	NA
	30	NA	2.47 ± 0.15	NA	NA	1.30 ± 0.20	NA
	40	7.23 ± 0.31	3.77 ± 0.35	NA	5.97 ± 0.59	2.17 ± 0.31	NA
100	10	NA	4.53 ± 0.67	NA	NA	4.50 ± 0.26	NA
	20	NA	5.53 ± 0.15	NA	NA	10.60 ± 1.57	NA
	30	NA	6.80 ± 0.60	NA	NA	17.17 ± 1.07	NA
	40	71.83 ± 2.28	9.33 ± 0.15	14.83 ± 3.12	65.73 ± 2.64	21.37 ± 1.96	7.30 ± 1.41
1,000	10	NA	51.97 ± 2.52	NA	NA	34.17 ± 1.33	NA
	20	NA	66.30 ± 4.52	NA	NA	88.27 ± 3.56	NA
	30	NA	89.47 ± 4.52	NA	NA	121.67 ± 6.50	NA
	40	718.93 ± 5.75	104.20 ± 1.95	48.87 ± 3.31	606.47 ± 24.45	228.73 ± 5.42	33.35 ± 1.20

NA, not analyzed.
Results are the mean of two separate experiments performed in triplicate ± SD.

could lead to the induction and/or persistence of human chronic intestinal inflammatory diseases (Maresca and Fantini, 2010). Moreover, existing data demonstrate that several mycotoxins, at realistic doses, are able to affect key intestinal and immune functions such as composition of the intestinal microflora (Tenk et al., 1982; Waché et al., 2009), production of mucus (Obremski et al., 2008), epithelial barrier function (Gratz et al., 2007; Lambert et al., 2007; McLaughlin et al., 2009; Pinton et al., 2009; Van De Walle et al., 2010), bacterial translocation (Maresca et al., 2008), and innate and adaptive gut immunity (Fukata et al., 1996; Oswald et al., 2003; Li et al., 2005, 2006; Bouhet et al., 2006).

AFM$_1$, present in milk and dairy products, is of great importance because of the high consumption of these products by humans, especially children. Human exposure to AFM$_1$ through milk and dairy products has been shown in several studies (Sassahara et al., 2005; Unusan, 2006).

The intake of AFM$_1$ from milk is calculated to be 6.8 ng/person/day in the European diet but it is interesting to note that if all milk consumed were contaminated with AFM$_1$ at the proposed maximum EU levels of 50 ng/kg, the intake of AFM$_1$ from milk in the European regional diet would be 15 ng/person per day [Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2001]. Considering a tolerable daily intake (TDI) of 0.2 ng/kg b.w. (14 ng/person with a mean weight of 70 kg) as calculated by Kuiper-Goodman (1990), an intake of 15 ng/person

per day as estimated in the European regional diet could represent a significant dose (Prandini et al., 2009). In the assessment of human exposure to mycotoxins in dairy milk carried out by Coffey et al. (2009) AMF$_1$ resulted as the toxin of greatest concern as, in certain circumstances, its concentration exceeded the EU limit in milk (Commission of the European Communities (CEC), 2004, 2006, 2010). Infants, considering their milk-based diet, represent the population most exposed to this toxin (Turconi et al., 2004).

The toxicological effects of AFM$_1$ are much less investigated than the ones caused by AFB$_1$ and limited data are reported in literature regarding its absorption and metabolism, particularly in humans.

A previous study (Caloni et al., 2006) demonstrated a higher toxicity of AFM$_1$ in Caco-2 undifferentiated cells than in differentiated ones, in which GSH transferase enzyme is highly expressed. This suggests, as reported by Neal et al. (1998), a phase II conjugation mechanism. Roda et al. (2010) seem to confirm this detoxification pathway, as AFM$_1$ was seen to affect the immature human erythroid progenitor cells more markedly than the respective more mature cells.

AFM$_1$ absorption was previously evaluated in Caco-2 cells cultured in monolayer (Caloni et al., 2006) demonstrating a dose-dependent passage of the mycotoxin, particularly evident in 21-day differentiated cells.

In the present paper, we investigated the absorption profile of AFM$_1$ and possible damage to TJ of Caco-2/TC7 cells cultured on microporous filter supports for 21 days. The Caco-2/TC7 cell line is as suitable as the parental Caco-2 line as an intestinal model for studying absorption. Furthermore, due to its clonal origin, the TC7 cell line shows a less heterogenic cellular population, which can result in better reproducibility of results (Chantret et al., 1994; Turco et al., 2011).

In epithelial tissue the initial toxic effect of several substances seems to be directed at the molecules involved in the junctional complexes (tight and adherens junctions); for this reason changes in the permeability of epithelial barriers can be considered as early indicators of adverse effects after chemical exposure (Sambuy, 2009).

In this study, the effects of AFM$_1$ on TEER were initially studied. The TEER quantifies ion movement across a monolayer and is considered to be a good indicator of the integrity of epithelial barrier. A slight (15–20%) but significant ($P < 0.01$) TEER decrease, unrelated to the dose, was reported starting from the sixth hour of treatment. This decrease could indicate an alteration in paracellular permeability in the presence of AFM$_1$. A reduction in TEER can however be caused by different events including: (i) increase in paracellular permeability to ions; (ii) changes in transcellular ion flux through altered plasma membrane channels or pumps; or (iii) uncontrolled cell death within the monolayer (Madara, 1998). In the present work, the third option must be excluded since the cellular monolayer was completely intact at the end of the experiments.

Modulation of barrier properties is often mirrored by changes in specific TJ protein components, since TJ dynamic structures respond quickly to several physiological and pathological stimuli. We therefore examined whether the AFM$_1$-induced reduction of TEER could be due to changes in the expression of certain TJ proteins. We focused our attention on the expression of two TJ proteins located in different cellular compartments: ZO-1 interacting in the cytoplasm with actin cytoskeleton and occludin interacting throughout its extracellular domain with neighboring cells (McLaughlin et al., 2004; Schneeberger and Lynch, 2004). As expected, the localization of TJ proteins showed strong peripheral labeling in control Caco-2/TC7 cell monolayers. The overall morphology of cells treated with AFM$_1$ remained unchanged.

Treatment with AFM$_1$ did not affect ZO-1 or occludin staining or localization. Nuclear staining was also performed in Caco-2-TC7 cells and in this case monolayers exhibited characteristically uniform fluorescent nuclear staining throughout all nuclei indicating no apoptotic changes induced by AFM$_1$ at any of the concentrations tested.

The AFM$_1$ absorption profile was evaluated on the insert culture system. In this condition the cells, after about 3 weeks of culture, were able to polarize and fully differentiate according to the enterocytic pathway, with apical microvilli and a differentiated basolateral surface, similar to the cellular surface in contact with sub-epithelial tissue. In both treatments, a very low concentration of mycotoxin was detected in the cells, indicating that AFM$_1$ was poorly absorbed by these cells. Under these experimental conditions, AFM$_1$ passage through the Caco-2/TC7 layer was observed at all tested concentrations after both Ap and Bl exposure and the P_{app} value confirmed AFM$_1$ to be a molecule highly absorbed by the intestine (Prieto et al., 2010; Turco et al., 2011).

In particular, its passage was greater in the Bl-Ap direction than in the Ap-Bl one. The presence of asymmetric passage through Caco-2 monolayer usually suggests involvement of transporter pathways. This cell line expressed most of the known intestinal transporters overseeing influx/efflux carrier mediated processes, in a pattern similar to that reported for the small intestine (Sun et al., 2008). The AFM$_1$ absorption profiles in both experiments were similar to the ones reported with zearalenone (Videmann et al., 2008) and fumonisin B$_1$ metabolites (De Angelis et al., 2005), where the involvement of an active mechanism of transport was hypothesized. Otherwise the calculated AFM$_1$ uptake and efflux ratios (<2), suggested the inclusion of this mycotoxin in the group of compounds passively transported by paracellular or intracellular route, since xenobiotics generally considered active or carrier-mediate transported show an efflux or an uptake ratio >2 (Zhang et al., 2007).

In conclusion, our results pointed out that AFM$_1$: (i) was poorly absorbed in Caco-2/TC7 cells under the present experimental conditions, (ii) passed across the monolayer in both directions (from Ap to Bl and from Bl to Ap), even if to a different extent, (iii) did not cause viability impairment or barrier damage. Further studies need to be conducted in order to better understand the AFM$_1$ transport mechanism.

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