Effect of pO_2 on Antitumor Drug Cytotoxicity on MDR and Non-MDR Variants Selected from the LoVo Metastatic Colon Carcinoma Cell Line

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Abstract. *Two chemosensitive cell lines, LoVo-fusoid (LoVo-f) and LoVo-small cells (LoVo-sc) were derived from the original LoVo cell line. These two variants and the multidrug-resistant (MDR) cell line LoVo-Dox were screened for various properties. In non-permeabilized cells, only LoVo-sc showed mucin-2 staining whereas labelling was positive in all permeabilized cell lines. As shown by electron microscopy screening and by relative resistance to trypsin detachment, only LoVo-sc cells showed strong mucus secretion. All three cell lines displayed strong staining for P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and lung-resistance-related protein (LRP) in different locations according to the drug resistance state. The three cell lines showed intracellular labelling of LRP and MRP. The sensitive cells showed P-gp in a large perinuclear ring and in the cytoplasm, but little (LoVo-sc cells) or no staining (LoVo-f cells) was shown at the plasma membrane level. For the Lovo-Dox cells, P-gp was located in the plasma membrane, in cellular anchorages and in the cytoplasm as well. Cell resistance against antineoplastic agents often results from mobilization of various factors, the modulation of which is linked to the culture conditions. As most of the protocols utilize cells growing in (air+5-10% CO_2) atmosphere e.g. 20% O_2, balance of the respective participants in the MDR multi-modal mechanism may not be representative of the in vivo situation and may lead to erratic pharmacological response. Indeed, cells within solid tumours were exposed to low pO_2, most of them being under hypoxic condition (0.1-5% O_2). In the absence of anticancer drugs, all LoVo cell lines grew notably faster at 20% O_2 than at 5% O_2. Moreover, respective sensitivities of both non-MDR variants to doxorubicin were altered according the pO_2. Whatever the pO_2 was, virtually none of the antioxidants tested affected the cytotoxic activity of doxorubicin for the three cell lines. By contrast, trolox showed a strong inhibitory effect on doxorubicin activity. These results underline the importance of evaluating the role of hypoxia on the cytotoxic effect of chemotherapeutic agents used either as single drugs or in combination therapy.*

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The LoVo cell line was established by Drewinko *et al.* (1) from a metastatic tumor nodule fragment in the left supraclavicular region of a male patient with a histologically proven diagnosis of adenocarcinoma of the colon. As for many cell lines isolated from a tumor, the LoVo cell line constitutes a heterogeneous cell population at the origin of numerous chemosensitive or chemoresistant subclones, such as LoVo DR5 (2) LoVo H (3), LoVo ADR1,2 and SRA1,2 (4) LoVo E2 and C5 (5), LoVo 24517 (6), LoVo CP2.0 and LoVo RT (7), and LoVo C1.5 and LoVo C1.7 (8).

Using the chemosensitive LoVo cell line, two subclones named LoVo-fusoid (LoVo-f) and LoVo-small cells (LoVo-sc) were selected based on their morphology and growth patterns. In this paper, we report on the methodology used to isolate these two variants. Comparison of their respective features was made together with those of the multidrug resistant LoVo-Dox cell line that was derived from the original heterogeneous LoVo cell line by growth in increasing concentration of doxorubicin (9). Some of the pharmacological properties of these subclones have already been reported (10, 11). Here we present their basic

characteristics and emphasize the impact of the oxygen level on the properties of LoVo-f, LoVo-sc and LoVo-Dox variants. Indeed, solid tumour oxygen pressure analysis has shown that most of the cells comprising a tumour are subjected to a strong hypoxic environment (12). Comparing the effect of vinblastine and doxorubicin, two chemotherapeutic agent substrates for the ABCB1 multidrug transporter, we confirm our previous data (13) showing that the anticancer drug level of cytotoxicity was depending on the pO_2 used to grow the cells.

Materials and Methods

Chemicals. Doxorubicin hydrochloride (D-1515), (±) verapamil hydrochloride (V4629), crystal violet (C6158), L-carnitine hydrochloride (C0283) and niacin (N5410) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Doxorubicin concentrated solutions were prepared in dimethyl sulfoxide (DMSO) of ≥99.9% ACS spectrophotometric grade and kept as aliquots in dark glass vials. Phosphate-buffered saline, pH 7.4, (P-3813; PBS) and bovine serum albumine (A-4503; BSA) were obtained from Sigma. Bodipy® FL verapamil hydrochloride (BV, B7431) was from Molecular Probes (Invitrogen, Cergy Pontoise, France). Taurine (CAS 107-35-7) was from Janssen Chimica (Noisy le Grand, France). All chemicals were of analytical grade. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble derivative of vitamin E, was from Calbiochem (a brand of EMD Biosciences Inc., an associate of Merck KGaA, Darmstadt, Germany).

Cell lines: selection of the LoVo cell line variants. Two original LoVo cell lines were obtained from Pharmacia & Upjohn Cie (Experimental Oncology Department, Nerviano, Italy). The anticancer drug-sensitive LoVo was the cell line established by Drewinko *et al.* (1). The LoVo-Dox variant resistant to doxorubicin was derived by Grandi *et al.* (9). The LoVo-Dox cell subline presented a typical MDR phenotype with MDR-1 gene overexpression and was 25-30 times more resistant to doxorubicin than its LoVo counterpart (9). To prevent reversal of the MDR phenotype, LoVo-Dox was continuously maintained in 0.1 μg/ml doxorubicin as recommended by Grandi *et al.* (9). Cells were thereafter increased in resistance in our laboratory, by increasing doxorubicin concentration up to 0.8 μg/ml. This subline was thereafter subjected to treatment with 0.3 μg/ml doxorubicin in combination with 0.5 μg/ml vinblastine. This resistant LoVo cell population was termed LoVo-Dox and was thereafter continuously maintained at 0.4 μg/ml doxorubicin. Two variants were isolated from the LoVo drug sensitive cell line that differed in morphology and degree of differentiation from others – LoVo-f and LoVo-sc. Selection of these two variants was achieved *via* a sequential release of cells monitored with 0.25% trypsin-5 mM EDTA at pH 8 from a confluent monolayer of the original LoVo chemosensitive cell line. Following addition of trypsin and immediate removal of the proteolytic solution, rapid release of a population composed of larger cells was obtained whereas numerous smaller cells remained attached to the Costar plastic culture plate. Repeated differential trypsinization permitted isolation of two pure cell populations that were named in relation to their aspect: LoVo-f for the variant with cells of a spindle shaped morphology that were easily detachable from the substratum and LoVo-sc for the variant with polygonal shaped cells with strong adherent properties due to mucus secretion.

Cell culture conditions. The drug-sensitive variants LoVo-f and LoVo-sc, and the drug-resistant LoVo-Dox cell lines were grown in 75 cm^2 vented flasks (Costar n°3376; Dutscher, Brumath, France) in plain medium composed of DMEM-Ham F12 medium (Dulbecco's modified Eagle medium/Ham F12 nutrient mixture 1/1, F-4815; Seromed Biochrom AG, Berlin, Germany) supplemented with sodium pyruvate (1 mM final concentration), 365 mg/ml glutamine and 5% selected heat-inactivated (56°C, 30 min) fetal calf serum from Dutscher (Brumath, France). LoVo-Dox plain medium was supplemented with 0.4 μg/ml doxorubicin. Osmolarities of all the media and solutions used for cell culture and experiments were checked using a Roebling® osmometer (Hermann Roebling, Berlin, Germany). Osmolarities were all in the physiological range (290-315 mOsm). Cells were passaged at 3/4 confluence. Absence of mycoplasma contamination was routinely checked using Hoechst 33258 (Sigma); the presence of mycobacteria was excluded *via* screening by optical microscopy observation at high magnification (14). Cell viability was assessed by Trypan blue dye exclusion, or by MTT test as described elsewhere (15).

Cell cultures were maintained in a water saturated atmosphere at 37°C, either under standard conditions, *e.g.* 5% CO_2/95% air (pO_2 20%) or under physiological reduced oxygen pressure, *e.g.* 5% CO_2/5% O_2/90% N_2 (pO_2 5%) achieved by mean of a Sanyo multi-gas incubator (Fisher Scientific Bioblock, Illkirch, France).

Cell proliferation assays. Cells were seeded in 35 mm Petri dishes (Costar n°3035) at a density of 400 cells /2 ml of plain culture medium. As cell attachment is not identical at 5% and at 20% oxygen, cells were allowed to attach first for 12 h at 5% O_2. The culture medium was thereafter removed and replaced by 2 ml fresh plain medium containing or not agents to be tested (chemotherapeutic agents, antioxidants). Doxorubicin, or vinblastine (15 ng/ml for non-MDR variants, 1 μg/ml for MDR cells, respectively) were added together with or without antioxidant (2 mM). Anticancer drug concentrations were chosen according previous half maximal inhibitory concentration results (IC_{50}) (13) in order to enable cell growth. Cells were then switched to the required oxygen levels. Culture media were changed every other day. Control cells were treated with an equal concentration of the solvents used to dissolve drugs (DMSO, water or ethanol). In all experiments, the final concentration of solvent never exceeded 0.2%.

IC_{50} determination for doxorubicin and vinblastine. Approximately 400 cells were seeded in 35 mm Petri dishes in 2 ml of DMEM/Ham F12 + 5% (v/v) FCS (+0.4 μg/ml doxorubicin for LoVo-Dox cells) at 5% O_2. Two days later, the culture medium was removed and replaced by 2 ml of fresh plain medium containing or not increasing concentrations of vinblastine and/or of doxorubicin. Cells were grown for 36 h in these culture media either at 5% O_2 or at 20% O_2. Cell proliferation was determined as described below. Cell proliferation in the presence of drugs was achieved using a 0-30 ng/ml concentration range of doxorubicin and/or vinblastine for the LoVo non-MDR variants and a concentration range of 0-4 μg/ml of doxorubicin and/or vinblastine for the LoVo-

Dox type. When used in combinations, doxorubicin and vinblastine were added together to the cultures at the same concentration.

Cell proliferation determination. Cell growth was determined using crystal violet staining method as described by Kueng *et al.* (16) and optimized by Lelong and Rebel (15). Briefly, after removal of the culture medium, attached cells were washed twice with isoosmotic saline (NaCl 0.9%) then were fixed for 15 min with freshly prepared 1% glutaraldehyde in 0.9% NaCl. The fixative solution was removed and fixed cells were kept at 4˚C until completion of all series. Fixed cells were thereafter stained with 1 ml crystal violet solution (0.1% w/v in twice-distilled water) for 30 min, at room temperature. After removal of the staining solution, cells were washed three times with 2 ml bi-distilled water. Stain was desorbed with 3 ml 33% acetic acid solution and the absorption at 600 nm of stain aliquots was determined.

Preparation for scanning electron microscopy (SEM). Cells were replicated and transferred to a 35mm diameter Petri dish on a thin glass slide and thereafter grown as described above. After 8 days of cell growth, the culture medium was gently removed and immediate fixation was performed for two hours in 2.5% glutaraldehyde, 0.1 M cacodylate balanced solution followed by washing with cacodylate solution. Cultures were post-fixed in osmium tetroxide (1%). After alcoholic progressive dehydration to absolute alcohol, the pieces were dried at the critical point of hexamethyldisilazane and stuck with a very fine layer of conductive carbon glue for SEM (Leit-C®; Fluka, Buchs, Switzerland) to a metallic support for SEM, the surface exposed at the top. Preparations were thereafter gilded in a vacuum evaporator SCD 040 (Balzers, Meudon, France), and observed with a Cambridge 360 SEM.

Immunochemistry - Confocal microscopy. LoVo-sc, LoVo-f and LoVo-Dox cells, grown on 22x22 silanized coverslips in 6-well plates (Costar 3335), were fixed for 1 h at room temperature with PBS containing 2% paraformaldehyde and 0.05% glutaraldehyde. Fixed cells were thereafter washed with PBS. To detect intracellular epitopes, fixed cells were permeabilized for 3 min at room temperature with Triton X-100 0.1% in PBS, pH 7.4 and washed 3 times with PBS and incubated for at least 1 h with PBS containing 5% BSA to prevent non-specific binding. Cells were then incubated for 3 h at room temperature or overnight at 4˚C in a humid chamber with specific antibodies diluted in PBS containing 1% BSA, washed with 1% BSA in PBS solution and incubated for 2 h with the appropriate secondary antibody labelled either with Alexa Fluor® 488 (Molecular Probes, Invitrogen, Cergy Pontoise, France) or Alexa Fluor® 546 (Molecular Probes). Coverslips were mounted in moeviol (Sigma, Saint Quentin Fallavier, France) and labelled cells were examined using a Zeiss LSM 510 confocal laser microscope (Carl Zeiss S.A.S., France).

The presence of three proteins associated with a multidrug-resistant phenotype was tested. Screening comprised two ABC transporters: P-glycoprotein (Pgp), the ABCB1 transporter responsible for the MDR-1 phenotype and MRP1, the ABCC1 transporter responsible for the non-Pgp-associated MDR phenotype, showing some overlapping substrate specificity with P-glycoprotein. Screening also addressed the lung resistance protein (LRP) involved in nucleocytoplasmic transport and thereby associated with the emergence of non-Pgp-associated multiple resistance has also been tested. To determine the MDR-1 morphophenotype linked to Pgp of the LoVo-Dox cell line, MRK16 monoclonal antibody (MC-012; Kamiya Biomedical Company, Interchim, France) that binds to a 3-dimensional extracellular epitope, was used. The 4007 polyclonal antibody, (a generous gift of Dr M.M. Gottesman, NCI-NIH, Rockville Pike, MD, USA) recognizing peptide epitope 919-1280 at the COOH terminus of Pgp was also used (17). Multidrug-associated protein (MRP) was tested with monoclonal antibody MRPm6 (Monosan, Tebu, France) whereas LRP was detected using LRP-56 monoclonal antibody (Monosan). Bodipy-verapamil (BV), a fluorescent substrate competitor for other Pgp substrates such as doxorubicin (18) was used at 2.5 μM.

Sucrase isomaltase (SI) and other brush-border enzymes are considered as differentiation markers for the crypt-to-villus differentiation process *in vivo* (19). The primary antibody for sucrase isomaltase detection was the monoclonal Caco-3/73 antibody, a generous gift of Dr A. Quaroni (20) and was used in the form of ascite fluids diluted 1/50 with PBS as described elsewhere (21).

Mucin synthesis and secretion are features of glandular epithelial tissues with differential expression according to tissue type, such as mucin 2 (Muc-2) which coats epithelia of the intestine and airways and is associated with colonic tumors (22-24). Muc-2 mouse monoclonal antibody (Ccp58; Santa Cruz Biotechnology, Tebu, France) was used according to the manufacturer's instructions.

Statistical analysis. Each measurement was performed at least in quadruplicate. The standard error of the mean (SEM) was expressed as the percentage of error for each experimental condition. Results are expressed as means±SEM. Statistical significance of the experimental results for cell proliferation studies expressed±SEM as optical densities at 600 nm was obtained by variance analysis with a Fisher's test using the StatView computer software (Abacus Concepts, Inc., Berkeley, CA, USA). Statistical significance of the experimental results for proliferation and cell viability experiments expressed as percentages was obtained with a Bonferoni test. $P<0.05$ was considered to denote statistical significance.

Results

Morphological characteristics of LoVo variants. Scanning electron micrographs of the three cell lines and standard observations made with a Leica inverted microscope at several magnifications up to x400 of nearly confluent cultures (Figure 1) show that LoVo-sc cells are clearly smaller than the two other LoVo cell types. LoVo-sc cells show a morphology typical of epithelial cells and rapidly form dense islets which join to create a dense, tight monolayer. These cells exhibit a relatively highly differentiated morphotype with numerous tonofilaments implicated in junction complexes and in desmosomes. LoVo-sc cells present numerous intracellular vacuoles, typical brush border structures and some secretion granules evoking mucus secretion. Evidence for this mucin secretory activity is evidenced during trypsin dissociation of the monolayer, giving rise to a dense viscous aggregate. LoVo-

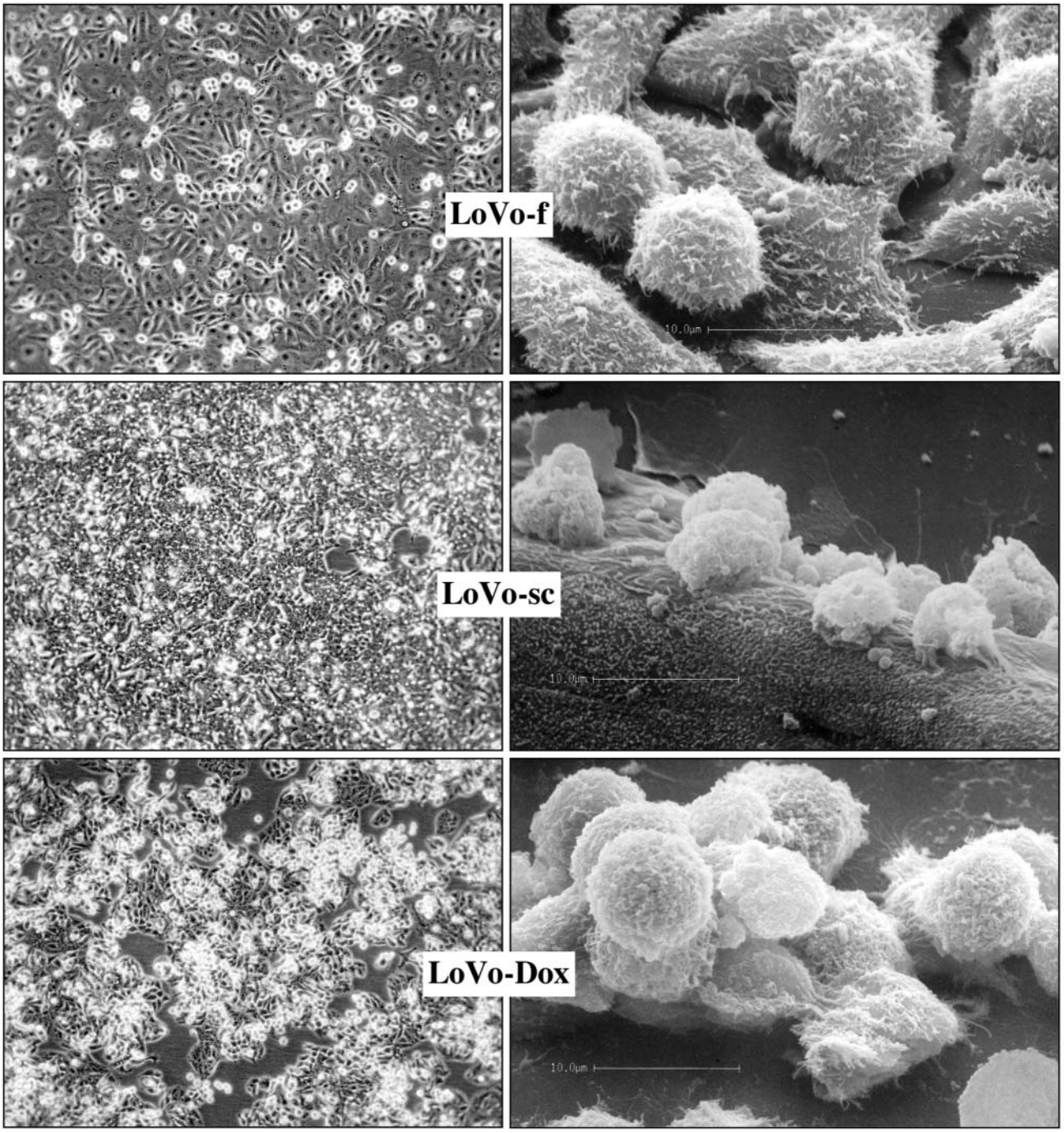

Figure 1. *Morphology of the LoVo cell lines. Phase-contrast microscopy of LoVo cell lines at high confluence (left hand panel) and corresponding scanning electron micrographs (right hand panel). White bar 10 μm.*

sc cells are capable of building multilayers after reaching confluence and enable formation of spheroids under particular conditions (data not shown).

LoVo-f cells exhibit a fusiform nature very similar to fibroblastic morphology at low-density culture, changing to a more polygonal epitheloid form thereafter. This cell line forms confluent monolayers with low adhesiveness due to labile intercellular adhesion easily cleavable by trypsin. Numerous microvilli are present at the cell surface, without exhibiting a brush border. Mild endocytic activity, together

with numerous active lysosomes is seen, but without evidence of mucus secretory activity.

LoVo-Dox cells show a morphology very similar to the LoVo-sc cell line. This cell line also exhibits a high capacity to form dense monolayers and thereafter to grow in multilayers. Although basal cells are partially polarized, brush border structures appear quite disorganized. A lack of mucus granules tends to show a cell differentiation trend rather towards an endocytic pathway than an exocytic one.

Cell properties – specific markers. Sucrase isomaltase is present in the three variants. However the labelling intensity differed among cell variants, being strong in the small cell type, medium in the fusoid type and low in the MDR type. When tested on non-permeabilized cells, Muc-2 labelling was low in the three variants with some exception for some LoVo small cells being individually strongly labelled. After permeation, the three cell lines were clearly positive for Muc-2 expression.

The three cell lines displayed a strong intracellular staining for MRP and LRP under confocal microscopy (data not shown). In chemosensitive cells, Pgp localisation with 4007 polyclonal antibody was exclusively cytoplasmic, whereas in the LoVo-Dox MDR variant, strong staining with 4007 was observed in the cytoplasm and plasma membrane. Colocalisation of 4007 and MRK16 staining was observed in the plasma membrane of MDR cells but was totally absent from the plasma membrane of non-MDR variants. Detection of Pgp with MRK16 fluorescent immunolabelling was observed in the form of a strong large pericellular ring in the LoVo-Dox MDR variant, whereas in LoVo-f cells and LoVo-sc cells, Pgp immunostaining was granular and was intracellularly localised (Figure 2).

A cytoplasmic localisation of Bodipy-verapamil (BV) in vesicle-like structures was observed in the two sensitive cell lines, whereas in the LoVo-Dox cells, BV staining was localized at the plasma membrane and overlapped with pericellular Pgp staining (Figure 3). When LoVo-Dox cells were treated with a combination of BV + doxorubicin, BV export was inhibited as a result of competition between the two drugs, which are both substrates for Pgp (18). With this combination, BV was detected in vesicle-like structures in the LoVo-Dox cells, in a location similar to that observed in LoVo sensitive cells incubated with sole BV alone (Figure 4).

Effect of pO_2 on LoVo cell growth. Growth of the three cell lines was affected by the oxygen pressure applied in the incubator, being higher at 20% then at 5% O_2 (Figure 5). The doxorubicin inhibitory effect on the three cell lines was slightly higher at 20% O_2 compared to 5% O_2. This effect is more marked in sensitive cells than in Lovo-Dox cells (Figure 6). By contrast, the vinblastine inhibitory effect was not affected by pO_2 (Figure 6).

Effect of antioxidants on LoVo cells growth. Figure 7 shows the effect of antioxidants on the growth of LoVo cells in the presence of doxorubicin. Among the various compounds tested (*e.g.* trolox, taurine, carnitine, *N*-acetylcysteine, and niacin), only *N*-acetyl-cysteine affected the growth of LoVo cells in the absence of doxorubicin. With the exception of trolox, none of the antioxidant tested affected the growth inhibitory activity of doxorubicin. Trolox notably reduced the inhibitory effect of doxorubicin on the growth of the three LoVo lines. A 50% reduction of the anticancer drug efficacy was observed in cells grown at 20% O_2 whereas a 92% decrease was obtained with cells grown at 5% O_2.

Discussion

The LoVo original cell line was isolated from a supraclavicular metastatic nodule of a colorectal adenocarcinoma (1). From these LoVo cells, numerous cell lines have been subsequently derived, most of them selected by increasing concentrations of anticancer drugs are chemoresistant (2-4, 6-9). To the best of our knowledge, few authors addressed the population heterogeneity of the original chemosensitive LoVo cell line isolating chemosensitive variants such as LoVo E2 and LoVo C5 (5). LoVo-sc and LoVo-f cells were isolated by repetitive selection related to their trypsin sensitivity. This procedure linked to differential cell detachment by trysin is similar to that which permitted selection of epitheloid and fusiform subpopulations from human retinal pigmented cells (25). Both LoVo-sc and LoVo-f cells reacted positively with sucrase isomaltase antibody, a marker for normal and tumoral colonic cells (19). Screening for secreted gel-forming mucins was focused on mucin-2, as Muc-2 and Muc-3 are prominent mucin genes expressed in the human intestinal tract (26, 27). Although expression of a particular type of gel-forming secreted mucin is rather complex and not restricted to any histopathological type, even considering all components present in a given tumor, Muc-2 epitopes are frequently expressed in well- and moderately well-differentiated colon cancer (28, 29). Only few intestinal cell lines secrete notable amounts of mucus (28-30). None of the LoVo cell lines already described in the literature has been reported as a mucus-secreting type. Although a positive stain for Muc-2 as an indicator for mucus synthesis was observed for our three LoVo cell lines, only LoVo-sc showed actual mucus secretion. However, the pattern of Muc-2 reactivity of LoVo-sc layers indicates heterogeneity of the secretion level. We do not know presently whether this LoVo-sc line or the high mucus secreting cells that would be derived from it could be useful for addressing polarized drug transport studies as reported for HT29X (29, 30).

ABC transporters Pgp and MRP play an important role in the regulation of intestinal drug adsorption (31, 32). The presence of the multidrug resistant transporter protein Pgp

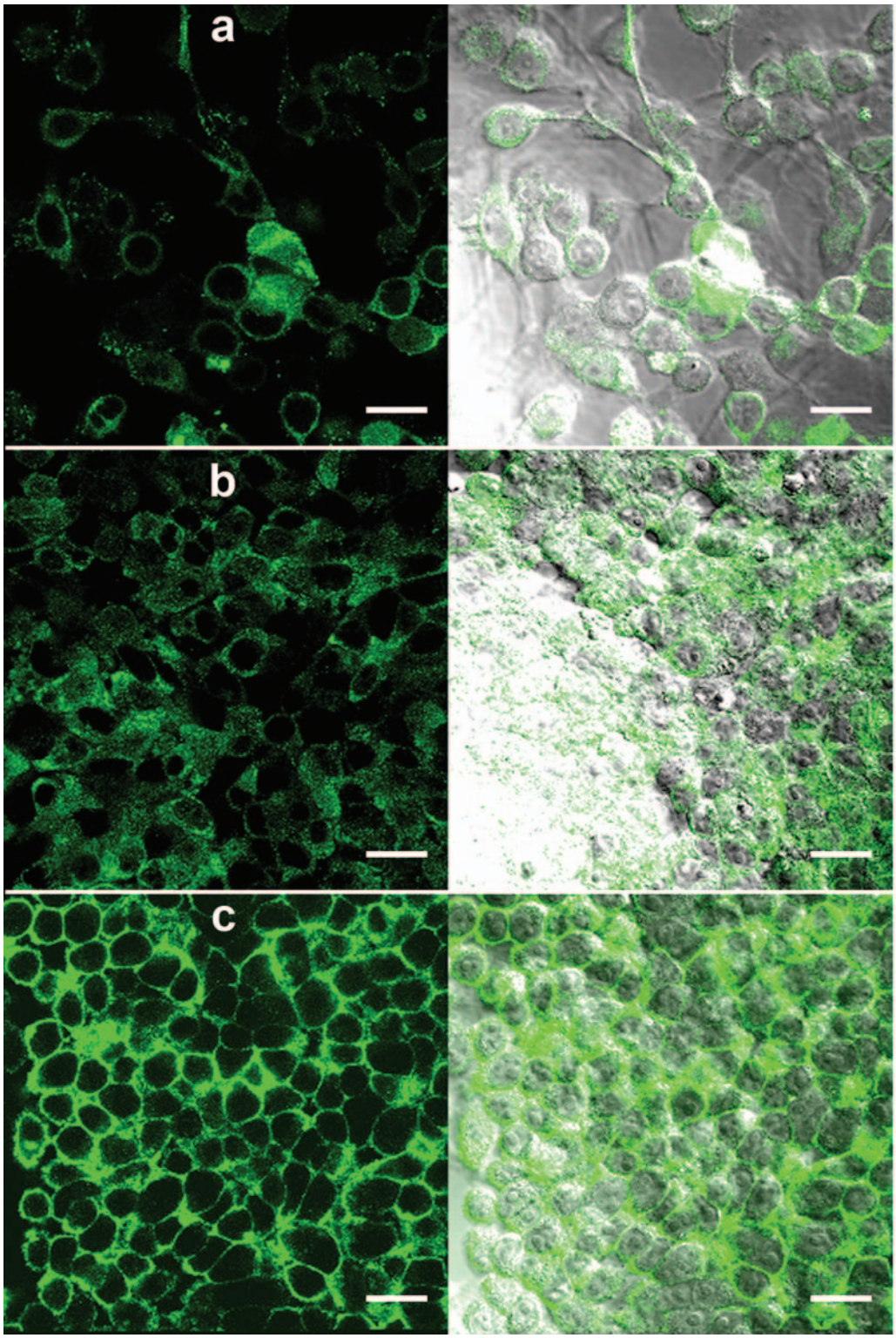

Figure 2. *Localisation of P-glycoprotein by confocal immunofluorescence microscopy in the LoVo cell lines. LoVo-f (a), LoVo-sc (b) and LoVo-Dox (c) grown on glass coverslips were fixed and fluorescent immunolabeling with MRK16/Alexa fluor® 488 was achieved for P-glycoprotein localisation, as described in Materials and Methods. Images composing the right part of the photomontage are stacked images obtained by differential interference contrast mode used for image acquisition while scanning the samples by confocal immunofluorescence microscopy, results of which are shown on the left. White bar: 20 μm.*

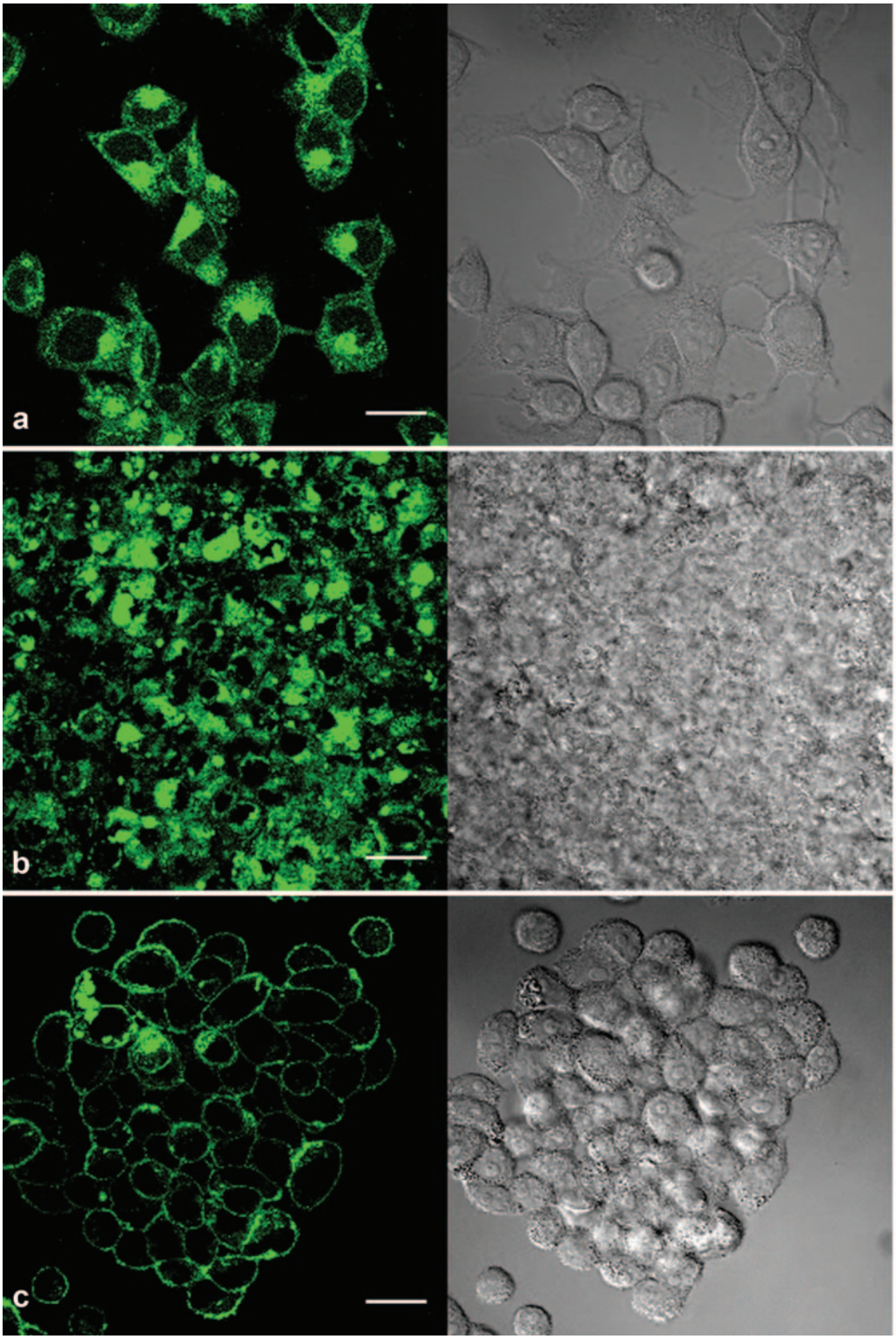

Figure 3. *Localisation of Bodipy-verapamil by confocal immunofluorescence microscopy in the LoVo cell lines. LoVo-f (a), LoVo-sc (b) and LoVo-Dox (c) grown on glass coverslips were incubated for 12 hours in the presence of 2.5 μM Bodipy-verapamil in plain medium and were fixed as described in Materials and Methods. Left panel shows Bodipy-verapamil localisation by confocal immunofluorescence microscopy. Corresponding images in the right panel were obtained by the differential interference contrast mode used for image acquisition while scanning the samples by confocal immunofluorescence microscopy. White bar: 20 μm.*

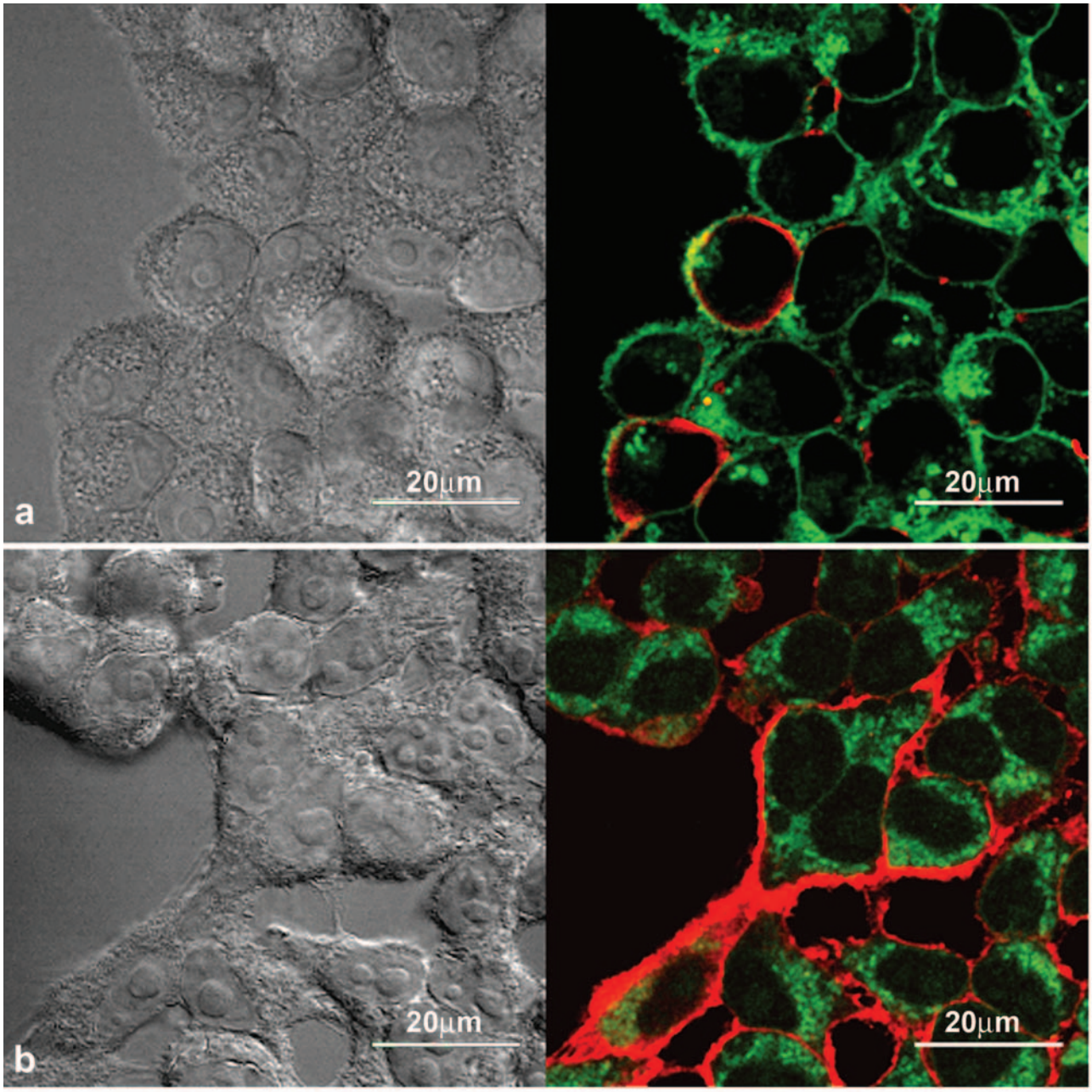

Figure 4. *Effect of doxorubicin on Bodipy-verapamil localisation in the LoVo-Dox cell line. LoVo-Dox cells grown on glass coverslips were incubated overnight either with (a) 2.5 μM Bodipy-verapamil or (b) with (2.5 μM Bodipy-verapamil+0.4 μg/ml doxorubicin). Cells were thereafter fixed and fluorescent immunolabelling with Alexa fluor® 546 as secondary antibody was achieved for P-glycoprotein detection, as described in Materials and Methods. The right panel shows Bodipy-verapamil (green labelling) and Pgp localisation (red) by confocal immunofluorescence microscopy. Corresponding images of the left panel were obtained by the differential interference contrast mode used for image acquisition while scanning the samples by confocal immunofluorescence microscopy.*

was reported for the original LoVo cell line and for some of its derived clones (2, 4, 7, 9, 33, 34). We also observed intracellular positive staining for Pgp in LoVo-sc, LoVo-f and LoVo-Dox cells. Our data are similar to those which have shown intracellular localisation of Pgp in chemosensitive LoVo wild-type cells (33, 34) and its two selected variants, variant 5 and 7, respectively. However, variant 5 showed a low level of Pgp whereas a greater level was observed in variant 7 (34). Intracellular localisation of P-gp was also reported for other colon tumorous cells (33).

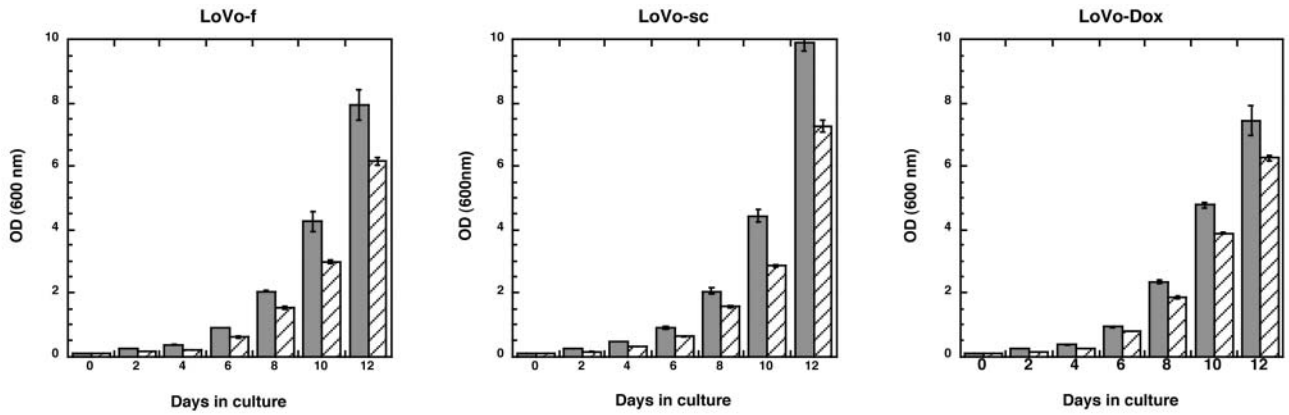

Figure 5. *Effect of pO_2 on the growth of the three LoVo variants. LoVo-f, LoVo-sc and LoVo-Dox cells were seeded at the same cell density and were cultured as described in Materials and Methods. Cultures in quadruplicates were grown at 5% O_2 (hatched columns) and at 20% O_2 (dark columns). Cell growth was determined with crystal violet staining as described in Materials and Methods.*

As also shown by Meschini *et al.* (34), we detected MRP in all of our cell lines in a cytoplasmic localisation. For the first time, LRP was detected in LoVo cell lines, a feature that may be encountered in other tumourous intestinal cells. Our results together with the data by Meschini *et al.* (34) show the importance of intracellularly localized multidrug resistant proteins in MDR cells, as well as in non-MDR cells. The presence of cytoplasmic P-glycoprotein has been reported in various chemoresistant cell lines (35-37) but few have related this localisation to any drug resistance status (37). Presently, no explanation has been found for a particular role of intracellular localised Pgp in non-MDR cells. Pgp was detected in plasma membranes and cytoplasm of normal brain capillaries. In these cells, the presence of cytoplasmic Pgp was restricted to a glycoprotein synthesis locus (38). Cytoplasmic location of Pgp and of MRP in chemosensitive cells could indicate the ability of these cells to synthesize MDR proteins dedicated for other functions such as intracellular trafficking. However, the synthesis of non-functional transporters, *e.g.* with a folding defect or which are unable to reach the plasma membrane, is not excluded but is rather speculative.

Growth of the three LoVo variants was affected by pO_2, being faster when cultured in air-CO_2 (20% O_2) than in 5% O_2. Growth at low oxygen levels has been addressed, as pO_2 of solid tumors is notably lower than the 20% O_2 widely used for cell culture. Whereas tumoral cells close to functional blood vessels are subjected to 8-10% O_2, most of the cells within a tumour are subjected to 0.1-5% O_2 according to localization and tumour compaction (39-42). Our results agree with the data published by several authors on the effect of pO_2 on tumoral cell growth, whether grown in monolayer or in suspension cultures (43-48). In contrast to tumoral cells, normal (untransformed) cells exhibit the opposite behaviour as the numerous available data obtained with various cell types showing that normal cell growth was notably faster at low pO_2 levels than at 20% O_2 (49-50). This behaviour was also demonstrated with megakaryocytes (51), macrophages (52), retinal cells (53), mammary cells (54), and cardiac cells (55) beside others.

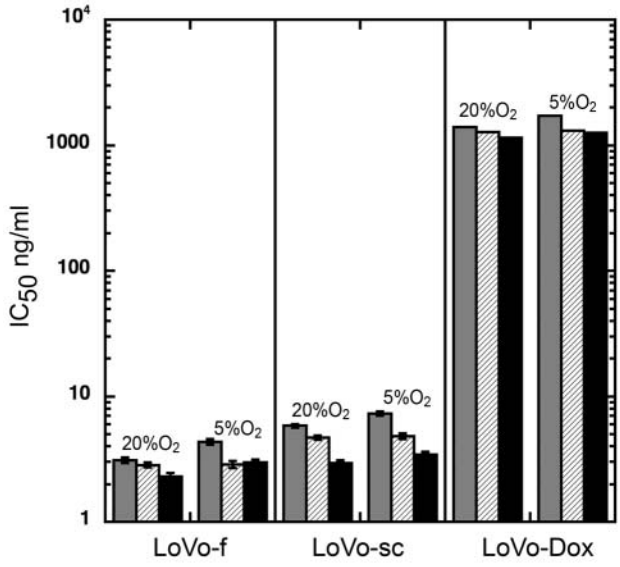

Figure 6. *Effect of pO_2 on doxorubicin and vinblastine cytotoxicity on LoVo cells. The three LoVo cell types were seeded at the same cell density and were processed thereafter as indicated in Materials and Methods. Cultures in quadruplicates were grown at 5% O_2 and at 20% O_2, respectively, in the presence either of doxorubicin (grey columns) or of vinblastine (hatched columns) or of the doxorubicin+vinblastine combination (black columns). Corresponding IC_{50}s were determined for quadruplicates of each drug treatment set. When not shown, error bars are smaller than can be shown in figure.*

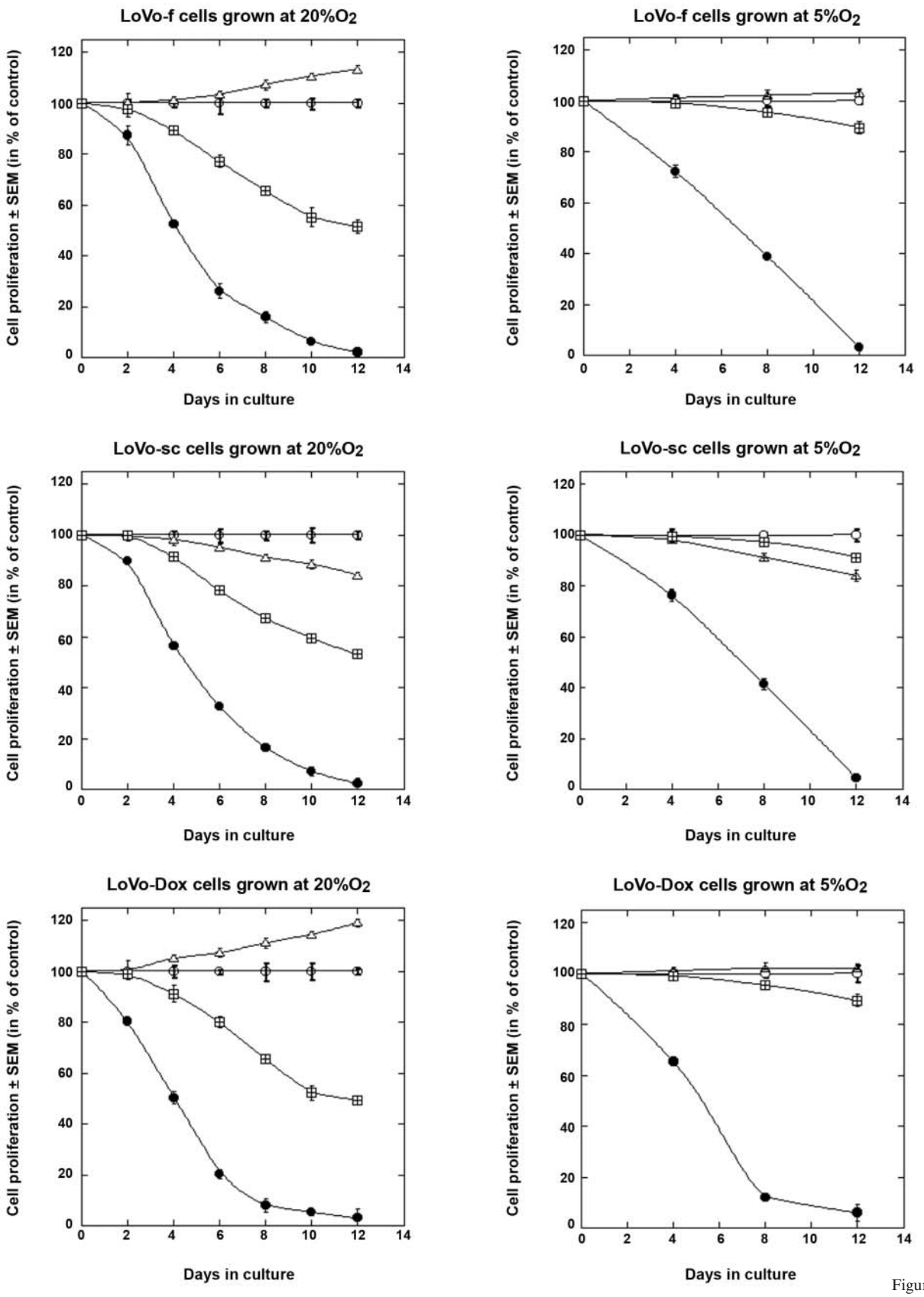
LoVo-f cells grown at 20%O2
LoVo-f cells grown at 5%O2
LoVo-sc cells grown at 20%O2
LoVo-sc cells grown at 5%O2
LoVo-Dox cells grown at 20%O2
LoVo-Dox cells grown at 5%O2
Cell proliferation ± SEM (in % of control)
Days in culture

Figure 7.

Metabolism and the mechanism of action of doxorubicin partly involve the formation of free radicals. The chemical structure of this drug implicates a redox system producing a semi-quinone free radical that in presence of O_2 enables the formation of reactive oxygen species (ROS). This is partly responsible for the anticancer activity of the drug when tested on cells grown in an air/CO_2 atmosphere (56-63). Of interest is the observation that doxorubicin cytotoxicity towards cultured cardiomyocytes increased with the oxygen level (64). In contrast to doxorubicin, the mechanism of action of vinblastine is not affected by oxygen levels (65). Our results showing increased cytotoxicity of doxorubicin at 20% O_2 compared to 5% O_2, corroborates the role of free radicals as one of the causal effects in the anticancer activity of doxorubicin.

Our present results, confirming our earlier reported findings (13) in showing that the three LoVo cell lines are more resistant to doxorubicin when grown under hypoxia than at 20% O_2, whereas no significative proliferation change in relation to oxygen level was observed with vinblastine. Similarly, Chinese hamster V79 cells are also more resistant to adriamycin under hypoxia, compared to air-CO_2 growth conditions. This is not related to a decrease of the drug uptake by the hypoxic cells (63). These observations indicate that in order to get the correct picture of an anticancer agent's activity, it is advisable to test the activity of the drug under hypoxic conditions that should be as close as possible to the *in vivo* situation.

These results are in contradiction with those obtained with rat liver microsomes by Vile and Winterbourn (64) describing an increase in lipid peroxidation by adriamycin under hypoxic conditions. However, this discrepancy can be linked to the differences in adriamycin metabolism when tested in hypoxic cultured cells *versus* isolated organelles maintained in saline buffer. The free radicals generated by anthracyclines are at the origin of cardiotoxicity and nephrotoxicity (61, 62, 65-67).

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Figure 7. *Effect of antioxidants on the cytotoxicity of doxorubicin in LoVo cell lines grown either at 5% O_2 or at 20% O_2. Different antioxidants such as carnitine, N-acetyl-cysteine, niacin, taurine or trolox were used at 2 mM concentration in combination with or without doxorubicin. Doxorubicin concentrations used were 15 ng/ml for the sensitive variants LoVo-f and LoVo-sc, and 1 μg/ml for the MDR LoVo-Dox variant, respectively. Cell proliferation was measured using crystal violet stain. Identical proliferation curves shown by ○-○ were obtained for control cultures (e.g. with no agent added) and for cells cultured in presence of either trolox, taurine, carnitine, or niacin. The proliferation curve △-△ was obtained in the presence of N-acetyl-cysteine. Identical proliferation curves shown by ●-● were obtained either with doxorubicin±taurine, doxorubicin±carnitine, doxorubicin±N-acetyl-cysteine, or doxorubicin±niacin combinations. The proliferation curve ⌗-⌗ was obtained with the doxorubicin+trolox combination. Results are expressed as mean values of quadruplicates. When not shown, error bars are smaller than symbols.*

Testing the effect of direct or indirect antioxidants, which are known to protect cardiac cells from adriamycin cytotoxicity, we have observed that neither taurine, *N*-acetyl cysteine, niacine, nor carnitine affect the level of cytotoxic activity of doxorubicin in any of our LoVo variants whether grown at 5% or at 20% O_2. Comparison of our data with those of others showed some discrepancies in the antioxidant protection towards the cytotoxic effect of anthracyclines. This could be related to the antioxidant concentration used in the protocol. In contrast to our data achieved with 2 mM *N*-acetyl cysteine, a fifty-fold higher concentration of the antioxidant (100 mM) reduced adriamycin cytotoxicity towards human ovarian carcinoma A2780 cells (68). Other sources of discrepancy could also arise from the endogenous capacity of cells to destroy ROS linked to variability of superoxyde dismutase (SOD), glutathione peroxidase, catalase activities or to the variability of glutathione concentration (69). With the exception of trolox, the fact that none of the other antioxidants affected anthracycline activity under the dose used may also be related to their respective mechanisms of action. For instance, taurine reduces the level of some lipid peroxidation end-products, such as malondialdehyde and 4-hydroxy-nonenal (70, 71) whereas niacin protects against ROS-induced damage by regenerating sufficient nicotinamide adenine dinucleotide (NAD^+) to sustain poly(ADP-ribose) polymerase (PARP) activity and to regenerate sufficient amounts of ATP (72). Carnitine protects the heart from oxidative stress by inhibiting lipid peroxidation (73), whereas *N*-acetyl cysteine improves synthesis of glutathione and consequently the activity of glutathione peroxidase, a scavenger of hydroxyl free radicals (74). The effect obtained with trolox, a water-soluble derivative of α-tocopherol, which strongly inhibited the cytotoxic effect of doxorubicin was surprising. This inhibition was by far more efficient at 5% O_2 than at 20% O_2. Since vitamin E is a chain breaking antioxidant (75, 76), prevention of ROS formation might be part of the mechanism responsible for the observed inhibition. However, as the trolox effect was more efficient at 5% O_2 compared to 20% O_2, this suggests the existence of other mechanisms rather than a pure antioxidant pathway. The higher activity of trolox at 5% O_2 may also be related to a stimulation of its uptake by cells when grown at this pO_2. This possibility was for example validated for taurine, as this amino acid is the only antioxidant whose uptake has already been tested under hypoxic conditions. Uptake of this compound, which operates in mammalian cells *via* a transporter (77), is higher in Ishikawa endometrial cancer cells grown at 5% O_2 than at 20% O_2 (78). Most of the data available on the effect of antioxidants towards anthracycline activity have been obtained with animal models, therefore involving numerous homeostatic regulations, thus being difficult to compare with *in vitro* results. It has been shown that vitamin E reduces anthracycline cytotoxicity in human

fibroblasts and melanoma cells (79) and in murine macrophages (80). In contrast, it does not modulate the effect of anthracyclines in Ehrlich ascitic cells (81). Use of antioxidant either as a preventive anticancer drug, or as adjuvant protective therapy, has been previously suggested. However, due to the heterogeneity among cell models and protocols used, a rather more standardized approach using methodologies as close as possible to the real *in vivo* parameters should be considered in order to be able to efficiently transpose *in vitro* anticancer pharmacology studies into clinical data.

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