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 some novel aspects of drug–metal induced reactivity with unsaturated lipids under oxidative conditions. In cell cultures, 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 a reducing agent that by incubation at 37 °C gave rise to the thiy 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 understanding 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 Fig. 1 summarizes the BLM behaviour in the presence of Fe(II) and O2, 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

So far, the molecular basis of the drug activity has focused on DNA damage especially at the mitochondria level, determining pulmonary side toxicity 7 and kindling 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(II) to BLM-Fe(II) (see Fig. 1). 9 During the reductive process the production of thiy radicals can occur, which are known to react with double bonds and catalyse the isomerisation of unsaturated fatty acid residues (in blue colour).

![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 cause PUFA consumption; in the presence of thiols, the reduction of Fe(II) 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).](image-url)
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 an efficient catalysis for C–H activation and C=C oxidation, whereas the reactivity towards double bonds and the isomerisation represent new aspects to be developed.

After our preliminary experiments evidencing the trans fatty acid formation using bleomycin treatment, we wished 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, compared 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 the formation of thyl radicals responsible of the cis–trans lipid isomerisation, 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 of incubation with bleomycin. To our knowledge, there is only one study on the fatty acid composition of this cell type, 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$^{-1}$. Details of the incubation and the fatty acid composition of cell membranes are given in the Materials and methods section as well as in ESI. Briefly, after incubation the 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 using known methods.

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 consider-able percentage of trans MUFA isomers (trans-16:1 and trans-18:1), identified by appropriate libraries, was found, equivalent to 24% isomerisation of the total MUFA content. A significant diminution of the corresponding cis-MUFA (cis-16:1 and cis-18:1) was correspondingly 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 formation of trans PUFA isomers, identified as mono-trans linoleic acid isomers (see ESI, Fig. 3s†), achieving ca. 20% isomerisation of the 18:2 content, together with mono-trans arachidonic acid isomers, achieving 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 underscoring that membrane lipid reactivity with the change of naturally occurring cis geometry is a new aspect of the free radical-based antitumoral drug effect. Other details concerning the fatty acid transformations are reported in ESI,† which showed also a profound membrane lipid remodelling with the change of the membrane fatty acid asset.

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

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 (IC$_{50}$ = 400 μg mL$^{-1}$).†

| FAME$^{bc}$ | 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***     |
| 6cis-16:1  | 2.0 ± 0.5      | 0.8 ± 0.4*       |
| 9cis-16:1  | 1.4 ± 0.4      | 0.5 ± 0.04*      |
| 18:0       | 35.8 ± 5.9     | 50.4 ± 0.6*      |
| 9trans-18:1| 0.1 ± 0.03     | 2.2 ± 1.1***     |
| 9cis-18:1  | 15.8 ± 2.5     | 6.8 ± 0.6*       |
| 11cis-18:1 | 4.3 ± 0.7      | 2.1 ± 0.1*       |
| mono-trans 18:2<sup>‡</sup> | 0.3 ± 0.3 | 0.1 ± 0.0 |
| 18:2 06b   | 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 06b   | 0.4 ± 0.1      | 1.5 ± 0.9*       |
| mono-trans 20:4<sup>‡</sup> | 0.04 ± 0.03 | 0.1 ± 0.03 |
| 20:4 06b   | 6.8 ± 2.2      | 3.4 ± 0.1*       |

$^{a}$The values are reported as relative percentage (% rel) of the total fatty acid content, together with mono-trans isomers of 20:4.$^{b}$Values significantly different from the control, p < 0.05. $^{c}$Values significantly different from the control, p < 0.001.
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 ESI†). The reactivity of unsaturated fatty acids, studied by us in other biomimetic models in the presence of thiols, can involve MUFA and PUFA residues: the MUFA moiety of oleic acid (9cis-18:1) is not so prone to oxidation as PUFAs are, whereas it can be the target of the thyl radical catalysed isomerisation, being transformed into elaidic acid (9trans-18:1). On the other hand PUFA residues of soybean lecithin liposomes can be partitioned between oxidative and isomerisation pathways. In particular, linoleic acid (9cis,12cis-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: 9trans,12cis-18:2, 9cis,12trans-18:2, and 9trans,12trans-18:2, which can be clearly separated by GC analysis (see Fig. 3s in ESI†). We were aware of lipid oxidation in the mechanism of bleomycin, as depicted in Fig. 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 the 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. It is worth noting that the isomerisation of double bonds requires catalytic thyl radicals, so that appreciable quantities of trans isomers are formed also at very low radical concentration. For this reason trans lipids can be used as sensitive markers of thyl radical generation in complex systems. Using the palmitic acid moiety of phospholipids as the 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 the oxidative process under our experimental conditions.

MUFA-containing vesicles

The results for the reaction of POPC vesicles are summarized in the graph of Fig. 2 (green and violet bars). The trans isomer formation was detected after 24 h of 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 lower 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 (Fig. 2). Comparing the presence and the absence of the drug under the same oxygen conditions, a 5.5 to 7-fold decrease of isomerisation can be observed. Therefore, bleomycin makes a substantial contribution to the generation of thyl radical species and trans fatty acid formation under aerobic conditions.

In the liposome model design, 2-mercaptopethanol was chosen as the thiol for its amphiphilic character, in order to diffuse freely in the heterogeneous environment of the 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, 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 the bleomycin–iron complex was chosen as that reported for lipid peroxidation experiments of chemical and biological models. The steps in the formation of the iron–drug complex with oxygen and the role of thiol (RSH) during the formation of the Fe(II)–bleomycin–thiol complex are depicted in Fig. 1. UV-vis spectra were recorded to ascertain the formation of the absorbance band of the BLM-Fe-SR complex at 580 nm, as described in the literature (see ESI†).

![Fig. 2](image-url) trans 18:1 isomer formation (% rel., see Table 1s in ESI†) 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 ESI†).
The effect of thiol concentration, which in living organisms can reach mM concentrations, was assayed by increasing 2-ME by ten-fold (100 μM), keeping the presence of BLM-Fe(II) at 10 μM concentration each (data not shown). Under these conditions, 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 Fig. 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 for this behaviour and the connection of bleomycin reactivity with the variation of local co-reactant concentrations are a subject for 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 bilayers, as already reported. 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 compared 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 recommend further investigations toward the identification of possible biological candidates for the generation of thyl 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, oleic and linoleic transformations to the corresponding geometrical isomers (9trans-18:1 and trans 18:2 isomers) were followed up, estimating also the PUFA consumption by oxidative degradation (see Fig. 3s in ESI†). The BLM–thiol triad, each at 10 μM concentration, produced a trans 18:1 isomer (for example, ca. 8.5% at 5% oxygen; see Fig. 2, red bars). In these experiments PUFA content strongly diminished after the first 2 h of incubation (consumption >92%, not shown), and thus isomerisation of linoleic acid residues was not detectable. The PUFA oxidative pathway depicted in Fig. 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 Fig. 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 of 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 the thiol–metal complex, led to an even lower isomerisation (<1%) and no PUFA consumption. From these experiments it can be highlighted that bleomycin is responsible for 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: 9trans,12cis > 9trans,12trans > 9cis,12trans. This shows that the isomerisation occurs as a regioselective process, which is expected as a consequence of the supramolecular arrangement of fatty acids in the bilayer (see ESI, Fig. 3s and 4s†).

In anaerobic experiments the PUFA consumption (reaching 60% at higher thiol concentration), not attributable to oxidative processes, was also preliminarily investigated by 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 ESI, Fig. 5s†).

**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-mercaptoethanol (2-ME). The mean ± sd values were calculated from triplicates of the same experiment.

| BLM (μM) | 2-ME (μM) | trans 18:1* (%) | trans 18:2* (%) | PUFA consumpt.** (%) |
|---------|----------|----------------|----------------|---------------------|
| 0       | 0        | 2.3 ± 0.1      | 2 ± 0.2        | 14 ± 0.2            |
| 10      | 10       | 0.9 ± 0.1      | 0.7 ± 0.1      | 0                   |
| 0       | 100      | 30.4 ± 0.9     | 41.2 ± 0.8     | 65.2 ± 0.7          |
| 10      | 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 a peak of the GC analysis of the corresponding FAME. For the isomer recognition see Table 1. The PUFA consumption is calculated using the linoleic acid GC peak areas (cis + trans isomers) before and after 24 h of incubation calibrated vs. palmitic acid used as the internal standard (see ESI). *See Fig. 4s in ESI for the GC analysis relative to the identification of each trans 18:2 isomer. **See Fig. 5s in ESI for the identification of thyl–linoleic acid adducts in the reaction mixture.
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.

Conclusions

The general aim of our study was to obtain molecular insights into 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 isomerisation. Although the bleomycin–iron complex was reported to interact with cell membranes by a fluorescent probe technique in intact cells, this is the first report to highlight that membranes are 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 with oxidative stress.

As a chemical biology model for antitumoral strategies, liposomes highlight the role of cell membranes, which are not spectators but important targets of the drug effect, with synergistic roles for chemotherapeutic effects. Indeed, fatty acid recruitment and membrane formation are attracting a lot of interest in cancer, and in this context the loss of the natural cis geometry and the oxidation-induced lipid remodelling are worthy 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 ESI†

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 per mL of penicillin and 100 µg per mL of streptomycin under a 5% CO₂ atmosphere at 37 °C as described in the manual of the cell line bank. We used the half maximal inhibitory concentration (IC₅₀) of bleomycin (400 µg mL⁻¹) for 24 h incubations, determined in our previous studies and published.

After incubation the cells were detached using accutase, thoroughly washed with phosphate buffer, and pelleted by centrifugation at 14 000g for 40 min at 4 °C after adding water. The pellet was resuspended in pure water and centrifuged for a second washing and then dissolved in 2:1 chloroform–methanol to extract phospholipids. The phospholipid extract was treated with 0.5 M 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. 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 the 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 the 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 phosphatidylcholines from soybean lecithin (Sigma-Aldrich, Milan), as described elsewhere. The LUVET stock suspensions (70 mM) were transferred into a vial and stored at 4 °C for a maximum of 2 weeks. From a stock solution prepared as a 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 the 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 using GC for the cis–trans isomer content. To apply different oxygen concentrations, the appropriate mixtures of nitrogen–oxygen containing 5%, 10% and 20% oxygen were obtained by regulating the flux using a precision gas blending apparatus. The atmosphere was maintained during the 24 h incubation period by attaching a balloon (wall thickness 12 mm) filled with an appropriate gas mixture to the reaction vial. Under similar conditions also blank experiments were carried out. Fig. 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 on the reaction yields. PUFA consumption was calculated by quantitating linoleic acid, based on the calibration of its GC peak area using palmitic acid as the internal standard, at the beginning and after incubation (see ESI†).
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