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SUPPLEMENTARY INFORMATION

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

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MATERIALS and METHODS

Ferrous ammonium sulfate $Fe(NH_4)_2(SO_4)_2$ 6 H_20 (Fe^{II} AS, Carlo Erba, Milan), bleomycin sulfate (BLM, Cayman Chemical, USA) 2-mercaptoethanol (2-ME, Fluka, Sigma-Aldrich, Milan), commercially available *cis* and *trans* FAME, 1-palmitoyl-2-oleoyl phosphatidyl choline (POPC, Avanti Lipids, USA) and the phospholipids from soybean lecithin (Sigma-Aldrich, Milan), were used without further purification. Chloroform, methanol, *n*-hexane (HPLC grade, Merck KGaA, Germany) were used without further purification.

Incubations were carried out in an incubating orbital shaker (Carlo Erba, Milan) keeping the temperature at 37 °C.

When necessary silica gel thin-layer chromatography (analytical) was performed on Merck silica gel 60 plates (0.25 mm thickness, Merck KGaA, Germany) and the spots were detected by spraying the plate with cerium ammonium sulfate/ammonium molybdate reagent.

Fatty acid methyl esters were analyzed by GC (Agilent 6850, Milan) equipped with a 60m × 0.25mm × 0.25µm (50%-cyanopropyl)-methylpolysiloxane column (DB23, Agilent, USA), and a flame ionization detector with the following oven program: temperature started from 165 °C, held for 3 min, followed by an increase of 1 °C/min up to 195 °C, held for 40 min, followed by a second increase of 10 °C/min up to 240 °C, and held for 10 min. A constant pressure mode (29 psi) was chosen with helium as carrier gas. Methyl esters were identified by comparison with the retention times of authentic samples.

When necessary the reaction mixtures were analysed by GC-MS (Thermo Scientific Trace 1300, USA) equipped with a 15m × 0.25mm × 0.25µm TG-SQC 5% phenyl methyl polysiloxane column, with helium as carrier gas, coupled to a mass selective detector (Thermo Scientific ISQ, USA) with the following oven program: temperature started at 80 °C, maintained for 2 min, increased at a rate of 15 °C/min up to 140 °C, increased at a rate of 5 °C/min up to 280 °C and held for 10 min.

UV-*vis* spectra were recorded in water on a Cary100 spectrophotometer (Agilent, USA).

Preparation of bleomycin-Fe(II)-thiol complex and UV monitoring

Ferrous ammonium sulfate $Fe(NH_4)_2(SO_4)_2$ 6 H_20, bleomycin sulfate and 2-mercaptoethanol were prepared as stock solutions in water and mixed with the desired final concentrations.

UV spectra were registered and follow up was carried out of the band at 580 nm reported for the formation of the BLM-Fe-thiol complex.[1]

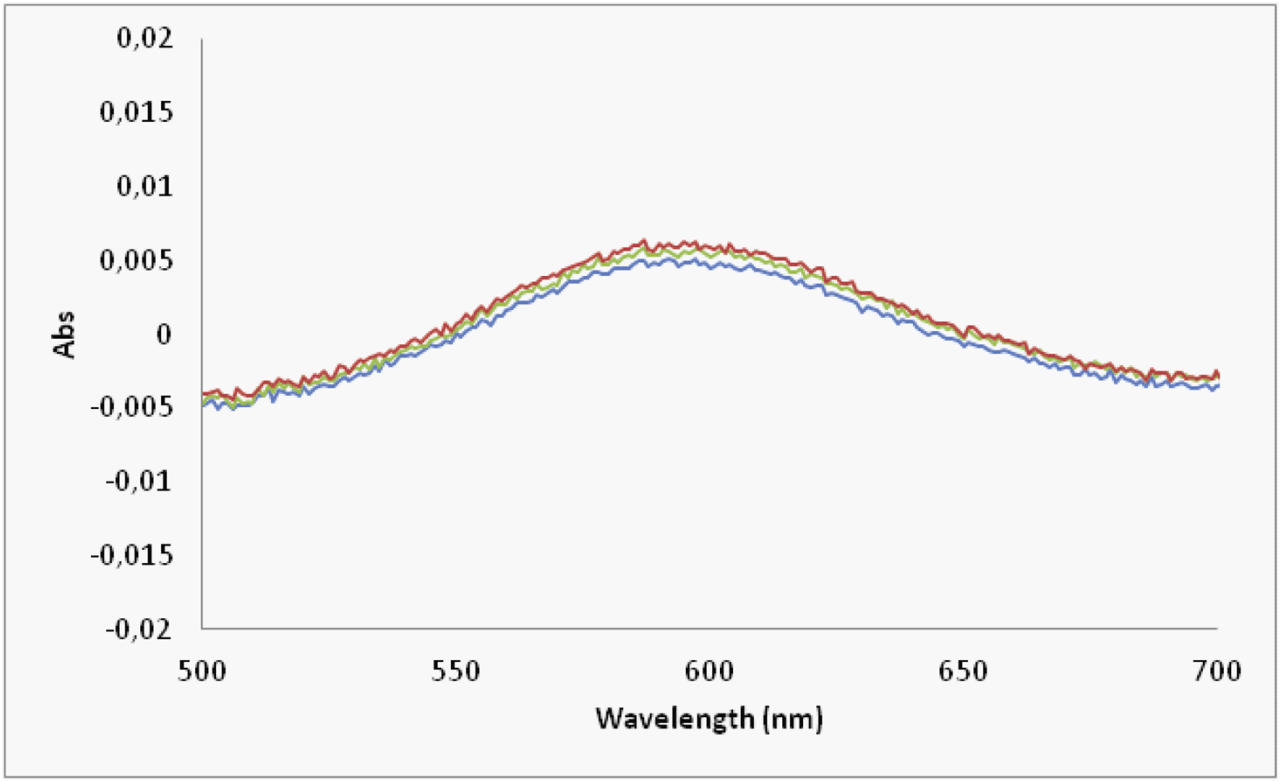


Figure 1s – Absorbance of the band at 580 nm at 0 min (blue), at 2.3 min (reddish brown), at 20 min (green)

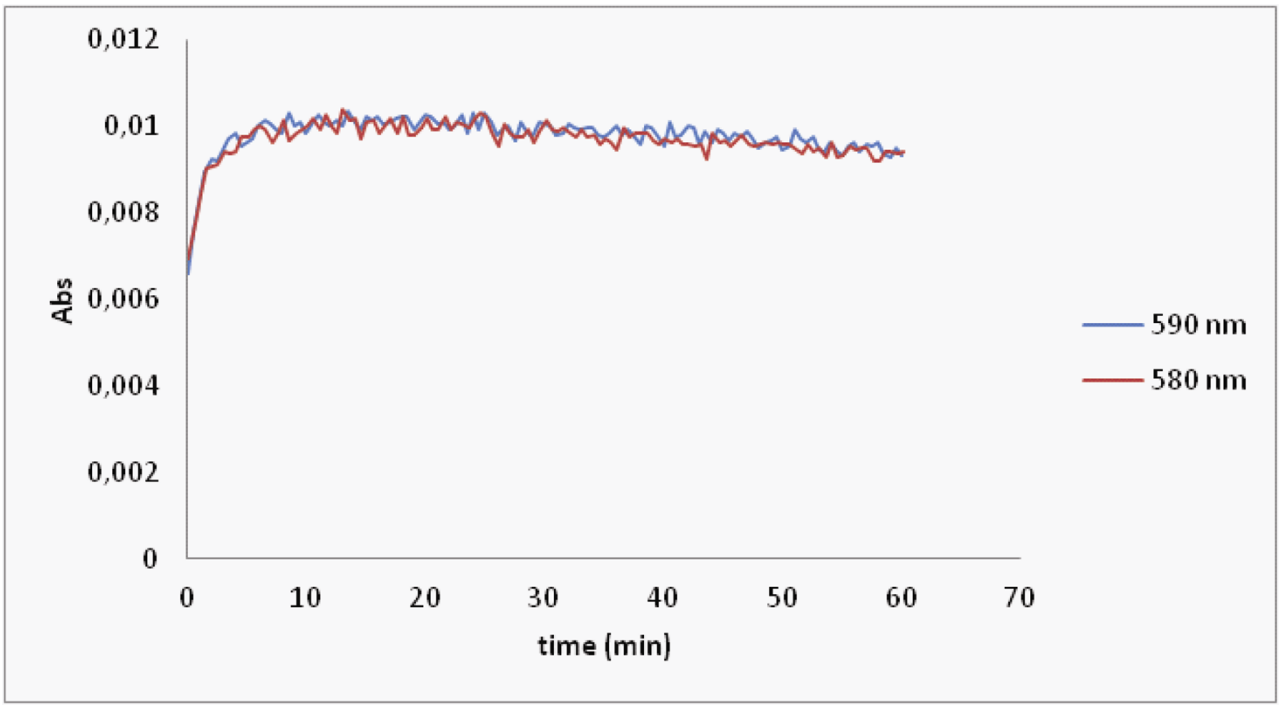


Figure 2s – Monitoring of the band at 580-590 nm for 1 hr

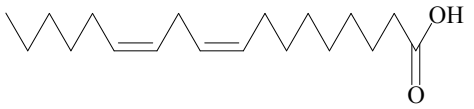
NTera-2 cell membrane fatty acid analysis

Table 1 in the main text shows the fatty acid residues found in treated cells 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. It is worth noting that in control cells the omega-3 fatty acid series was found in traces (<0.1% of the total fatty acid content, not reported in Table 1), whereas primary human testicular germ cells are reported to contain these fatty acids (ca. 4% of the total fatty acid content).[2] Such starting PUFA content derives from culture conditions, as already reported by us for leukemia[3] and neuroblastoma[4] cell lines, and is not further explored in this work. After drug incubation, most of membrane fatty acids were changed significantly compared to controls. Bleomycin treatment resulted in: *i)* the increase of saturated fatty acids (in particular stearic acid,18:0 and eicosanoic acid , 20:0; $p<0.05$); *ii)* the diminution of *cis* MUFA, such as, 6*cis*-16:1 (sapienic), 9*cis*-16:1 (palmitoleic), 9*cis*-18:1 (oleic), 11*cis*-18:1 (vaccenic) ($p<0.001$; $p<0.05$) identified as reported previously;[5] *iii)* the formation of *trans* MUFA isomers (*trans*-16:1 and *trans*-18:1), identified by appropriate libraries corresponding to a 24% isomerisation of the total MUFA content; *iv)* the diminution of omega-6 PUFA, in particular linoleic (18:2) and arachidonic (20:4) acids levels, compared to controls ($p<0.05$). It is worth noting that not all PUFAs were diminished during bleomycin treatment, since the omega-6 eicosatrienoic acid (8*cis*,11*cis*,14*cis*-20:3) increased significantly compared to controls; *v)* finally, the *trans* PUFA isomers were identified as mono-*trans* linoleic acid isomers,[6] (Figure 3s) reaching ca. 20% isomerisation of the 18:2 content, and mono-*trans* arachidonic acid isomers,[7] reaching ca. 2% of the total 20:4 content. In this work we focused on the bleomycin-induced membrane fatty acid changes, whereas the biochemical pathways and the influence of cultivation conditions will be reported elsewhere.

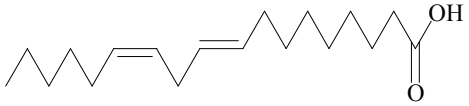
Lecithin composition and isomerization

The phospholipids from soybean lecithin were examined by GC after transesterification to fatty acid methyl esters (FAME) under known conditions.[5-7] The fatty acid composition resulted to be: palmitic acid, 16:0, 34%; stearic acid, 18:0, 14.22%; oleic acid, 9*cis*-18:1, 30.3%; vaccenic acid, 11*cis*-18:1, 1.3%; linoleic acid 9*cis*,12*cis*-18:2, 17.5%; arachidonic acid, 5*cis*,8*cis*,11*cis*,14*cis*-20:4, 2.5%. We decided to monitor the 18:1 and 18:2 fatty acids under different liposome incubation conditions since these two fatty acids are the most representative unsaturated moieties of the mixture. The calibration of the gas chromatographic peak areas was effected by using the peak area of palmitic acid as the internal standard to quantify the corresponding presence of the unsaturated fatty acids and establish their reaction yields after the experiments.

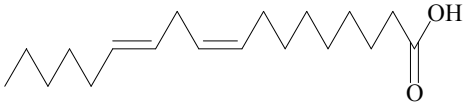
Linoleic acid (9*cis*,12*cis*-18:2) is expected to be transformed by thiyl radical-catalysed isomerisation into its corresponding mono-*trans* isomers (9*trans*,12*cis*-18:2; 9*cis*,12*trans*-18:2) and into the di-*trans* isomer (9*trans*,12*trans*-18:2). The four linoleic acid isomers are shown in Figure 3s.



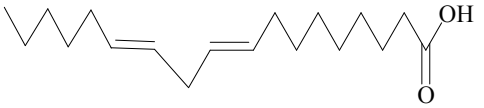
Linoleic acid 9*cis*,12*cis*-18:2



9*trans*,12*cis*-18:2



9*cis*,12*trans*-18:2



Linolelaidic acid 9*trans*,12*trans*-18:2

Figure 3s – Structures of linoleic acid, the two mono-*trans* isomers, and the all-*trans* isomer

The results of the isomerization of monounsaturated residues in POPC and lecithin vesicles are shown in Figure 2 and the related data are reported here below in Table 1s. The mean ±sd values are obtained from n=3 repetitions of the same experiment.

	9t–18:1 (%)			
	POPC (1 mM)		Lecithin (1 mM)	
	Fe(II)-2-ME (10 μM)	Fe(II) (10μM); -2-ME (10μM); BLM (10 μM)	Fe(II)-2-ME (10 mM)	Fe(II) (10μM); -2-ME (10 μM); BLM (10 μM)
N_2:O_2 (95:5)	**5.4 ± 0.4**	**26.9 ± 0.6**	**5.2 ± 0.5**	**8.5 ± 0.3**
N_2:O_2 (90:10)	**1.5 ± 0.2**	**13.4 ± 0.1**	**4.4 ± 0.4**	**6.9 ± 0.2**
N_2:O_2 (80:20)	**1.2 ± 0.1**	**7.3 ± 0.2**	**2.9 ± 0.2**	**4.7 ± 0.4**

In the lecithin experiments, the PUFA consumption was calculated by the difference between the starting linoleic acid quantity (determined by the calibration of the peak area in the control) and the sum of *cis* and *trans* isomer of linoleic acid peak areas found after the incubation, expressed as percentage related to the starting concentration. In all the aerobic experiments the PUFA moiety of lecithins was found to be >92%, so that the isomerization of linoleic acid could not be detected.

Gas chromatographic separation of FAME

After the reaction under the conditions described in the Material and Methods, GC was used to separate *cis* and *trans* isomers.

In particular, Figure 2 in the text shows the result of the experiment under anaerobic conditions with BLM-Fe(II) and thiol (2-mercaptoethanol) reported in Table 2 of the manuscript. The isomerization of oleic and linoleic acids occurred and it was gratifying to see that the *trans* 18:2 isomers (see Figure 4s) were formed in different amounts.

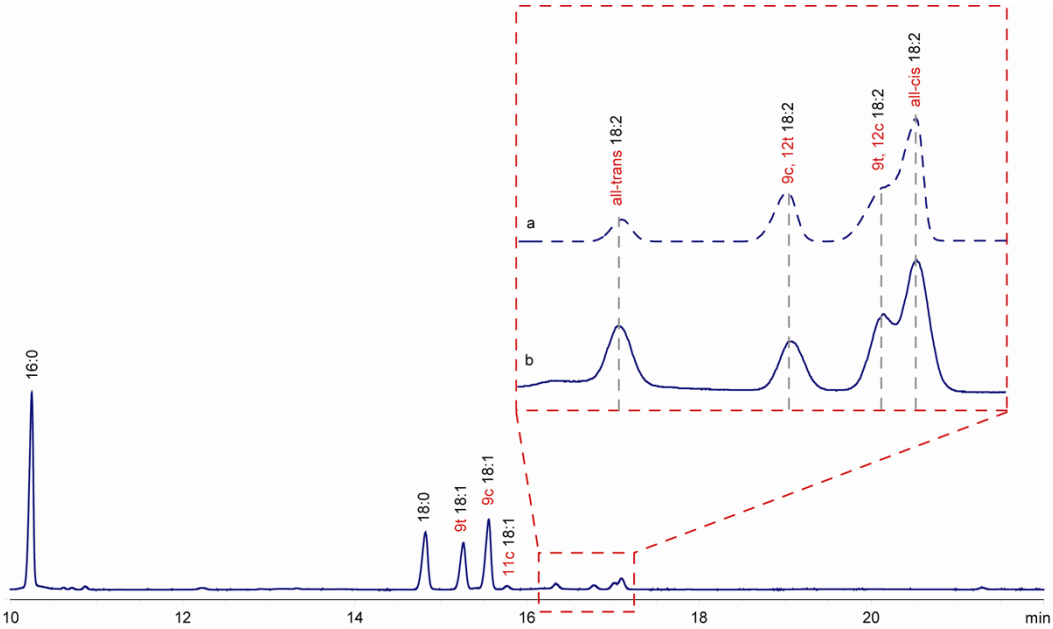


Figure 4s. GC chromatogram of FAMEs obtained after incubation of lecithin liposomes at 37 C° under anaerobic condition (N_2, 0% O_2) in the presence of 10μM of Fe(II) and bleomycin added with 100μM 2-ME; in the red box the GC chromatograms of **a**) 18:2 isomer references and **b**) the 18:2 peaks formed in the experiment.

These results confirmed that the isomerization process occurred by diffusion of thiyl radical into the lipid bilayer, as previously reported for other liposome systems.[6]

Isomerization in lecithin vesicles with formation of the thiol:linoleic acid adducts.

The experiments carried out with 10 and 100 µM 2-mercaptoethanol and 10 µM bleomycin-Fe(II) in lecithin liposomes showed similar PUFA disappearance. Therefore, the crude phospholipid mixture after the isolation and transesterification was examined by GC/MS using appropriate elution conditions. The formation of a more polar product was highlighted by gas chromatography and the mass fragmentation gave results similar to those described for the addition products between the thiol and linoleic acid, used as reference.[6]

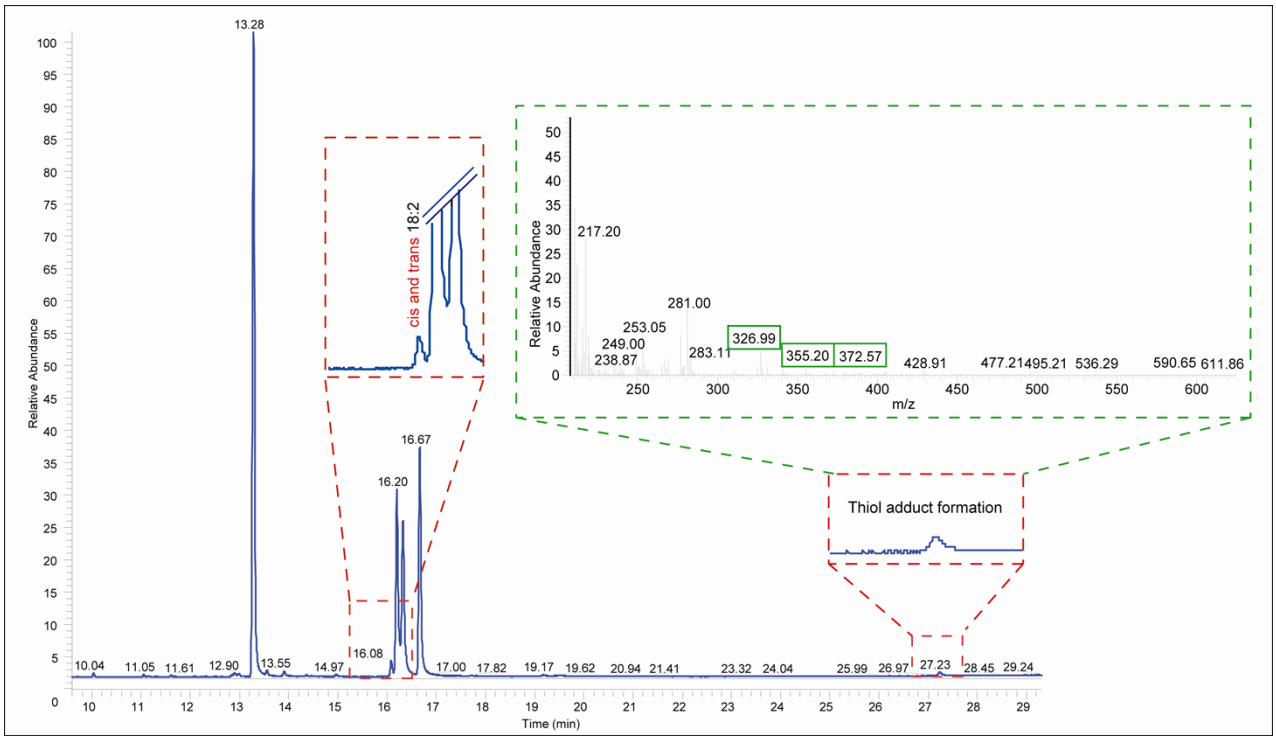


Figure 5s. GS/MS of the FAME mixture obtained after incubation of lecithin liposomes at 37 C° under anaerobic condition (N_2, 0% O_2) in the presence of 10µM of Fe(II) and bleomycin added with 100µM 2-ME; in the red box the enlargement of the GC chromatogram area showing the 18:2 fatty acid as a unique peak of *cis* and *trans* isomers; in the red box on the right the GC area enlargement corresponding to the thiol adduct, confirmed by the MS spectrum (in the green box) as reported.[6]

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