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Antiproliferative activity of levobupivacaine and aminoimidazole carboxamide ribonucleotide on human cancer cells of variable bioenergetic profile

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ABSTRACT

We assessed the impact of ten mitoactive drugs on the viability and the proliferation of human cancer cells of variable origin and bioenergetics. A validated chemotherapeutic drug, doxorubicin, was used as a gold-standard for comparison. We also looked at the effect of these drugs on Rho^0 cells and on embryonic fibroblasts, both of which rely mainly on glycolysis to generate the vital ATP. The statistical analysis of the area under the curves revealed a cell-type specific response to mitodopant and mitotoxic compounds, in correlation with the contribution of glycolysis to cellular ATP synthesis. These findings indicate that the bioenergetic state of the cell determines in part the impact of mitodopants and mitotoxics on cancer cells viability.

1. Introduction

Bioenergetic studies have long used, and still actively utilize, specific chemical inhibitors of the mitochondrial respiratory chain complexes to study the fundamental mechanisms of energy production (Chance and Williams, 1956). Yet, rotenone, antimycin and potassium cyanide are highly cytotoxic and cannot be used safely for the clinical modulation of mitochondrial activity. In the last decade, numerous pharmacological compounds used in clinical practice were shown to inhibit mitochondrial respiration *in vivo* and *in vitro*, so that more significance was given to mitochondrial testing in pharmacological drug development (Dykens and Will, 2007). Famous examples are the use of statins to treat hypercholesterolemia, which unexpectedly block coenzyme Q biosynthesis and alter mitochondrial respiration (Folkers et al., 1990), or the use of NRTIs (nucleoside reverse transcriptase inhibitors, such as AZT) which inhibit mitochondrial DNA replication and further reduce OXPHOS capacity by other mechanisms (Benbrik et al., 1997). Several compounds used for local anesthesia also demonstrated a mitotoxic effect, as widely reported for bupivacaine which can induce muscle ultrastructural alterations and associated pain (Nouette-Gaulain et al., 2010b). On the other hand, drugs used for the treatment of type 2 diabetes, such as metformin, where shown to activate AMP-activated protein kinases (AMPK) (Lee et al., 2010) and to stimulate mitochondrial biogenesis (Suwa et al., 2006). A comparable compound named AICAR (aminoimidazole carboxamide ribonucleotide), recently evaluated in a clinical trial for the treatment of the Lesch-Nyhan syndrome, also activates AMPK and initiates mitochondrial biogenesis (Canto et al., 2009). Lastly, resveratrol (Lagouge et al., 2006), bezafibrate (Wenz et al., 2008), pioglitazone (Bogacka et al., 2005) and rosiglitazone (Wilson-Fritch et al., 2003) can also be classified as mitodopants, as they induce mitochondrial biogenesis *in vitro* and *in vivo*.

Lately, a growing interest has been given to all these mito-active compounds for developing anti-cancer metabolic therapeutic approaches

Abbreviations: ADP, adenosine diphosphate; AICAR, 5-amino-4-imidazolecarboxamide ribonucleoside; ANT, adenine nucleoide translocator; ATP, adenosine triphosphate; COX, cytochrome c oxidase; Cyt c, cytochrome c; CoQ, coenzyme Q; OXPHOS, Oxidative Phosphorylation; RCR, respiratory control ratio; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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(D'Souza et al., 2010; Fulda et al., 2010). On the one hand, mitodopants can mimic a low-energy state in cancer cells and activate mitochondrial biogenesis, leading to cell proliferation arrest or apoptosis. Such artificial increase in mitochondrial mass might destabilize the remodeled energetic machinery of cancer cells and annihilate the malignant disequilibrium between glycolysis and OXPHOS as well as perturbate the deviant glycolysis-linked anabolic processes. On the other hand, mitotoxics could directly reduce the cellular ATP supply and eventually induce ROS generation, leading to cell proliferation arrest and apoptosis. Hypothetically, the therapeutic success to either one or the other approach is likely to depend on the type of cancer cells which widely differ in their capacity and modality of energy production as well as ROS scavenging, cell cycle control and apoptosis (dys)regulation. Specifically, bioenergetic studies indicate the existence of two main classes of tumors, deriving their vital ATP mainly from glycolysis or from OXPHOS (for review see Jose et al., 2010a) which may respond differently to mito-active compounds.

Here, we investigated (i) the specific impact of five mitodopants and of five mitotoxics on cancer cell viability of variable bioenergetic profile and (ii) the correlation between the magnitude of this effect and the steady-state cellular ATP content. A validated chemotherapeutic drug, doxorubicin, was used as a gold-standard for the evaluation of both the specificy and the potency of the cancer-killing effect of the mitoactive compounds assayed in our study. We also looked at the impact of these drugs on cells lacking mitochondrial DNA (Rho^0 cells), which rely exclusively on glycolysis to generate the vital ATP and on embryonic cells which are thought to behave energetically as cancer cells (Kim et al., 2010). Our comparative analysis revealed that the sole intracellular ATP level does not determine the cancer specific impact of AICAR. Conversely, we observed a strong correlation between levobupivacaine anti-cancer effect and the extent of glycolytic ATP production.

2. Methods

2.1. Chemicals

All the reagents were purchased from Sigma-Aldrich, with the exception of the ATP monitoring kit (ATP Bioluminescence Assay Kit HS II from Roche). The local anesthetic dilutions were prepared as for the clinical practice: 0.5% bupivacaine hydrochloride, 0.75% ropivacaine hydrochloride, 1% lidocaine hydrochloride and 1% mepivacaine hydrochloride (purchased from AstraZeneca; Rueil-Malmaison, France). 0.5% levobupivacaine hydrochloride was obtained from Abbott (Rungis, France).

2.2. Cell types and culture conditions

HeLa, DU145, HEPG2, 143B, HTB126 and MRC-5 cells were purchased from ATCC. The primary monolayer epithelial cell line EM64, the primary monolayer fibroblasts FM14 and DF, the human skeletal muscle cell line (HSMM) and the primary fibroblasts derived Rho^0 cells were prepared in our laboratory. A description of each cell line is given in Table 1. Cells were grown in Glucose Dulbecco's Modified Eagle Media (DMEM) containing 25 mM glucose supplemented with 10% fetal bovine serum (*PAA*), 100 U/ml penicillin, and 100 U/ml of streptomycin. All cells were kept in 5% CO_2 at 37 °C. For all experiments cells were harvested during exponential phase of growth at 70% confluency.

2.3. Cell treatment with mitodopant and mitotoxic compounds

Stock solutions of pioglitazone and rosiglitazone were prepared in DMSO at a concentration of 2.5 mM and 7 mM respectively. Resveratrol was diluted in acetone at 12 mM, bezafibrate in methanol at 51 mM. AICAR and doxorubicin were diluted in sterile water at a concentration of 14.8 mM and 1.7 mM, respectively. Stock solutions of local anesthetics were provided in hydrochloride solution of 5 mg/ml (see above). All the subsequent dilutions were done in DMEM before each treatment. The final concentration of solvents used for the cell treatments did not exceed 0.5%.

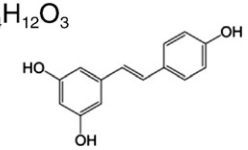
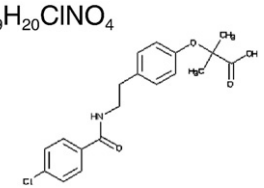
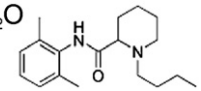
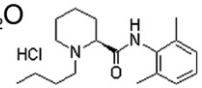
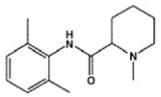
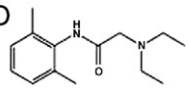
2.4. ATP measurements and cell respiration

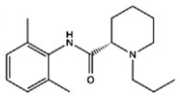
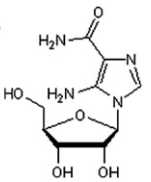
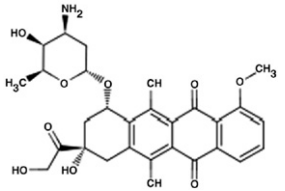
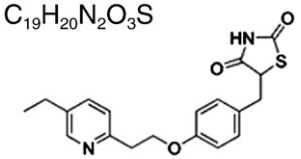
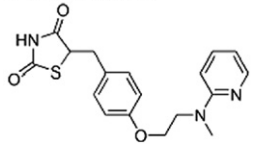
The intracellular ATP content was measured by using the bioluminescent ATP kit HS II (Roche Applied). A suspension of 1×10^6 cells/ml in DMEM was mixed with lysis buffer and incubated for 5 min at room temperature to release the intracellular ATP. 100 µl of cell lysate and 100 µl of luciferase were injected in a bioluminometer multiplate reader (Luminoskan) and after 10 s of incubation, bioluminescence was read (1 s integration time). Standardization was performed with known quantities of standard ATP provided with the kit and measured in the same conditions. The contributions of glycolysis and of mitochondrial oxidative phosphorylation to cellular ATP synthesis were obtained by measuring the total ATP content (as described above) in cells incubated at 37 °C in DMEM for 20 min with

Table 1
Cell lines description and bioenergetic profile characterization. For each cell line, the measurements of cell proliferation, cell respiration, ATP content and contribution by OXHOS and glycolysis were performed with a minimum of N≥4 independent experiments, as indicated in the table. The results are expressed as mean (standard deviation). n.d stands for not determined.

Cell line	Organ	Cancer	Tissue	Age	Gender	Ethnicity	Proliferation (t_{48}/t_0)	Routine respiration (ng·atom O/min/10^E6 cells)	Total ATP (pmol per 10E6 cells)	% Glycolytic ATP
Tumor derived cell lines										
HeLa	Cervix	Adenocarcinoma	Epithelial	31	Female	Black	3.76	6.75 (0.35)	0.53 (0.10)	79
143B	Bone	Osteosarcoma	Epithelial	15	Female	Caucasian	n.d.	4.39 (0.82)	0.71 (0.03)	94
HTB126	Breast	Carcinoma	Epithelial	74	Female	Caucasian	n.d.	2.01 (0.07)	0.47 (0.14)	86
DU145	Prostate	Carcinoma	Epithelial	69	Male	Caucasian	6.38	2.96 (0.36)	0.63 (0.09)	96
HEP G2	Liver	Hepatocellular carcinoma	Epithelial	15	Male	Caucasian	1.68	4.13 (0.21)	0.61 (0.10)	76
Adult normal cell lines										
EM64	n.c.	Normal	Epithelial	Adult	n.d.	n.d.	2.18	2.66 (0.78)	0.96 (0.08)	71
FM14	Skin	Normal	Fibroblast	Adult	Female	Caucasian	1.30	4.40 (1.09)	1.40 (0.16)	48
DF	Skin	Normal	Fibroblast	Adult	n.d.	n.d.	n.d.	10.20 (0.49)	0.32 (0.02)	56
HSMM	Striated muscle	Normal	Smooth Muscle	Adult	n.d.	n.d.	n.d.	8.26 (0.28)	0.73 (0.13)	54
Manipulated and embryonic cell lines										
Rho^0	Skin	BET treated	Fibroblast	Adult	Female	Caucasian	2.06	0.84 (0.31)	0.71 (0.09)	100
MRC-5	Lung	Embryonic	Fibroblast	Fetal	Male	Caucasian	3.08	6.92 (1.18)	0.63 (0.12)	95

Table 2
Metabolic profile of the primary cells and of the tumor-derived cell lines.

Molecule	Structure and formula	MW g·mol^{-1}	Solubility	Half-life	Molecular targets	Drug category	Indications
Resveratrol	$C_{14}H_{12}O_3$ 	228.24	Acetone, ethanol, DMSO, water	9 h	Sirtuin 1, PGC-1α	Antioxidant, mitodopant, antiapoptotic	Anti-aging
Bezafibrate	$C_{19}H_{20}ClNO_4$ 	361.8	methanol	1–2 h	PPAR-α	Antilipemic agent, mitodopant	Hyperlipidemia
Bupivacaine	$C_{18}H_{28}N_2O$ 	288.4	Water, PBS, DMSO	(IV) 162 min ou 1.2–2.9 h	Sodium channel	Local anesthetics, mitotoxic	Regional anesthesia and wound infiltration
Levobupivacaine	$C_{18}H_{28}N_2O$ 	288.4	Water, PBS, DMSOs	(IV) 157 ± 77 min	Sodium channel		
Mepivacaine	$C_{15}H_{22}N_2O$ 	246.3	Water, PBS, DMSO	(IV) 1.9–3.2 h	Sodium channel		Regional anesthesia
Lidocaine	$C_{14}H_{22}N_2O$ 	234.3	Water, PBS, DMSO	(IV) 1.0–2.2 h	Sodium channel		

Ropivacaine	$C_{17}H_{26}N_2O$ 	274.4	Water, PBS, DMSO	(IV)111 ± 62 min	Sodium channel		Regional anesthesia and wound infiltration
AICAR N1-(b-D-Ribofuranosyl)-5-aminoimidazole-4-carboxamide	$C_9H_{14}N_4O_5$ 	258.2	Water, DMSO	12–18.5 h	AMP-activated protein Kinase activator	Adenosine regulating agent , mitodopant	Acute lymphoblastic Leukemia and diabetes
Doxorubicin	$C_{27}H_{29}NO_{11}$ 	543.5	Water	55 h	DNA (intercaling agent) Topoisomerase II-α	Antibiotics, antineoplastic agent (gold standard)	Cancer
Pioglitazone	$C_{19}H_{20}N_2O_3S$ 	356.4	DMSO, dimethyl formamide	3–7 h	PPAR-α PPAR-γ	Antihyperglycemic, antidiabetic agent, mitodopant	Diabetes mellitus type 2
Rosiglitazone	$C_{18}H_{19}N_3O_3S$ 	357.4	DMSO, ethanol	3–4 h	PPAR-γ	Antidiabetic agent, mitodopant	Diabetes mellitus type 2

specific inhibitors of these pathways, 200 μM iodoacetate or 10 μM antimycin, respectively. Mitochondrial oxygen consumption was monitored at 37 °C in a 1 ml thermostatically controlled chamber equipped with a Clark oxygen electrode (Oxy 1, *Hansatech*). The routine (endogenous) respiration was measured on 1×10^6 cells placed in DMEM with 5.5 mM glucose.

2.5. Cell enumeration and cell viability

Proliferation rate was evaluated on the different cell lines by counting the cells after 48 h of growth in DMEM using a Malassez haemocytometer. Cells were seeded in 6 well plates (100 000 cells per well) and grown for 48 h, then trypsinized and counted manually ($N>3$ for each condition). The cytotoxicity of the 11 drugs was evaluated on the different cell lines using the neutral red assay as detailed by Borenfreund (Borenfreund and Puerner, 1985; Nouette-Gaulain et al., 2009a). Absorbance was measured in a multi-well scanning spectrophotometer (MP96 from SAFAS) at a wavelength of 540 nm with a reference set at 630 nm. For each condition, multiple replicates ($n\geq6$) were performed. The results were expressed as percent value of the control absorbance ratio.

2.6. Statistical analysis

All the data presented in this study correspond to the mean value of N experiments ±SD, with $N\geq3$. It was not always possible to determine the IC_{50} since in some cases cytotoxicity was very low (even at the highest drug concentrations), while in other cases cytotoxicity was already high at the lowest drug concentrations. In those instances, the IC_{50} was poorly estimated. The area under the curve (AUC) index gives an integrative view of the effect of drugs because it takes into account the inhibition of cells viability at all the tested doses of drugs. This gives a more complete information on drug sensitivity at lower and higher dose levels. The AUC has been calculated for each cell lines for each treatment with Prism 5 Software. Comparisons of AUCs of the cancer versus non-cancer groups of cell lines were performed with the Student's *t* test, using Excel Software (Microsoft). Two sets of data (cancer group versus non-cancer group, or treated cells versus untreated ones) were considered statistically different when $P<0.05$. The correlation studies were performed as follow: we performed first a series of linear regressions between the AUCs obtained in the different cell lines treated with the 11 drugs and three parameters (total cellular ATP level, glycolytic ATP and respiratory rate). The Pearson's correlation coefficient (R^2) was determined from these linear regressions using Prism 5. The test of significance was also performed using Prism 5 and calculated a p value for each correlation. It was based on the assumption that the distribution of the residual values (i.e., the deviations from the regression line) for the dependent variable followed the normal distribution, and that the variability of the residual values was the same for all values of the independent variable.

3. Results

3.1. Metabolic profile of the tumor-derived cell lines and of the human primary cells

The tissue of origin and the type of tumor from which the cancer and the non-cancer cells used in this study originated are specified in Table 1. We evaluated first the rate of cell proliferation by measuring the net increase in cell number after 48 h of growth during the exponential phase. We expressed growth rate as the ratio of cells counted after 48 h over the number of cells seeded at t_0 (Table 1). DU145 can be considered as a fast-growing cancer cell line followed by HeLa cells. In contrast, HEPG2 and the group of control cells (FM14 and EM64) showed lower rates of cell proliferation. The embryonic cell line MRC-5 presented a relatively high proliferation rate, while the Rho^0 cells presented a low rate of growth. The rate of routine respiration measured in DMEM (same conditions as cell growth and ATP content evaluation) was not statistically different ($p=0.114$) in cancer cells versus non-cancer cells. The total ATP content was generally higher in most non-cancer cells tested (EM64, FM14 and CML) as compared to cancer cells, at the exception of the dermo-fibroblasts cell line (DF) which presented a low intracellular ATP content (0.32 pmol/10^6 cells). Yet, these cells were used at late passage (8–13) which might coincide with the onset of senescence and alter both growth and bioenergetic properties of these cells (Hutter et al., 2004). The relative contribution of sole glycolysis and of oxidative phosphorylation (OXPHOS) to the cellular ATP production was measured by using specific inhibitors of glycolysis (200 μM iodoacetate) and of OXPHOS (10 μg/ml antimycin A), respectively. It can be seen in Table 1 that all the tumor-derived cell lines used in this study produced their vital ATP mainly through glycolysis (from 76 to 96% of the total ATP produced), while non-cancer cells relied on glycolysis to a significant ($p=0.0011$) lesser extent (from 48 to 71%). As expected, the Rho^0 cells, depleted of mitochondrial DNA, relied exclusively on glycolysis to produce ATP. The embryonic lung fibroblasts (MRC-5) also used glycolysis to a larger extent (95%) as compared to adult non-cancer cells.

3.2. Impact of mitodopants on cancer cells viability

In the group of mitodopants, we included drugs which were previously shown to stimulate mitochondrial biogenesis *in vitro*: AICAR, resveratrol, bezafibrate, pioglitazone and rosiglitazone. The molecular structure and the chemical properties of these drugs are summarized in Table 2. We treated the cancer and the non-cancer cells with 6 clinically relevant concentrations of each of these drugs during 48 h and measured cell viability by using the neutral red assay. For levobupivacaine, lidocaine, bupivacaine, mepivacaine and ropivacaine, we tested the following concentrations: 10, 100, 500, 1000, 3000 and 5000 μM; for doxorubicin: 0.1, 1, 5, 10, 25 and 50 μM; for bezafibrate: 50, 100, 200, 500, 1000 and 1350 μM; for resveratrol: 10, 20, 50, 100, 200 and 500 μM, for AICAR: 50, 100, 250, 500, 750 and 1000; for rosiglitazone: 0.1, 0.2, 2, 5, 10 and 20 μM and for pioglitazone: 2.5, 5, 10, 25, 50 and 100 μM. The neutral red assay gives a measure of the active (ATP-dependent) uptake of neutral red inside the cell. Previous experiments indicated that the neutral red assay can be used as a metabolic index which directly depends on cellular ATP production (Nouette-Gaulain et al., 2009a; Smolkova et al., 2010). In our study, the cell lines were regrouped in two categories, cancer-derived or primary non-cancer cells (Table 1). The Rho^0 and the MRC-5 cells were analyzed separately due to their specific characteristics. An example of the impact of a mitodopant (AICAR) on cell viability is illustrated in (Fig. 1A–I) for the 9 cell lines tested. The shape of the drug–response curves varies widely according to the type of compound used for treating the cells, as well as with the type of cells, so that a unique appropriate drug–response model could not be used to fit these curves and to calculate an IC_{50}. In these conditions, it was more appropriate to calculate the area under the curve (AUC) in order to compare the impact of the different mitodopants on the two groups of cell lines. The AUC values obtained for AICAR and for the other drugs tested are listed in Table 3, as well as the student-test *p* values obtained from the comparison of the two groups of cells. Only one compound, AICAR, revealed a significant cancer-specific toxicity ($p=0.0089$). As expected, doxorubicin reduced cell viability with a high significance in the cancer group as compared to the non-cancer cells ($p=0.0002$). Moreover, we observed different sensitivities to doxorubicin among the different cancer cells (Table 3). For instance 143B cells (AUC = 4.0) were more severely affected than DU145 cells (AUC = 12.1). Interestingly, the impact of AICAR on Rho^0 cells was quasi-null (Fig. 1I), indicating that the sole property of deriving ATP exclusively from glycolysis cannot explain why cancer cells are more

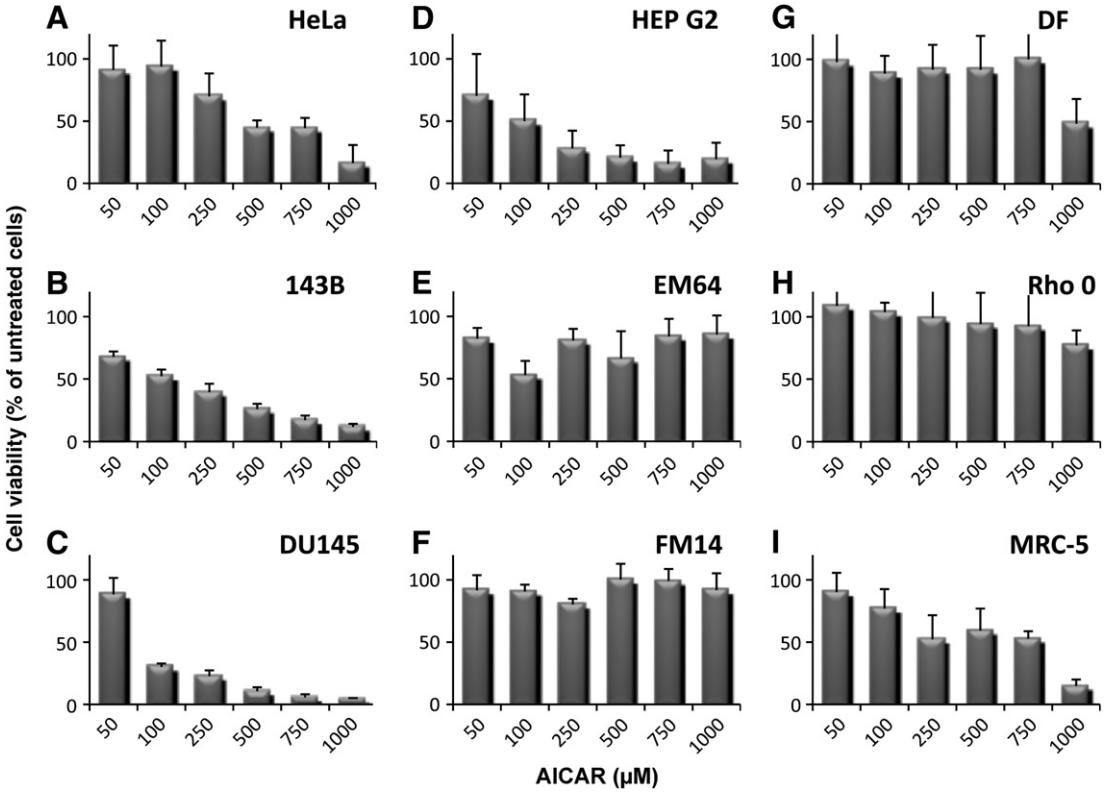


Fig. 1. Effect of the mitodopant AICAR on different cancer and non-cancer cell lines after 48 h treatment with 6 concentrations. The values are expressed as means ± SD.

sensitive to this drug. MRC-5 cells which derive ATP mainly by glycolysis were also less sensitive to AICAR as compared to cancer cells.

3.3. Impact of mitotoxics on cancer cells viability

The group of mitotoxic drugs included the following local anesthetics: bupivacaine, levobupivacaine, lidocaine, mepivacaine and ropivacaine (see formulas and drug-properties in Table 2). We focused on local anesthetics since previous studies indicated an impairment of OXPHOS in tissues and cells treated with some of these molecules (Galbes et al., 2010; Nouette-Gaulain et al., 2009a, 2009c, 2010a, 2010b). An example of titration curves of cell viability with local anesthetics (levobupivacaine) is illustrated in (Fig. 2A–J). As for mitodopants, we calculated the AUC for all the tested drugs to compare the impact of the different treatments on the group of cancer-cells versus the group of non-cancer cells (Table 3). The results indicate that levobupivacaine triggered a cancer-specific reduction of cell viability with $p = 0.0379$. Ropivacaine was the second more effective anti-cancer drug, at the limit of significance ($p = 0.0527$). The two cancer cell lines HEPG2 and DU145 were particularly sensitive to the levobupivacaine treatment with AUC values of 96.92 and 68.16, respectively, as compared to 185 ± 23 for the non-cancer group. As discussed above doxorubicin showed a strong anti-cancer specificity ($p = 0.0002$).

Table 3
AUCs values of the 11 drugs for each group of cells. The titration curves were repeated with a minimum of 4 independent experiments for each drug and for each cell line. The Student's t-test (cancer versus non-cancer cells) are indicated in italics for each drug. Significant p ($p \leq 0.05$) are bolded.

	AICAR	Resveratrol	Bezafibrate	Rosiglitazone	Pioglitazone	Doxorubicine	Bupivacaine	Levobupivacaine	Lidocaine	Mepivacaine	Ropivacaine
Cancer cells											
HeLa	89.2	46.7	77.5	61.8	115.4	11.7	168.0	191.3	179.6	243.2	166.3
143B	51.7	65.1	100.2	98.9	112.0	4.0	166.1	141.4	263.8	245.7	182.5
HTB126		111.5	129.6				176.9	151.7	237.9	258.7	197.4
DU145	35.3	78.7	106.4	106.4	89.2	12.1	174.5	68.2	161.6	126.6	175.3
HEP G2	46.2	60.9	106.9	59.8	47.4	8.1	99.2	96.9	124.6	129.8	170.6
Adult non-cancer cells											
EM64	92.7	67.4	115.7	69.5	99.9	40.2	151.3	158.8	203.6	208.0	149.5
FM14	118.0	101.4	121.8	90.0	130.7		165.0	185.2	239.3	261.5	226.2
DF	117.5	101.0	103.8	99.8	101.3	45.5	154.5	215.1	281.3	207.3	247.2
HSMM		97.8	97.1				177.6	180.9	222.6	191.7	254.1
P values	**0.0089**	0.1107	0.3117	0.3909	0.1896	**0.0002**	0.3872	**0.0379**	0.1110	0.3333	**0.0527**
Transformed and embryonic cells											
Rho^0	128.4	84.5	86.2	75.8	77.1		56.9	60.8	205.7	242.2	81.7
MRC-5	81.7	63.3		101.4	133.0	74.8	157.5	138.9	204.9	234.2	165.5

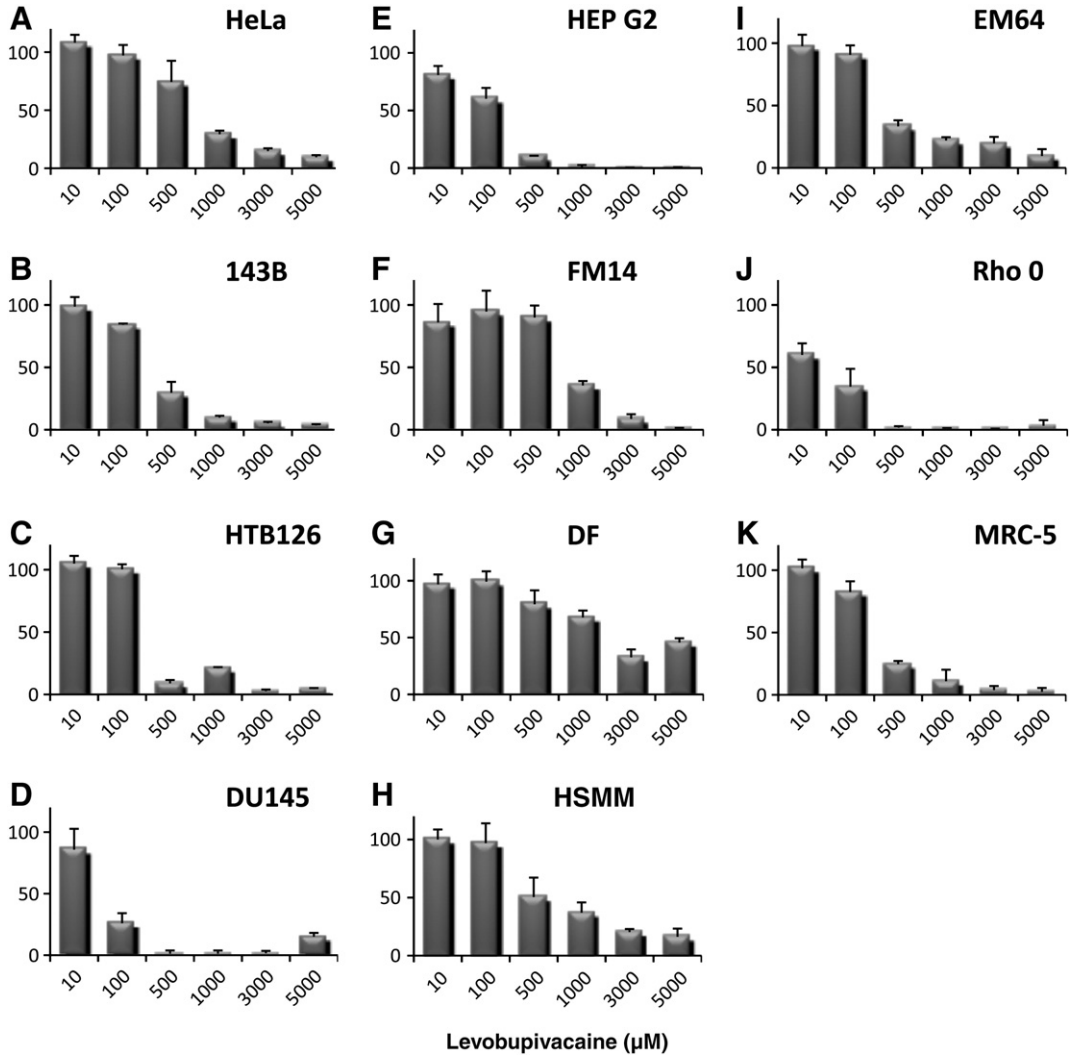


Fig. 2. Effect of the mitotoxic levobupivacaine on different cancer and non-cancer cell lines after 48 h treatment with 6 concentrations. The values are expressed as means ± SD.

3.4. Impact of AICAR and of levobupivacaine on cell proliferation

The viability studies detailed above require additional cell enumeration analyses to understand whether mitodopants or mitotoxics inhibit cell proliferation or solely impact energy metabolism. As AICAR and levobupivacaine demonstrated the more significant anti-cancer effects, we analyzed the impact of these two compounds on the proliferation of different types of cell over 48 h of growth. We chose a concentration of AICAR (750 μM) and of levobupicavaine (1 mM) which allowed to discriminate the two groups of cells (at this concentration the reduction of cell viability is stronger in the group of cancer cells as compared to the non-cancer cells with $p<0.05$). A comparison of the impact of AICAR or levobupivacaine on neutral red uptake versus cell growth (expressed as percentage of viability and percentage of growth measured in the untreated cells) is given in (Fig. 3). For AICAR (Fig. 3A) the changes in cell viability are paralleled by comparable changes in cell number, indicating that AICAR reduces cell proliferation in cancer cells. In DU145, the impact of AICAR was stronger on cell viability as compared to cell number, indicating an additional effect on energy metabolism. For levobupivacaine and ropivacaine (Fig. 3B), there was also a close correlation between the reduction of cell viability and cell growth, indicating an anti-proliferative effect of levobupivacaine on DU145 cancer cells. No sign of cell death was noticed during the counting procedure in cells treated with AICAR and levobupivacaine.

3.5. Importance of the cellular bioenergetic properties for mito-active drugs

To assess a possible correlation between the observed anti-cancer effect of a given compound and the cellular bioenergetic profile we performed a test of correlation (Pearson's) between the impact of a given compound on cell viability, as determined by the AUC, and the following parameters: (i) total steady-state ATP content, (ii) glycolytic ATP synthesis and (iii) routine respiratory rate. The results of this correlative analysis are given in Table 4. It can be seen that for two local anesthetics, levobupivacaine and ropivacaine, a significant correlation exists between the impact of these drugs on cancer cells proliferation and both the level of glycolytically produced (positive correlation) ATP and cell respiration (negative correlation). These results suggest that levobupivacaine could be an effective anti-proliferative drug against predominantly glycolytic cancer cells.

4. Discussion

A wide metabolic remodeling occurs during tumorigenesis to adapt energy production to cancer specific anabolic needs. Particularly, mitochondria undergo a series of modifications which vary among tumors and throughout carcinogenesis (Bellance et al., 2009; Jose et al., 2010a). Therefore, adapted metabolic therapies necessitate to determine first the “bioenergetic stage” of the tumor, as determined by the

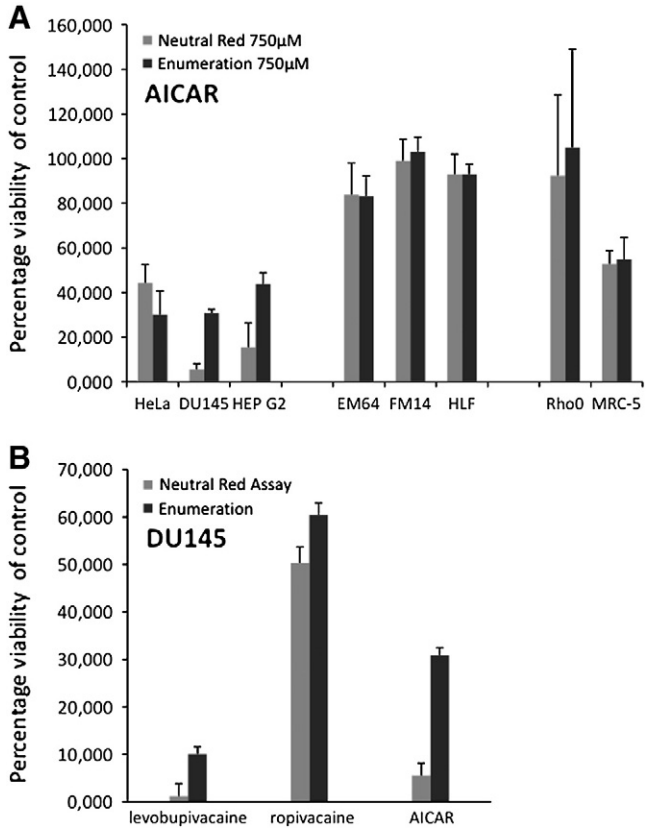


Fig. 3. Comparison between the results of neutral red assay and the manual enumeration performed after 48 h of treatment of: A) different cancer cells and non cancer cells with AICAR and B) the cancer cell DU145 with levobupivacaine, ropivacaine and AICAR. The values are expressed as means ± SD.

modality of energy substrate utilization and the molecular changes that occurred in the metabolic machinery (Jose et al., 2010a). According to the bioenergetic profile of the tumor, adapted pharmacological approaches could be developed to block specifically the predominant mechanisms of energy production and the linked anabolic pathways. The diversity of the molecular changes observed between different types of cultured cancer cells also exists between tumors, and even inside a tumor (Kallinowski et al., 1989; Vaupel and Hockel, 2000; Zu and Guppy, 2004). Here, we compared two types of human cells in culture, either derived from tumors or obtained from normal adult tissues. In addition, we looked at cells of embryonic origin and at cells devoid of mitochondrial DNA. To evaluate the impact of the sole pharmacological treatment on cell viability we used the same growth medium for all cell types despite their different nature (cancer, adult non-cancer, transformed and embryonic). This situation might not provide optimal growing conditions for all the cells, and could have impacted the sensitivity of the different cell lines to mitodopants and mitotoxics.

The first part of our work established the bioenergetic profile of these cells under comparable conditions of energy substrate availability. The cancer cells presented a higher dependency on glycolysis to generate ATP, but the non-cancer cells also used glycolysis to a large extent. This is not surprising as previous studies indicated that the sole fact of plating primary cells in culture dishes triggers a metabolic remodeling toward a more glycolytic phenotype (Gstraunthaler et al., 1999). Yet, the sole percentage of ATP produced by glycolysis permitted to separate the two groups of cells with a p value of 0.0011, while the values of cell proliferation rate or of total intracellular ATP levels did not show a significant difference between cancer and non-cancer cells.

We assayed different pharmacological compounds capable of either stimulating OXPHOS, the mitodopants, or to inhibit mitochondrial energy production, the mitotoxics. Hence, AICAR, resveratrol and bezafibrate are classified as mitodopants since previous studies demonstrated their stimulatory effect on mitochondrial biogenesis and consecutive cellular respiration. Some of these molecules were also tested for their anti-cancer potential in parcellar analyses (Guo et al., 2009; Tenenbaum et al., 2008; van Ginkel et al., 2007), but their potency was not evaluated as regard to the bioenergetic profile of the treated cells. Our results show that AICAR alters strongly and specifically the viability of cancer cells, while the two other tested mitodopants had no specificity of action. The underlying mechanisms by which AICAR reduces cell proliferation are multiple and might depend on cancer cell physiology (Guo et al., 2009; Jung et al., 2004; Rattan et al., 2005; Sengupta et al., 2007; Su et al., 2007; Woodard and Platanias, 2010; Yu et al., 2009). Interestingly, the Rho^0 cells which solely rely on glycolysis to survive were not affected by the AICAR treatment, indicating that the pathway primarily used for energy production does not determine by itself the sensitivity to AICAR. In a recent study, we showed that AICAR cytotoxicity involves three modes of action which include (i) the stimulation of mitochondrial apoptotic pathway with compensatory activation of Akt and upregulation of oxidative phosphorylation, (ii) reduction of Akt phosphorylation and (iii) activation of apoptosis by different pathways (Jose et al., 2010b). The use of AICAR in therapy is impeded by a high toxicity, as shown in clinical trials (Dixon et al., 1991). Undesired metabolic effects of AICAR also limit its utilization in cancer therapies. Yet, the strategy of mimicking a low-energy state in cancer cells to reduce cancer cell viability is still valid, and the research for identifying novel energy restriction-mimetic agents (ERMAs) (Wei et al., 2010) capable of reducing human tumor growth will undoubtedly benefit from a better bioenergetic characterization of the different cancer cell lines, in particular for what concern their response to challenging energy conditions as occurs in hypoxia and aglycemia (Smolkova et al., 2010).

The mitotoxic compounds tested in our study were local anesthetics as bupivacaine was shown to inhibit OXPHOS by two additive mechanisms: kinetic inhibition of oxidative phosphorylation

Table 4
Correlation analyses. The Pearson's correlation coefficient were calculated from the linear regressions between the AUC values obtained for a each drug on different cell lines (N = 4) and different parameters related to these cells: the proliferation ratio (N = 8), the total ATP content (N=8), the glycolytic ATP (N = 8), and the endogenous respiration (N = 5). Pearson's coefficients are expressed as R2 and significant correlations ($p \leq 0.05$) are bolded. * indicates $p \leq 0.005$. N indicates a negative correlation.

	AICAR	Resveratrol	Bezafibrate	Rosiglitazone	Pioglitazone	Doxorubicine	Bupivacaine	Levobupivacaine	Lidocaine	Mepivacaine	Ropivacaine
Proliferation/drug	0.61 N	0.22 N	0.24 N	0.51	0.05	0.23 N	0.48	0.30	0.33 N	0.46 N	0.03
Total ATP/drug	0.23	0.09	0.32	0.06 N	0.32	0.04 N	0.02	0.04	0.02	0.23	0.00
Glycolytic ATP/drug	0.44 N	0.42 N	0.26 N	0.13	0.19 N	0.14 N	0.30	**0.74*** N	0.30 N	0.03 N	**0.75*** N
Respiration/drug	0.14	0.01	0.28 N	0.27	0.39	0.44	0.41	**0.71**	0.33	0.04 N	0.70

(Nouette-Gaulain et al., 2007, 2009b) and ii) reduction of mitochondrial content (Benard et al., 2006). The different modes of bupivacaine inhibition on mitochondrial energy metabolism include: the specific inhibition of mitochondrial respiratory chain complex I (as observed on isolated mitochondria) (Sztark et al., 1998, 2000), ii) OXPHOS uncoupling (Schonfeld et al., 1992; Terada et al., 1990), iii) the specific inhibition of the mitochondrial F_1-F_0 ATP synthase, iv) the decrease of mitochondrial membrane electric potential (Nouette-Gaulain et al., 2009a; Sun and Garlid, 1992), v) fragmentation of the mitochondrial network (Nouette-Gaulain et al., 2009a), vi) the possible onset of mitoptosis and vii), the reduction of the respiratory chain protein content, which can be observed for long-lasting exposure to bupivacaine. In our study, we observed that levobupivacaine induced a specific loss of cancer cells viability which was positively correlated to their dependency of using glycolysis to synthesize ATP. These cells presented a low routine respiratory rate, suggesting that cancer cells with low mitochondrial oxidative capacity might be more affected by levobupivacaine which further reduces respiration and ATP production. Accordingly, it was shown that bupivacaine toxicity is stronger in conditions of chronic hypoxia (Nouette-Gaulain et al., 2002) where energy is preferentially produced by glycolysis. The advantage of using local anesthetics for eventual anti-cancer therapy is the possibility of delivering these molecules inside the surgical site after tumor removal, at the vicinity of eventual residual cancer cells, to maximize the specificity of action of these drugs and to minimize their potential cytotoxic effect on surrounding tissues.

5. Conclusion

Tentative bioenergetic therapy of cancer could use pharmacological modulators of energy metabolism to perturbate the peculiar modalities of cancer cells energy production, in order to alter cell proliferation and eventually trigger specific cancer cell death. It would require first a biochemical evaluation of the tumor's energy metabolism and a clinical measurement of tumor size and growth rate. Our preliminary studies exemplify the use of previously validated modulators of energy metabolism, classified as mitodopants and mitotoxics, to reduce cancer cells proliferation rate. Our results demonstrate a strong and specific cytotoxic effect of AICAR and of levobupivacaine on cancer cells viability. Yet, these two compounds trigger opposite effects on energy metabolism since AICAR typically stimulate OXPHOS while levobupivacaine generally inhibits mitochondrial energy production. Furthermore, these two compounds have pleiotropic effects on cultured cells, and we cannot simply conclude from our results that cancer cell proliferation is reduced as a direct consequence of changes in energy metabolism. Our findings further indicate that the sole intracellular ATP level does not determine the cytotoxic effect of AICAR, while a low respiratory rate associates with a strong sensitivity to levobupivacaine cytotoxicity. These observations might stimulate further research for testing combinations of metabolic drugs, as well as discovering new compounds aiming at increasing both the specificity and the potency of bioenergetic therapy.

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