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ARTICLE TYPE

Bleomycin-induced *trans* lipid formation in cell membranes and in liposome models†

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Cell cultures of NTera-2 cells incubated with bleomycin and liposomes as biomimetic models of cell membranes were used for examining novel aspects of drug-metal induced reactivity with unsaturated lipids under oxidative conditions. In cell culture, bleomycin was found for the first time to cause the formation of *trans* fatty acids. The chemical basis of this transformation was ascertained by liposome experiments, using bleomycin-iron complexes in the presence of thiol as reducing agent, that by incubation at 37 °C gave rise to the thiyl radical-catalysed double bond isomerisation of membrane phospholipids. The effect of oxygen and reagent concentrations on the reaction outcome was studied. An interesting scenario of free radical reactivity is proposed, which can be relevant for the role of membrane lipids in antitumoral treatments and drug carrier interaction.

Introduction

The glycopeptide antibiotic bleomycin (BLM), used in chemotherapy against neck, head and testicular cancers as well as Hodgkin lymphoma, has a long-studied mechanism of action, based on the formation of a complex with metals, such as iron, which under aerobic conditions generates reactive oxygen species (ROS) thus causing DNA damage.[1-3] Figure 1 summarizes the BLM behaviour in the presence of Fe(II) and O_2, to produce activated forms of the drug and hydroxyl radicals.[2,4,5] In the presence of polyunsaturated fatty acids (PUFA) their consumption via peroxidation is known to occur.[1,3,6]

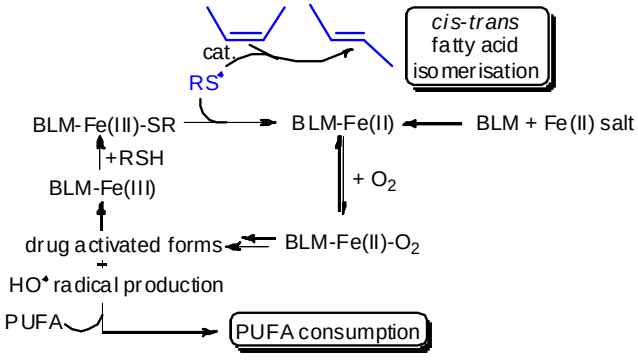

Fig. 1 Reactivity pathways of the BLM-iron complexes with unsaturated fatty acids. In the presence of oxygen, drug activated forms and hydroxyl radicals can be formed and produce PUFA consumption; in the presence of thiols, the reduction of Fe(III) to Fe(II) occurs with formation of thiyl radicals, which can catalyse the *cis-trans* double bond isomerisation of unsaturated fatty acid residues (in blue colour).

So far, the molecular basis of the drug activity are focused on DNA damage especially at mitochondria level, determining pulmonary side toxicity[7] and motivating interest in possible antioxidant combinations.[8] On the other hand, redox-based antioxidants, such as thiols, are known to increase the drug effectiveness, their effect being explained by the recycling of BLM-Fe(III) to BLM-Fe(II) (see Figure 1).[9] During the reductive process the production of thiyl radicals can occur, which are known to react with double bonds and catalyse the isomerisation of the natural *cis* unsaturated fatty acids to the corresponding geometrical *trans* isomers (see Figure 1, blue colour). This reaction can involve monounsaturated fatty acids (MUFA) and PUFA and is particularly relevant in biological systems, when affecting membrane lipids.[10] It is not known whether lipid isomerisation is connected with the effects of metals and generation of reactive oxygen species that cause cell death.[11] The perturbation produced by the unnatural *trans* geometry to membrane properties[12,13] is a dramatic event for the eukaryotic cell survival. Its potential as a strategy for antitumoral activity has not yet been evaluated.

It is worth noting that drug-metal complex chemistry provides efficient catalysis for C-H activation and C=C oxidation,[14] whereas the reactivity towards double bonds and the isomerization represent new aspects to be developed.

After our preliminary experiments evidencing the *trans* fatty acid formation by bleomycin treatment,[15]

we thought to apply an original chemical biology model for the investigation of the drug-induced chemical reactivity by combining the data of the cell culture experiments with biomimetic chemistry in liposome vesicles. Our aim was to furnish a comprehensive scenario of the drug behaviour with membrane fatty acids. Here we report the fatty acids of human testicular cancer cell membranes (NTera-2) incubated with bleomycin under standard conditions, paralleled with those forming liposomes under similar incubation conditions. Using liposome models more detailed chemical information could be gathered varying the reaction conditions, for the oxygen, drug and thiol concentrations. We anticipate that bleomycin-induced thiyl radical reactivity was discovered causing *cis-trans* lipid isomerization, and the chemical behaviour of membrane lipids in liposomes helped to understand also the outcome in the cellular model.

Results and Discussion

Cell cultures

The fatty acid composition of human testicular cancer cell membranes (NTera-2) was examined before and after 24 h incubation with bleomycin. To our knowledge, there is only one study on the fatty acid composition of this cell type,[16] and the fatty acid profiles of testicular cancer cells in response to antitumoral drugs are unknown. Bleomycin was used at the IC_{50} dose of 400 μg/mL. Details of the incubation and fatty acid composition of cell membranes are given in the Materials and Methods section as well as in Supplementary Information. Briefly, after incubation cells were thoroughly washed in order to obtain samples of 4×10^6 cells suspended in phosphate buffer, followed by membrane phospholipid isolation, transformation of the fatty acid residues in fatty acid methyl esters (FAME) and gas chromatographic (GC) analysis, in order to examine the fatty acid moieties detached from the phospholipids by known methods.[17-19]

Table 1 shows the fatty acid residues found in treated *versus* control cells. Results are reported as percentage of fatty acids over the total fatty acid content and as means ± sd with statistical significance of n repetitions. In cultured cells a considerable percentage of *trans* MUFA isomers (*trans*-16:1 and *trans*-18:1), identified by appropriate libraries,[20,21] was found, equivalent to a 24% isomerisation of the total MUFA content. A significant diminution of the corresponding *cis*-MUFA (*cis*-16:1 and *cis*-18:1) was correspondently observed. For the omega-6 PUFA, the diminution of *cis* isomers (18:2 and 20:4 with $p<0.05$) was also accompanied by the *trans* PUFA isomers formation, identified as mono-*trans* linoleic acid isomers,[18] (see Supplementary Information, Figure 3s) reaching ca. a 20% isomerisation of the 18:2 content, together with mono-*trans* arachidonic acid isomers,[17] reaching ca. 2% of the total 20:4 content.

The formation of *trans* isomers was not known previously in the mechanism of action of antitumoral drugs, and it is worth underlining that membrane lipid reactivity with change of naturally occurring *cis* geometry is a new aspect of the free radical-based antitumoral drug effect. Other details connected with the fatty acid transformations are reported in Supplementary Information, which showed also a profound membrane lipid remodelling with change of the membrane fatty acid asset.

This behaviour inspired a biomimetic chemistry study in membrane models, constituted of liposomes containing MUFA and PUFA residues, using bleomycin as complex with iron and a reducing agent such as thiols, to create the recycling of the redox state of the complexes, which is at the basis of the activity as depicted in Figure 1.

Set-up of the liposome experiments

Unilamellar liposomes (Large Unilamellar Vesicle by extrusion technique, LUVET) were designed since they represent the best biomimetic model for investigating cell membrane phospholipid behaviour. Experiments were carried out with liposomes of two different lipid compositions at 1 mM concentration: the first one made of the synthetic phospholipid, 1-palmitoyl-2-oleoyl phosphatidyl choline (POPC), whereas the second one made of natural phospholipids such as soybean lecithin, containing different percentages of saturated, mono- and polyunsaturated (MUFA and PUFA) fatty acids (see Supplementary Information). The reactivity of unsaturated fatty acids, studied by us in other biomimetic models in the presence of thiols,[17,18,22] can involve MUFA and PUFA residues: the MUFA moiety of oleic acid (9*cis*-18:1) is not so prone to oxidation as PUFAs are, whereas it can be the target of the thiyl radical catalysed-isomerisation, being transformed into elaidic acid (9*trans*-18:1). On the other hand PUFA residues of soybean lecithin liposomes can be partitioned between oxidative and isomerisation pathways. In particular, linoleic acid (9*cis*,12*cis*-18:2), which is the most representative PUFA in the lecithin (17.5% of the total fatty acid composition), can give three geometrical *trans* isomers: 9*trans*,12*cis*-18:2, 9*cis*,12*trans*-18:2, 9*trans*,12*trans*-18:2, which can be clearly separated by GC analysis (see Figure 3s in Supplementary Information). We were aware of lipid oxidation in the mechanism of bleomycin, as depicted in Figure 1, and we measured it by fatty acid consumption after the reactions, using the GC calibration curves *vs.* the saturated fatty acid residue of palmitic acid (hexadecanoic acid, 16:0). This fatty acid is present in both POPC and soybean liposomes and can act as

internal standard, being unreactive under free radical conditions. MUFA and PUFA yields were calculated from their peak areas in the GC analysis, which is the gold standard for fatty acid quantification. Control experiments included also the liposome reaction in the presence of the metal-thiol complex without bleomycin, in order to better estimate the drug contribution to lipid reactivity.

Table 1 Membrane fatty acid composition of NTera-2 cells (reported as fatty acid methyl esters (FAME, % rel. ± sd) incubated for 24 h with bleomycin (at IC_{50} = 400 μg/mL).

FAME [a,b]	Control (*n*=8)	Bleomycin (*n*=6)
14:0	2.4 ± 0.4	1.3 ± 0.1*
16:0	28.0 ± 0.7	27.1 ± 1.7
***trans*-16:1c**	0.1 ± 0.04	0.3 ± 0.2***
6*cis*-16:1	2.0 ± 0.5	0.8 ± 0.04*
9*cis*-16:1	1.4 ± 0.4	0.5 ± 0.04*
18:0	35.8 ± 5.9	50.4 ± 0.6*
9*trans*-18:1	0.1 ± 0.03	2.2 ± 1.1***
9*cis*-18:1	15.8 ± 2.5	6.8 ± 0.6*
11*cis*-18:1	4.3 ± 0.7	2.1 ± 0.1*
mono-*trans* 18:2[c]	0.3 ± 0.3	0.1 ± 0.0
18:2 ω6	0.7 ± 0.2	0.4 ± 0.05*
20:0	0.8 ± 0.3	1.3 ± 0.02*
20:1	0.9 ± 0.2	2.4 ± 1.2
20:3 ω6	0.4 ± 0.1	1.5 ± 0.9*
mono-*trans* 20:4[c]	0.04 ± 0.03	0.1 ± 0.03
20:4 ω6	6.8 ± 2.2	3.4 ± 0.1*

The values are reported as relative percentage (% rel) of the total fatty acid peak areas detected in the GC analysis (recognition > 98% of the peaks). The values are given as mean ± sd. n is the number of the tests repeated. Statistical comparisons were conducted using the SPSS software, version 13.0 (Chicago, IL), using t-test for group comparisons. Statistical significance was based on 95% confidence limits (p≤0.05). Comparison of the non-parametric data among the groups was performed using Mann-Whitney U test.

*[a] FAME (fatty acid methyl ester) were determined performing membrane phospholipid extraction, derivatization, and GC analysis. [b] The identification of the peaks was performed using authentic samples. [c] The sum of the geometrical isomers is considered, i.e., 6-trans and 9-trans for 16:1,[21] 9cis,12trans-18:2 and 9trans,12cis-18:2 for 18:2,[18] the four mono-trans isomers of 20:4.[17] * Values significantly different from the control, p<0.05. *** Values significantly different from the control, p<0.001.*

In the liposome model design 2-mercaptoethanol was chosen as the thiol for its amphiphilic character, in order to diffuse freely in the heterogeneous environment of liposome suspension and interact with the membrane compartment. Other thiols, such as cysteine, glutathione and N-acetylcysteine, were used in comparative experiments under aerobic conditions to estimate the effect of different partition coefficients (see below).

The bleomycin-iron complex was prepared as described in the literature,[23] directly in the liposome suspension, briefly by adding bleomycin sulphate, ferrous ammonium sulphate (10 μM each) and thiol at the desired concentration (10 and 100 μM) to the vesicle aqueous suspension (1 mM), keeping the test tubes under controlled aerobic conditions (mixture with nitrogen at 5, 10 and 20% oxygen) or under nitrogen (0% oxygen). The concentration of bleomycin-iron complex was chosen as the one reported in lipid peroxidation experiments of chemical and biological models.[3,9] The steps of formation of the iron-drug complex with oxygen, and the role of thiol (RSH) with the formation of the Fe(III)-bleomycin-thiol complex occur as depicted in Figure 1.[1-5] UV-*vis* spectra were carried out to ascertain the formation of the absorbance band of the BLM-Fe-SR complex at 580 nm, as described in the literature (see Supplementary Information).[9]

MUFA-containing vesicles

The results for the reaction of POPC vesicles are summarized in the graph of Figure 2 (green and violet bars). The *trans* isomer formation was detected after 24 h incubation with 10 μM bleomycin-iron(II) salt and 10 μM 2-ME at 37 °C, under the three different oxygen concentrations used (20%, 10% and 5%). The BLM-iron-thiol triad caused lipid isomerisation, inversely correlated with the oxygen presence. For example, in POPC liposomes oleic acid isomerisation went from ca. 27% *trans* isomer formation with 5% oxygen to ca. four-fold less in the presence of 20% oxygen. The same experiments were run without bleomycin, in order to define the contribution of drug reactivity to the lipid isomerisation. When only the iron-thiol complex is present, a decisive decrease in the isomerisation efficiency occurred, which remains inversely correlated with oxygen concentration. In this case *trans* isomers were formed in POPC in the range of 5%-1.2% going from 5% to 20% oxygen (Figure 2). Comparing the presence and the absence of the drug under the same oxygen conditions, a 5.5 to 7-folds decrease of isomerisation can be observed. Therefore, bleomycin gives a substantial contribution to the generation of thiyl radical species and *trans* fatty acid formation under aerobic conditions.

It is worth noting that the isomerisation of double

bonds requires catalytic thiyl radicals, so that appreciable quantities of *trans* isomers are formed also at very low radical concentration.[10] For this reason *trans* lipids can be used as sensitive markers of thiyl radical generation in complex systems. Using the palmitic acid moiety of phospholipids as internal standard, it was also possible to ascertain that the molarity of the 18:1 substrate did not change from the initial one (1 mM), showing that no MUFA consumption occurs by oxidative process under our experimental conditions.

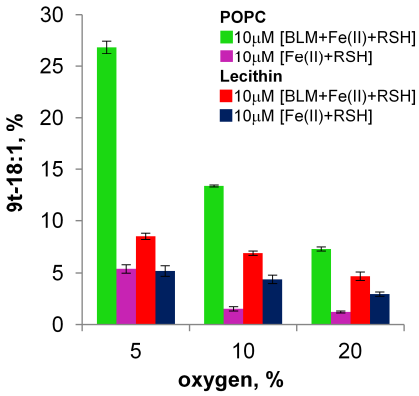

Fig. 2 *Trans* 18:1 isomer formation (% rel., see Table 1s in Supplementary Information) in POPC or lecithin vesicles treated with 10 μM Fe(II) and 10 μM 2-mercaptoethanol in the presence or absence of BLM, under variable oxygen concentrations and reagents. The mean ± sd values were calculated from triplicates of the same experiment (see Table 1s in Supplementary Information).

The effect of thiol concentration, which in living organisms can reach mM concentrations, was assayed by increasing 2-ME by ten-folds (100 μM), keeping the presence of BLM-Fe(II) at 10 μM concentration each (data not shown). Under this condition, *trans* isomer formation in POPC vesicles dropped (ca 3.7-1.1% depending on the oxygen concentration). On the other hand, in the absence of bleomycin, the increased concentration of thiol (100 μM) in the presence of 10 μM Fe(II) in POPC vesicles produced a higher isomerisation percentage (ca. 7.6-4% *trans* isomer depending on oxygen concentration). Therefore, in MUFA-containing liposomes the isomerisation process under aerobic conditions was more effective with the drug and 10 μM thiol concentrations (see Figure 2), than with a higher thiol concentration, which is even insensitive to BLM. Obviously, in the absence of thiol no isomerisation was detected. The reasons of this behaviour and the connection of bleomycin reactivity with the variation of local co-reactant concentrations are matter of further work, also in view of an extrapolation to factors altering the drug activity *in vivo*.

In POPC vesicles the efficiency of other thiol compounds was also tested (data not shown). Cysteine and glutathione, which are hydrophilic thiols, did not afford *trans* isomer. This can be easily explained on the basis of no thiol partition in membrane bilayer, as already reported.[18] The less hydrophilic N-acetylcysteine (10 and 100 μM concentrations) gave a low but detectable *trans* isomer formation after 24 h of incubation (up to 3-4% *trans* 18:1, data not shown). These results are intriguing, when paralleled with the cell models showing an efficient formation of *trans* isomers in NTera-2 cells treated with bleomycin and without specific thiol addition. These results suggest further investigations toward the individuation of possible biological candidates for the generation of thiyl radicals by drug interaction *in vivo*.

MUFA- and PUFA-containing liposomes

Liposomes were also prepared with soybean lecithin and used for analogous experiments with bleomycin. In such MUFA and PUFA-containing liposomes the oleic and linoleic transformations to the corresponding geometrical isomers (9*trans*-18:1 and *trans* 18:2 isomers) were followed-up, estimating also the PUFA consumption by oxidative degradation (see Figure 3s in Supplementary Information). The BLM-iron-thiol triad, each at 10 μM concentration, produced *trans* 18:1 isomer (for example, ca. 8.5% at 5% oxygen; see Figure 2, red bars). In these experiments PUFA content strongly diminished after the first 2 h incubation (consumption >92%, not shown), thus isomerization of linoleic acid residues was not detectable. The PUFA oxidative pathway depicted in Figure 1 is clearly operative. Further experiments were carried out to evaluate the roles of bleomycin and oxygen in the reaction outcome. Under aerobic conditions and in the absence of bleomycin, PUFA consumption generated by metal and thiols was always high (>91%), whereas the 18:1 isomerisation was less efficient (for example, ca. 4-5% at 5% oxygen, see Figure 2, blue bars).

The role of oxygen was exploited under anaerobic conditions (with nitrogen at 0% oxygen) in lecithin liposomes treated with different conditions of drug and thiol, and the results are reported in Table 2. After 24 h incubation with 10 μM drug-thiol-iron triad, the formation of *trans* MUFA and PUFA isomers (ca. 2.5% each) was observed, and was coupled with PUFA consumption of ca. 14%. The same experiment repeated without bleomycin, with thiol-metal complex, led to an even lower isomerisation (<1%) and no PUFA consumption. From these experiments it can be highlighted that bleomycin is the responsible of PUFA consumption also when oxygen is absent. The effect of higher thiol concentration was tested with 100 μM concentration

and Table 2 shows that under anaerobic conditions the PUFA reactivity is almost similarly partitioned between isomerisation and consumption, with comparable results in the presence and absence of the drug. Therefore, at higher thiol concentration the BLM effect for isomerisation can be considered negligible, whereas the system ensures that thiyl radical generation occurs for the consistent formation of *trans* 18:1 and 18:2 isomers.

A more detailed study was carried out on the structure of the fatty acid isomers produced in the experiments reported in Table 2. It was gratifying to see that the three *trans* 18:2 isomers were formed in the liposomes at different percentages and in the order: 9*trans*,12*cis* > 9*trans*,12*trans* > 9*cis*,12*trans*. This shows that the isomerisation occurs as a regioselective process, which is expected as consequence of the supramolecular arrangement of fatty acids in the bilayer (see Supplementary Information, Figures 3s and 4s).[17,18]

In anaerobic experiments the PUFA consumption (reaching 60% at higher thiol concentration), not attributable to oxidative processes, was also preliminary investigated examining the crude reaction mixture. The phospholipid extract was transformed into the corresponding FAME derivatives and analysed by GC/MS under appropriate conditions. The presence of thiol-linoleic acid adducts was ascertained by comparison with reference compounds (see Supplementary Information, Figure 5s).[18]

These results indicate an intricate relationship between the drug and thiol compounds to be extrapolated to the variability of drug effectiveness under different nutritional and oxygen conditions, that can influence the response of cancer tissues.

Table 2 Reactivity of 18:1 and 18:2 residues and PUFA consumption in lecithin liposomes under anaerobic conditions (nitrogen with 0% oxygen) incubated for 24 h at 37 °C with 10 μM Fe(II) salt, and in the presence/absence of bleomycin (BLM) and 2-mercaptoetanol (2-ME). The mean ± sd values were calculated from triplicates of the same experiment.

BLM μM	2-ME μM	*Trans* 18:1 %[a]	*Trans* 18:2* %[a]	PUFA consumpt.* *% [b]
10	10	2.3 ± 0.1	2 ± 0.2	14 ± 0.2
0	10	0.9 ± 0.1	0.7 ± 0.1	0
10	100	30.4 ± 0.9	41.2 ± 0.8	65.2 ± 0.7
0	100	41.4 ± 1.0	46.6 ± 0.8	62.3 ± 0.9

*[a]Each trans isomer is reported as percentage relative to the sum of the corresponding cis and trans isomers found as peak of the GC analysis of the corresponding FAME. For the isomer recognition see Table 1. [b]The PUFA consumption is calculated by using the linoleic acid GC peak areas (cis + trans isomers) before and after 24 h incubation calibrated vs. palmitic acid used as the internal standard (see Supplementary Information). *See Figure 4s in Supplementary Information for the GC analysis relative to the identification of each trans 18:2 isomers. **See Figure 5s in Supplementary Information for the identification of thiol-linoleic acid adducts in the reaction mixture.*

Conclusions

The general aim of our study was to get molecular insights of the drug-metal complexes acting via free radical production, examining a well known chemotherapeutic agent, such as bleomycin, and considering the novel aspect of membrane unsaturated lipid reactivity via double bond isomerization. Although bleomycin-iron complex is reported to interact with cell membranes by a fluorescent probe technique in intact cells,[24] this is the first report that highlights membranes as a relevant site of drug activity other than DNA to be explored.

Using cell and liposome models, it was observed that in both cases under aerobic conditions MUFA and PUFA residues partitioned between isomerisation and consumption processes, with the only difference that in living cells the final fatty acid composition is an effect of the phospholipid remodelling process associated to oxidative stress.[25]

As chemical biology model for antitumoral strategies, liposomes highlighted the role of cell membranes, which are not spectators but important targets of the drug effect, with synergic roles for chemotherapeutic effects. Indeed, fatty acid recruitment and membrane formation attract a lot of interest in cancer, and in this context the loss of the natural *cis* geometry and the oxidation-induced lipid remodelling are worth of deeper studies in antitumoral strategies. Furthermore, the interaction between drugs and lipids can be suggestive of novel aspects of chemical reactivity for liposome carriers when circulating *in vivo*.

Materials and methods

The materials and general methods used in this work are described in Supplementary Information.

Cell cultures, membrane phospholipid extraction and fatty acid analysis

NTera-2 human testicular germ cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated

fetal bovine serum (FBS), 100 units/mL of penicillin and 100 µg/mL of streptomycin in a 5% CO_2 atmosphere at 37 °C as described in the manual of the cell line bank. We used the half maximal inhibitory concentration (IC_{50}) of bleomycin (400 µg/mL) for 24 h incubations, determined in our previous studies and published.[26]

After incubation 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. The pellet was resuspended in pure water and centrifuged for a second washing, then, dissolved in 2:1 chloroform:methanol to extract phospholipids. The phospholipid extract was treated with 0.5M KOH in MeOH for 10 min at room temperature to convert fatty acid residues of phospholipids into their corresponding fatty acid methyl esters (FAME). After this transesterification step, FAMEs were extracted with *n*-hexane, and analyzed by gas chromatography (GC). Fatty acids (including *cis* and *trans* isomers) were identified by comparison with standard references used in previously reported studies.[17-21] Fatty acid compositions are calculated as relative percentages over the total fatty acid content and reported as means ± SD of a number of repetitions as indicated in Table 1. Statistical comparisons were conducted using the SPSS software, version 13.0 (Chicago, IL), using *t*-test for group comparisons. Statistical significance was based on 95% confidence limits ($p \leq 0.05$). Comparison of the non-parametric data among the groups was performed using Mann-Whitney U test.

Liposome experiments

Preparation of Large Unilamellar Vesicles by Extrusion Technique (LUVET) was carried out with 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC, Avanti Lipids) and with phospholipids from soybean lecithin (Sigma-Aldrich, Milan), as described elsewhere.[17,18] The LUVET stock suspensions (70 mM) were transferred in a vial and stored at 4 °C for a maximum of 2 weeks. From a stock solution prepared as LUVET suspension (70 mM phospholipid content) an aliquot (14.5 µL) was transferred in the reaction vial and tridistilled water was added to obtain a 1 mM phospholipid concentration (total reaction volume 1 mL). To the liposome suspension the bleomycin-iron complex (10 µM each) and the thiol (in 10 µM or 100 µM concentration from stock solutions of 2-mercaptoethanol, or other thiols such as cysteine, N-acetyl-cysteine and glutathione, prepared in tridistilled water) were added consecutively. 4 mL vials containing 1 mL reaction volume were prepared, one was used as control and the experiments were run in triplicates incubating at 37 °C, stopping the reaction after the desired time (24 h) for the work-up. Work-up was carried out as already reported in order to obtain the corresponding FAMEs examined by GC for the *cis-trans* isomer content.[17,18] To apply different oxygen concentrations, the appropriate mixtures nitrogen:oxygen containing 5%, 10% and 20% oxygen were obtained by regulating the flux by a precision gas blending apparatus. The atmosphere was kept during the 24 hrs incubation period by attaching a balloon (wall thickness 12 mm) filled with the appropriate gas mixture to the reaction vial. Under similar conditions also blank experiments were carried out. Figure 2 and Table 2 report the results of all experiments. Palmitic acid as the main saturated fatty acid residue in the vesicle was used as an "internal" standard, in order to obtain quantitative information of the reaction yields. PUFA consumption was calculated by quantitating linoleic acid, based on the calibration of its GC peak area using palmitic acid as internal standard, at the beginning and after incubation (see Supplementary Information).

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