Effects of bleomycin and antioxidants on the fatty acid profile of testicular cancer cell membranes

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Abstract

Bleomycin is used in chemotherapy regimens for the treatment of patients having testicular germ-cell tumor (TGCT). There is no study in the literature investigating the effects of bleomycin on membrane lipid profile in testicular cancer cells. We investigated membrane fatty acid (FA) profiles isolated, derivatized and analyzed by gas chromatography of Ntera-2 testicular cancer cells incubated with bleomycin (Bleo) for 24 h in the absence and presence of N-Acetyl-L-Cysteine (NAC) and curcumin (Cur) as commonly used antioxidant adjuvants. At the same time the MAPK pathway and EGFR levels were followed up. Bleomycin treatment increased significantly saturated fatty acids (SFA) of phospholipids at the expense of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Bleomycin also led to a significant increase in the trans lipid isomers of oleic and arachidonic acids due to its free radical producing effect. Incubation with bleomycin increased the p38 MAPK and JNK levels and downregulated EGFR pathway. Coincubation of bleomycin with NAC reversed effects caused by bleomycin. Our results highlight the important role of membrane fatty acid remodeling occurring during the use of bleomycin and its concurrent use with antioxidants which can adjuvate the cytotoxic effects of the chemotherapeutic agents.

1. Introduction

Testicular germ cell tumors (TGCTs) are the most frequent malignancy in males between 20 and 45 years of age. The current tumor incidence in the general population is \( \approx 0.005\% \) which is 50% higher in comparison to 30 years ago and the number of diagnosed cases keeps increasing gradually [1]. However, the causes of this increase remain unclear [2]. TGCTs represent the majority of testicular tumors (>95%). Men with TGCTs have a higher risk of developing a subsequent tumor and a second primary contralateral testis tumor may occur in up to 5% of men with a prior tumor [3]. Conventional cancer treatments with some therapeutic drugs and radiation might generate free radicals (ROS) which exert additional cytotoxic effects such as induction of apoptosis. Bleomycin is commonly used to treat patients with TGCTs [4]. Bleomycin binds to and cleaves DNA in the presence of ferrous ion and molecular oxygen [5] involving the following three actions: (i) recognition of a particular base or base sequence on a DNA double strand, (ii) formation of radical species that propagates a free radical based mechanism of action, and (iii) oxidation reactions leading to DNA strand scission [6]. Bleomycin generates the highest level of free radicals which act as one of the intracellular second messengers leading to induction of various proteins through transcriptional activation [7]. Under these conditions, the unsaturated fatty acid residues of cell membranes may undergo oxidation and isomerization. Indeed, in our recently published study performed in liposomes and cell cultures, we demonstrated that bleomycin–iron complex transformed membrane mono- and polyunsaturated fatty acid components (MUFA and PUFA) into trans geometric isomers together with a profound remodeling of the membrane fatty acid residues [8]. Transformation of cis to trans geometry of unsaturated lipids has been described in other conditions in cells, animal models, as well as in humans [9]. It is worth to underline that trans fatty acid isomers cannot be synthesized in eukaryotic cells, and an enzymatic cis–trans isomerization occurs only in some bacteria [9,10]. The presence and effect of trans fatty acids in dietary foods consumed have been thoroughly evaluated for health [9,11,12]. Antioxidants prevent excess free radical formation and reactions, and exert inhibitory activity in lipid isomerization [13]. Intrinsic antioxidants represent the only way to protect the lipid geometry. There are conflicting views for the use of antioxidants in cancer patients due to their potential interactions with radiation and chemotherapy induced ROS generation [14,15]. Focusing on cell membranes as the essential element of the cells and lipidomic monitoring of the fatty acid residues of membrane...
phospholipids in cell cultures have demonstrated the effect of dietary conditions during the chemotherapeutic treatments. Lipidomic monitoring has been shown to be a powerful tool to follow up the consequence of diet and treatment on membrane fatty acid reorganization [16,17].

In the present study, we aimed to analyze the fatty acid remodeling of human testicular cancer cell membranes (NTera-2) incubated with bleomycin in the absence and presence of antioxidant supplementation of curcumin and NAC. Curcumin is the principal curcuminoid of the popular Indian spice turmeric, which is a member of the ginger family (Zingiberaceae). N-acetylcyesteine (NAC) is a pharmaceutical drug used also as a nutritional supplement and is a cysteine source for the synthesis of glutathione. In addition to investigating the lipidomic monitoring, we determined also the EGF receptor, a transmembrane glycoprotein involved in the regulation of cell proliferation, differentiation, and survival; and mitogen-activated protein kinase (MAPK), one of the most important response to oxidative stress, as two important biomarkers of signaling pathways. We searched if the results of lipidomic analysis highlight the role of bleomycin induced fatty acid alterations in cancer cell membranes as well as the effect of combined antioxidant supplementation, in order to envisage possible interference or synergism with the effect of bleomycin.

2. Methods

2.1. Cell culture

NTera-2 human testicular germ cancer cells provide a model system for investigating potential mechanisms of testicular cell membrane alterations induced by bleomycin and antioxidants in vitro. NTera-2 cells were obtained from the ATCC. We did not detect any infection (mycoplasma or other pathogens) in the NTera-2 cells. According to the material transfer agreement form attached, ATCC guarantees the provision of infection free cells. Additional analysis in our laboratory verified that the cells were infection free. As proposed by the reviewer, we added these sentences in the manuscript. The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin in a 5% CO2 atmosphere at 37 °C. We incubated the cells with the IC50 concentrations of bleomycin (0.28 mM) and curcumin (20 μM) for 24 h [18]. We used 5 mM NAC dose for 24 h incubations [19]. The curcumin was purchased from Sigma-Aldrich (Cat No C7727). The standard protocols for cell culture were applied in all of the experiments and all the cell groups were prepared under the same conditions.

2.2. Phospholipid extraction and fatty acid analysis

Cells were detached using accutase, thoroughly washed with phosphate buffer, and pelleted by centrifugation at 14,000 × g for 40 min at 4 °C after adding water. Phospholipids were isolated from the pellet and fatty acids were derivatized as described [20]. Fatty acid compositions are given in Table 1 as relative percentages of the total fatty acid content.

2.3. Mitogen-activated protein kinase (MAPK) assay

Cells were lysed with Cell Lysis Buffer (9803; Cell Signaling Technology), then the levels of p38, p-MEK1/2, p-ERK1/2, and p-SAPK/JNK in the cell lysates were determined using the PathScan MAPK Multi-Target Sandwich ELISA Kit (7274; Cell Signaling Technology) according to the manufacturer’s instructions.

2.4. Epidermal growth factor receptor (EGFR) assay

Epidermal growth factor receptor levels were measured by sandwich ELISA kit (Calbiochem), a sensitive colorimetric assay, in accordance with the procedure recommended by the manufacturer. The total protein content in the samples was determined by the Bradford method [21] using bovine serum albumin (BSA) as a standard. The total protein content was used to normalize the EGFR and MAPK values of each sample.

2.5. Statistical analysis

Results were given as mean ± SD. Statistical comparisons were conducted using t-test and SPSS software; version 13.0 (Chicago, IL). Statistical significance was based on 95% confidence limits (p ≤ 0.05). Comparison of the non-parametric data among the groups was performed using the Mann–Whitney U test.

3. Results

3.1. Membrane fatty acid profile

Table 1 shows membrane fatty acid composition in the NTera-2 cells after incubations with bleomycin and antioxidants for 24 h. Fig. 1 depicts the main fatty acid changes detected in the NTera-2 cell membrane phospholipids after 24 h exposure to bleomycin, NAC, curcumin, bleomycin + NAC, and bleomycin + curcumin. Palmitic acid (16:0) level was found similar in all of the groups. A significant increase in the stearic acid (18:0) residue was observed in the cells incubated with bleomycin and bleomycin + NAC compared to the control cells, and NAC decreased stearic acid level compared to the cells incubated with bleomycin. Bleomycin + NAC significantly enhanced stearic acid compared to NAC. As shown in Table 1, incubation with bleomycin alone or in combination with NAC or curcumin diminished significantly (p < 0.05, p < 0.01) the levels of monounsaturated cis-fatty acids: 6c-16:1, 9c-16:1, 9c-18:1 (oleic), and 11c-18:1 (vaccenic). Incubation with bleomycin and its combination with NAC or curcumin decreased palmitoleic acid (9c-16:1) levels compared to the control cells (<0.05). Bleomycin + NAC significantly decreased palmitoleic acid compared to NAC (<0.05). The trans isomers 9t-16:1 and 6t-16:1, expressed as trans-16:1, significantly increased in the cells incubated with bleomycin, curcumin and bleomycin + curcumin compared to the control cells (p < 0.001). NAC decreased trans-16:1 compared to bleomycin. Bleomycin + NAC and bleomycin + curcumin increased trans-16:1 compared to NAC. Bleomycin + curcumin significantly increased also the trans-16:1 than curcumin (<0.001). The level of the omega-6 essential fatty acid, linoleic acid (9c,12c-18:2) decreased in all of the cell groups incubated with bleomycin and its combination with the antioxidants compared to the control group (p < 0.05). The level of arachidonic acid (20:4) significantly diminished in the cells incubated with bleomycin (58%), bleomycin + NAC (%54), and bleomycin + curcumin (%51) compared to the control cells as shown in Fig. 1. The levels of the precursor of arachidonic acid in the omega-6 pathway, i.e. eicosatrienoic (8c,11c,14c-20:3) acid increased in the cells incubated with bleomycin and its combination with curcumin (p < 0.05). Fig. 2 depicts the levels of the three fatty acid families; SFA, MUFA and PUFA under the six experimental conditions.

The PUFA percentage significantly decreased in the cells incubated with bleomycin, curcumin, bleomycin + NAC, and bleomycin + curcumin compared to the control as shown in Fig. 2. MUFA percentage significantly decreased and the SFA percentage significantly increased by the combination of bleomycin + NAC compared to NAC alone group (<0.01). A 24 hour incubation with different agents caused significant changes in the membrane fatty acid composition compared to the controls. Incubations with bleomycin, curcumin, bleomycin + NAC and bleomycin + curcumin increased SFA/MUFA ratios significantly. In contrast, incubation with NAC alone did not cause any change in the membrane fatty acid family compositions as well as in SFA/MUFA ratios compared to the control. The crucial modifications in the membrane fatty acid composition were observed following incubations with
bleomycin and curcumin. The results of our lipidomic monitoring studies showed that bleomycin incubation caused a significant increase in the SFA and a remarkable decrease in the MUFA and PUFA concentrations in the testicular cancer cell membranes. Curcumin increased SFA and decreased MUFA and PUFA concentrations in the testicular cancer cell membranes similar to bleomycin. Curcumin incubation increased more trans isomer formation compared to NAC (Fig. 3A). Concurrent use of curcumin with bleomycin maintained the observed remodeling with bleomycin. Incubation with NAC was almost ineffective on the membrane fatty acid families. It is interesting to see that 9t-18:1 levels

Table 1

<table>
<thead>
<tr>
<th>FAMEa</th>
<th>Controlb</th>
<th>Bleomycinb</th>
<th>NACb</th>
<th>Curcuminb</th>
<th>Bleo + NACb</th>
<th>Bleo + Curb</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>2.3 ± 0.4</td>
<td>1.33 ± 0.1†</td>
<td>2.01 ± 0.5</td>
<td>1.75 ± 0.2</td>
<td>1.10 ± 0.9**</td>
<td>1.62 ± 0.3</td>
</tr>
<tr>
<td>16:0</td>
<td>27.4 ± 1.5</td>
<td>25.94 ± 0.2</td>
<td>26.73 ± 2.2</td>
<td>27.3 ± 0.7</td>
<td>28.32 ± 0.2</td>
<td>26.43 ± 2.1</td>
</tr>
<tr>
<td>trans 16:1</td>
<td>0.03 ± 1.0***</td>
<td>0.45 ± 0.0***</td>
<td>0.13 ± 0.0**</td>
<td>0.24 ± 0.0**#</td>
<td>0.2 ± 0.0**#</td>
<td>0.72 ± 0.0**#</td>
</tr>
<tr>
<td>6c-16:1</td>
<td>1.6 ± 0.2</td>
<td>0.80 ± 0.0*</td>
<td>1.69 ± 0.8</td>
<td>1.05 ± 0.2</td>
<td>0.57 ± 0.1**</td>
<td>1.14 ± 0.2</td>
</tr>
<tr>
<td>9c-16:1</td>
<td>1.18 ± 0.2</td>
<td>0.51 ± 0.0*</td>
<td>1.17 ± 0.6</td>
<td>0.7 ± 0.1</td>
<td>0.39 ± 0.0*</td>
<td>0.64 ± 0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>38.52 ± 3.0</td>
<td>50.38 ± 0.66</td>
<td>41.22 ± 2.88</td>
<td>47.85 ± 2.16</td>
<td>54.78 ± 1.2**</td>
<td>46.73 ± 0.66</td>
</tr>
<tr>
<td>9t-18:1</td>
<td>0.11 ± 0.0</td>
<td>2.45 ± 0.3***</td>
<td>0.37 ± 0.1***</td>
<td>0.77 ± 0.2**</td>
<td>0.48 ± 0.2**</td>
<td>2.80 ± 0.9**</td>
</tr>
<tr>
<td>9c-18:1</td>
<td>14.77 ± 2.5</td>
<td>6.85 ± 0.6*</td>
<td>13.3 ± 7.1</td>
<td>10 ± 1.3</td>
<td>4.86 ± 0.3**</td>
<td>7.79 ± 0.8</td>
</tr>
<tr>
<td>11c-18:1</td>
<td>3.99 ± 0.7</td>
<td>2.09 ± 0.1*</td>
<td>3.5 ± 1.6</td>
<td>2.71 ± 0.4</td>
<td>1.52 ± 0.0**</td>
<td>2.16 ± 0.2**</td>
</tr>
<tr>
<td>trans-18:2</td>
<td>0.02 ± 0.0</td>
<td>0.29 ± 0.0***</td>
<td>0.14 ± 0.0***</td>
<td>0.16 ± 0.0***</td>
<td>0.13 ± 0.0***</td>
<td>0.14 ± 0.0***</td>
</tr>
<tr>
<td>9c,12c-18:2</td>
<td>0.65 ± 0.3</td>
<td>0.43 ± 0.1*</td>
<td>0.51 ± 0.1*</td>
<td>0.51 ± 0.1*</td>
<td>0.29 ± 0.0</td>
<td>0.54 ± 0.0</td>
</tr>
<tr>
<td>20:0</td>
<td>0.95 ± 0.1</td>
<td>1.11 ± 0.0</td>
<td>0.97 ± 0.2</td>
<td>1.23 ± 0.0</td>
<td>1.37 ± 0.0**</td>
<td>1.17 ± 0.1</td>
</tr>
<tr>
<td>20:1</td>
<td>1.06 ± 0.3</td>
<td>2.37 ± 1.2</td>
<td>2.07 ± 0.7</td>
<td>1.47 ± 0.6</td>
<td>1.46 ± 0.6</td>
<td>2.91 ± 0.8</td>
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<tr>
<td>8c,11c,14c-20:3</td>
<td>0.46 ± 0.1</td>
<td>1.5 ± 0.9*</td>
<td>1.25 ± 0.8</td>
<td>0.58 ± 0.1</td>
<td>1.28 ± 0.1</td>
<td>2.02 ± 2.2</td>
</tr>
<tr>
<td>5c,8c,11c,14c-20:4</td>
<td>5.87 ± 2.2</td>
<td>3.4 ± 0.1*</td>
<td>4.87 ± 1.6</td>
<td>3.61 ± 0.4</td>
<td>3.18 ± 0.2*</td>
<td>2.97 ± 0.2*</td>
</tr>
<tr>
<td>trans-20:4</td>
<td>0.03 ± 0.0</td>
<td>0.09 ± 0.0</td>
<td>0.05 ± 0.0</td>
<td>0.07 ± 0.0</td>
<td>0.07 ± 0.0</td>
<td>0.22 ± 0.0</td>
</tr>
<tr>
<td>% trans MUFA</td>
<td>0.14 ± 0.0</td>
<td>2.9 ± 0.2**</td>
<td>0.50 ± 0.1*</td>
<td>1.02 ± 0.2**</td>
<td>0.68 ± 0.2*</td>
<td>3.53 ± 1.0</td>
</tr>
<tr>
<td>% trans PUFA</td>
<td>0.05 ± 0.0</td>
<td>0.38 ± 0.0**</td>
<td>0.19 ± 0.0**</td>
<td>0.23 ± 0.1*</td>
<td>0.20 ± 0.0**</td>
<td>0.36 ± 0.0</td>
</tr>
<tr>
<td>% total trans</td>
<td>0.19 ± 0.0</td>
<td>3.28 ± 0.2**</td>
<td>0.69 ± 0.1**</td>
<td>1.24 ± 0.1</td>
<td>0.88 ± 0.3**</td>
<td>3.89 ± 1.0**</td>
</tr>
<tr>
<td>% trans/cis 18:2</td>
<td>3.66 ± 0.2</td>
<td>68.62 ± 8.3***</td>
<td>26.64 ± 9.3***</td>
<td>31.02 ± 6.3***</td>
<td>45.04 ± 17.1***</td>
<td>26.01 ± 4.7***</td>
</tr>
<tr>
<td>% trans/cis 20:4</td>
<td>0.61 ± 0.2</td>
<td>2.66 ± 0.1*</td>
<td>1.15 ± 0.7</td>
<td>1.83 ± 0.5*</td>
<td>2.38 ± 1*</td>
<td>7.52 ± 1.2*</td>
</tr>
<tr>
<td>SFA</td>
<td>70.22 ± 1.8</td>
<td>78.76 ± 0.9</td>
<td>70.94 ± 3.3</td>
<td>78.13 ± 2.4##</td>
<td>85.57 ± 1.3##</td>
<td>79.05 ± 3.0##</td>
</tr>
<tr>
<td>MUFA</td>
<td>22.61 ± 2.3</td>
<td>12.63 ± 0.4*</td>
<td>21.73 ± 4.4*</td>
<td>15.92 ± 2.2*</td>
<td>8.81 ± 0.9##</td>
<td>14.64 ± 0.4##</td>
</tr>
<tr>
<td>PUFA</td>
<td>6.98 ± 0.6</td>
<td>5.32 ± 0.8*</td>
<td>6.64 ± 1</td>
<td>4.70 ± 0.6*</td>
<td>4.75 ± 0.1*</td>
<td>5.52 ± 2*</td>
</tr>
<tr>
<td>SFA/MUFA</td>
<td>0.17 ± 0.0</td>
<td>0.62 ± 0.3*</td>
<td>0.59 ± 0.2</td>
<td>0.49 ± 0.2##</td>
<td>9.71 ± 1.2##</td>
<td>5.19 ± 0.2##</td>
</tr>
</tbody>
</table>

The values are reported as the relative percentage (% rel.) of the total fatty acid peak areas detected in the GC analysis. The values are given as mean ± SD; n is the number of the tests repeated.

a Fatty acid methyl ester (FAMEs) levels were determined performing lipid extraction, derivatization, and GC analysis.

b The identification of the peaks was performed using authentic standards and the peaks identified accounted for 98% of the total peaks.

* Values significantly different from the control p < 0.05.
** Values significantly different from the control p < 0.01.
*** Values significantly different from the control p < 0.001.
### Values significantly different from the control p < 0.0001.

Values significantly different from the bleomycin p < 0.05.

# Values significantly different from the bleomycin p < 0.01.
## Values significantly different from the bleomycin p < 0.001.

Values significantly different from the NAC p < 0.05.

Values significantly different from the NAC p < 0.01.

Values significantly different from the NAC p < 0.001.

Values significantly different from the curcumin p < 0.05.

Values significantly different from the curcumin p < 0.01.

Values significantly different from the curcumin p < 0.001.

Values significantly different from the bleomycin + NAC p < 0.05.

Values significantly different from the bleomycin + NAC p < 0.01.

Values significantly different from the bleomycin + NAC p < 0.001.

Values significantly different from the bleomycin + curcumin p < 0.05.

Values significantly different from the bleomycin + curcumin p < 0.01.

Values significantly different from the bleomycin + curcumin p < 0.001.

Values significantly different from the bleomycin + curcumin p < 0.0001.

Fig. 1. Main membrane fatty acid composition of the NTer-a-2 cells following incubations with bleomycin, NAC, curcumin, bleomycin + NAC, and bleomycin + curcumin for 24 h. Values are mean ± SD. Values significantly different from the control p < 0.05. Values significantly different from the control p < 0.01. Values significantly different from the bleomycin p < 0.05. Values significantly different from the bleomycin p < 0.01. Values significantly different from the bleomycin p < 0.001. Values significantly different from the NAC p < 0.05. Values significantly different from the NAC p < 0.01. Values significantly different from the NAC p < 0.001. Values significantly different from the curcumin p < 0.05. Values significantly different from the curcumin p < 0.01. Values significantly different from the curcumin p < 0.001.
were significantly higher in the bleomycin (22 fold), curcumin (7 fold) and bleomycin + curcumin (25 fold) groups compared to the control group (Fig. 3A). NAC, curcumin and bleomycin + NAC significantly decreased 9t-18:1 levels compared to the bleomycin alone group. Trans-20:4 arachidonic acid isomers were also produced, although to a minor extent reaching the highest level in the bleomycin + curcumin (7 fold) group compared to the control, bleomycin and curcumin groups (Fig. 3B).

3.2. MAPK pathway member levels

Absorbances of phospho-p44/42 MAPK (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), MEK1, phospho-MEK1 (Ser217/221), SAPK/JNK and phospho-SAPK/JNK (Thr183/Tyr185) in the cell lysates are shown in Fig. 4. Ntera-2 cells were incubated with bleomycin, NAC, curcumin, bleomycin + NAC and bleomycin + curcumin for 24 h. As shown in Fig. 4, bleomycin, curcumin and their combination led to the activation of JNK and p38 and inactivation of phospho-MEK and phospho-ERK (p < 0.01). In contrast to curcumin, NAC decreased the activation of p38 and JNK (p < 0.01) which were upregulated by bleomycin and led to enhancement in phospho-MEK and phospho-ERK levels. Incubation with NAC alone did not cause any change in p38-MAPK and JNK levels compared to the control cells. We did not observe any difference in the dephosphorylated-JNK and dephosphorylated-MEK levels among the groups incubated with different agents. Bleomycin, curcumin or their combination led to a decrease in phosphorylated-MEK and phosphorylated-ERK levels. Bleomycin and curcumin synergistically reduced phosphorylated-MEK and phosphorylated-ERK levels. In contrast, concurrent incubation of bleomycin with NAC caused an enhancement in phosphorylated-MEK and phosphorylated-ERK levels compared to cells incubated with bleomycin alone.
3.3. EGFR levels

As shown in Fig. 5, 24 h treatment of NTera-2 cells by bleomycin decreased the level of EGFR in testicular cancer cells. Curcumin or its combination with bleomycin also led to a decrease in EGFR levels. NAC treatment was also effective in decreasing the EGFR level.

4. Discussion

We reported effects of bleomycin on apoptosis in the NTera-2 testicular cancer cells in our previous publications, which lack information on membrane lipidome changes [22–26]. Therefore, we aimed to determine bleomycin induced changes in the NTera-2 cell membrane fatty acid profiles due to its free radical mediated mode of action. We also searched the effects of two potent antioxidants, curcumin and NAC namely, on NTera-2 membrane fatty acid profiles in the context of possible protection from lipid profile changes. Bleomycin may activate one of the two reactivity pathways as shown in Fig. 6.

Pathway A is the isomerization pathway associated with the known free radical mediated cis-trans double bond isomerization induced by the sulfur-centered radicals generated by bleomycin. This isomerization process occurs “in situ” provided that the free radical species are able to
reach the double bonds located in the hydrophobic region of the bilayer. Pathway B is the oxidative pathway associated with the known mode of action of bleomycin affecting oxidation of DNA and membrane fatty acids, especially causing their release from the membrane. In addition to the well-known free radical damage caused by bleomycin on the sugar units of the DNA, this is the first time that a parallel damage on membrane lipid geometry has been reported in the literature. This might be an adjuvant effect on the cell toxicity brought about by bleomycin. Arachidonic acid is the fundamental component of the membrane lipidome of cells, contributing to the lipid assembly and functioning [27]. Fatty acids available for the lipid remodeling play an important role in the membrane lipid asset and its properties. Changes in the membrane fatty acid composition alter membrane fluidity and permeability [28]. In our study, we observed that arachidonic acid was significantly affected by bleomycin as well as its combination with curcumin and with NAC (Table 1), whereas arachidonic acid levels were not affected in the cells incubated with either NAC or curcumin.

We found decreases in the PUFA family members (18:2 and 20:4) which might be due to the oxidative damage that is generated by the mode of action of bleomycin. Our results are in accordance with similar decreases demonstrated in model membrane studies [8]. It is interesting for us to observe that the omega-6 eicosatrienoic acid (20:3) was positively affected by bleomycin (Table 1). It is possible to envisage that under bleomycin incubation path A (Fig. 6) significantly involves oleic acid residues to be transformed to the corresponding trans isomers i.e. elaidic acid (Fig. 3A) by 30% amount. Cis to trans isomerization is integrated to dietary conditions which are part of the profound remodeling as a consequence of the drug administration. In contrast, under the same condition the behavior of arachidonic acid residues is affected by the oxidative path B (Fig. 6).

The effects of bleomycin on the fatty acid profiles (SFA, MUFA and PUFA) in membranes were not reverted by concurrent incubation with NAC or curcumin, although it is worth noting that incubation with NAC alone did not cause any profound effect compared to incubation with curcumin alone (Fig. 2). Saturated and monounsaturated fatty acids are produced endogenously in eukaryotic cells and the increased production is associated with a malignant and aggressive phenotype [29].

In this study, we determined also the level of trans isomers of oleic and arachidonic acids occurring due to the free radical stress generated by bleomycin and searched the effects of two different antioxidants on the trans fatty acids. We observed the highest level (22 fold) of elaidic acid (9t-18:1) in the bleomycin incubated cell group. Concurrent incubation of bleomycin with NAC incubation decreased elaidic acid by 4.4 fold. In our previous study published, we found that the co-incubation of bleomycin with NAC diminished the increased trans lipid isomers (9t-18:1 and trans-20:4) caused by incubation with bleomycin alone [8]. NAC might interact with the ROS species generated by bleomycin and NAC generates equilibrium between O- and S-centered radicals [13]. In contrast to NAC, bleomycin and curcumin synergistically increased trans lipid isomers (9t-18:1 and trans-20:4) in testicular cancer cell membranes (Fig. 3A and B). In our previous publication, we reported that curcumin enhanced the antioxidant capacity and significantly decreased all oxidative stress markers (LPO, 8-isoprostane, protein carbonyl content, TBARS levels) in the Ntera-2 cells incubated with bleomycin [22]. Therefore, we may conclude that curcumin diminishes bleomycin-induced oxidative stress in the Ntera-2 cells without being able to affect or suppress trans fatty acid formation caused by bleomycin. Elucidation of the behavior of curcumin will be provided by the ongoing experimental studies regarding the multiple roles played by this component [30,31]. It is interesting to note that curcumin alone induced more trans isomer formation than NAC, thus indicating a specific reactivity involving the generation of radical species [32]. The endogenous free radical mediated cis–trans lipid isomerization can give an estimation of the cellular stress referred to the radical species generated under the specific conditions within the cells [8]. In our previous publication involving biomimetic and cell culture models of bleomycin treatment, we showed that an extensive peroxidation process occurs in the PUFA residues, therefore they are rapidly consumed and the isomerization extent cannot be determined [8]. Determining the membrane fatty acid changes fully under the effect of chemotherapeutic agents needs further studies.

Our results proved that bleomycin, curcumin and NAC induced a profound remodeling of membrane lipidome profile in the human testicular cancer cell line. The fatty acid changes observed in our study confirm that oxidative stress pathway is accompanied by an extensive membrane remodeling which can modify the cellular signaling pathways. In order to clarify this possibility, we investigated the MAPK pathway and EGFR activity. There is no study in the literature investigating the effects of bleomycin and concurrent use of bleomycin with an antioxidant on MAPK signaling pathway and its relation with lipidome monitoring in testicular germ cell tumors.

MAPKs are important mediators involved in the intracellular network of interacting proteins that transduce extracellular signals to intracellular responses. There are three well-defined subgroups of MAPKs which are extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. Each subgroup of MAPKs is activated through a cascade of sequential phosphorylation events, beginning with the activation of MAPK kinase kinases (MAP3K) which is induced by EGFR [33]. Activation of the ERK pathway is initiated by a ligand binding to receptor tyrosine kinases at the plasma membrane, such as EGFR, leading to activation of MAP3K, which phosphorylates

![Fig. 6. Reactivity pathways of membrane lipids (A) cis–trans isomerization of membrane fatty acids. (B) Oxidation of membrane fatty acids.](image-url)
and activates MEK, followed by phosphorylation and activation of ERK. Consistent with their known pro-survival role, ERK was shown to have proliferative and protective effects on cells exposed to oxidative stress [34]. JNK and p38 are classified together as stress-responsive kinases, which are involved in cell death [35].

In our study, we observed an interesting difference between the effects of two antioxidants incubated with bleomycin. Curcumin and bleomycin combination led to the activation of JNK and p38 and inactivation of phospho-MEK and phospho-ERK, whereas NAC and bleomycin combination decreased the activation of p38 and JNK and led to enhancement in phospho-MEK and phospho-ERK levels. Our results are in accordance with Yang et al. that reported an activation of JNK and inactivation of ERK after bleomycin A5 treatment [36] and with Lee et al. who showed that curcumin increased p38 and JNK activation [37]. Inhibition of p38 and JNK was reported to result in elevated survival of laryngeal carcinoma cells incubated with bleomycin [38]. It has been already underlined that plasma membrane fulfills a critical role in the propagation of tumorigenic or survival signals, whereby changes in membrane lipid content can either activate or silence relevant pathways. Bolognesi et al. reported that lipid composition of neuroblastoma cell membranes changes according to the fatty acid supplementation and has a clear relationship with cell viability [16]. The numerous statistically significant interactions between dietary factors, including antioxidant vitamins, and MAPK-signaling pathway members were observed after adjustment for multiple comparisons suggesting that dietary factors are involved in activation and regulation of the key MAPK-signaling pathways [39]. In our study bleomycin and antioxidants, alone or in combination, induced modification in the lipid content of NTER-2 cancer cells membranes, influencing the oxidation and isomerization processes which are involved in the lipid remodeling of NTER-2 cell membranes, thus activating p38 and JNK, the apoptotic MAPK pathway members, which in their turn inactivated the ERK and MEK survival MAPK family members.

Overexpression of EGFR has been demonstrated in a variety of cancers, including testicular germ cell tumors reported by Hechelhammer et al. [40]. EGFR becomes one of the potential targets for treating cancer. Our results are in accordance with Gong et al. that showed the downregulation of EGFR by bleomycin A5 in esophageal cancer cells [41], and with Lai et al. that reported the same effect by curcumin in breast cancer cells [42]. EGFR downregulation by bleomycin can be connected to the profound remodeling observed within the membrane lipid asset. This result evidences the impact on different cell metabolic pathways that is nowadays seen as a way to improve the responsivity to chemotherapy [43,44].

5. Conclusion

In conclusion, membrane lipidome monitoring is a potent tool in cell biology experiments for the characterization of membrane fatty acid organization. The results of this work provide important molecular evidence that in cells treated with bleomycin and antioxidants, alone or in combination, lipid isomerization and oxidation occur which induce a profound lipid remodeling and affect signaling pathways. These results can inspire further research to clarify the effect of chemotherapeutics on the phospholipid remodeling in cell membranes in order to develop a multitargeted drug design, including nutritional factors, to improve the success in inducing cancer cell death. Thus, the results of this study could be used to design more effective strategies for cancer treatment.

Transparency document

The Transparency document associated with this article can be found, in the online version.


A. Cort et al. / Biochimica et Biophysica Acta 1858 (2016) 434–441

441