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PRECLINICAL STUDIES

Screening of well-established drugs targeting cancer metabolism: reproducibility of the efficacy of a highly effective drug combination in mice.

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Summary Alterations in metabolic pathways are known to characterize cancer. In order to suppress cancer growth, however, multiple proteins involved in these pathways have to be targeted simultaneously. We have developed a screening method to assess the best drug combination for cancer treatment based on targeting several factors implicated in tumor specific metabolism. Following a review of the literature, we identified those enzymes known to be deregulated in cancer and established a list of sixty-two drugs targeting them. These molecules are used routinely in clinical settings for diseases other than cancer. We screened a first library in vitro against four cell lines and then evaluated the most promising binary combinations in vivo against three murine syngeneic cancer models, (LL/2, Lewis lung carcinoma; B16-F10, melanoma; and MBT-2, bladder cancer). The optimum result was obtained using a combination of α-lipoic acid and hydroxycitrate (METABLOC^{TM}). In this study, a third agent was added by in vivo evaluation of a large number of combinations. The addition of octreotide strongly reduced tumor development (T/C% value of 30.2 to 34.5%; $P<0.001$) in the same models and prolonged animal survival ($P<0.001$) as compared to cisplatin. These results were confirmed in a different laboratory setting using a human xenograft model (NCI-H69, small cell lung cancer). None of these three molecules are known to target DNA. The effectiveness of this combination in several animal models, as well as the low toxicity of these inexpensive drugs, emphasizes the necessity of rapidly setting up a clinical trial.

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Introduction

Almost 90 years ago the distinguished German biochemist, Otto Warburg, published his observations regarding a metabolic alteration frequently observed in cancer cells [1]. Warburg reported that the cancer cells he investigated metabolized glucose directly to lactic acid via pyruvate, as opposed to the pyruvate being converted to water and carbon dioxide in the mitochondria via the tricarboxylic acid (TCA) pathway. This metabolic property of cancer cells bears his name, that is, the Warburg effect. It is also referred to as aerobic glycolysis, as it takes place in cancer cells even under normoxic conditions.

Interest in the Warburg effect waned considerably for a long period of time. Part of the reason was the fact that Warburg was convinced that the altered glucose metabolism in cancer cells was actually the cause of cancer and that the most likely explanation for his observation was damage to the mitochondria [1]. Since then, modern molecular biology has demonstrated that cancer cannot originate without a change to a cell's genome and that, at least in most cases damage to the mitochondria is not the explanation for why many cancer cells adopt aerobic glycolysis as the principal pathway for glucose metabolism [2, 3]. However, during the last 15 years or so, there has been a considerable increase in interest regarding the Warburg effect and its role in cancer. As a result, some seminal publications have elucidated the role that the Warburg effect plays in cancer, and there are a number of recent excellent reviews as well [4–6].

There is considerable logic in targeting metabolic changes as an approach to the development of pharmaceutical agents to treat cancer despite the fact that these changes are not causal in nature. A relatively recent publication has shown that the genes involved in glycolysis are over-expressed in at least 24 different types of cancers that correspond to approximately 70% of all cancers [7]. It has been hypothesized that this widespread prevalence is because aerobic glycolysis provides a competitive advantage to cancer cells, allowing the synthesis of compounds (ribonucleotides and lipids) required for proliferation [8–10].

A number of specific inhibitors of key enzymes involved in the aerobic glycolytic pathway have been evaluated as potential anti-cancer drugs (see reviews [11–13]. However, with rare exceptions none of these compounds has been used clinically. Michelakis et al. [13] reported that treatment of five patients with glioblastoma multiforme using dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase, resulted in tumor regression in three individuals. Berkson et al. [14, 15] treated four pancreatic cancer patients with a combination of α-lipoic acid and naltrexone with excellent results. The first patient treated was still alive and well 78 months after presentation. Somewhat coincidentally, α-lipoic acid is also known to be an inhibitor of pyruvate dehydrogenase kinase as is dichloroacetate. Naltrexone, on the other hand, is an opioid receptor antagonist and is primarily used for the treatment of alcohol and opioid dependence, although there are limited data suggesting a potential role in cancer inhibition.

This relative lack of success suggested to us that a single inhibitor of cancer cell metabolism might be insufficient to significantly inhibit cancer proliferation. Given the extreme plasticity of malignant tissue, it seemed logical to attempt to use at least two different compounds, each one targeted to interact with enzymes catalyzing different steps. In addition, we adopted a strategy to use compounds already proven to be non-toxic in humans. Our conception of cancer and the details of the selection process are published elsewhere [16, 17].

In our first study [18], a detailed literature analysis was conducted from which a first library of twenty-seven drugs that are known to target pathways potentially implicated in cancer was developed (Table 1). We conducted in vitro tests on these molecules to determine their antiproliferative capacity on four cells lines at concentrations consistent with published human plasma levels. The data, summarized in Table 2, showed that 5 molecules were not effective, 11 molecules were weakly effective, while 11 molecules were significantly effective.

We then tested 15 combinations of two drugs based on the seven effective and least toxic molecules. Seven combinations showed a strong antiproliferative effect (< 20% of viable cells after 24 h). They were: acetazolamide and hydroxycitrate, lipoic acid and dichloroacetate, lipoic acid and hydroxycitrate, acetazolamide and miltefosine, albendazole and dichloroacetate, dichloroacetate and hydroxycitrate, lipoic acid and miltefosine.

We then proceeded to test these seven most effective combinations in vivo using mice bearing syngeneic MBT-2 bladder carcinoma. The majority of the combinations were not or only weakly effective (data not shown). The most effective treatment was hydroxycitrate and α-lipoic acid (designated as METABLOC™) [18]. The efficacy of this combination was confirmed in B16-F10 melanoma and LL/2 Lewis lung carcinoma. This combination both slowed growth of the tumor and increased survival with an efficacy similar to conventional cytotoxic chemotherapy. As already mentioned, α-lipoic acid is known to inhibit pyruvate dehydrogenase kinase [19], whereas hydroxycitrate inhibits ATP citrate lyase (ACL) a key enzyme involved in lipid synthesis and known to be frequently up-regulated in cancer [20].

Although the results obtained with the combination of α-lipoic acid and hydroxycitrate were promising, tumor growth was only retarded. As a consequence, we established a second

Table 1 List of the molecules used for the first screening and their putative mode of action

Drugs	Target and putative mechanism
Acetazolamide	Carbonic anhydrase inhibition
Albendazole	Phosphoenol pyruvate carboxykinase (PEPCK) inhibition
Amobarbital	NADH dehydrogenase
Amrinone	Pyruvate dehydrogenase activation
Betaine	Lipotropic factor
D-Fructose-1,6-bisphosphate	M2 isoform of pyruvate kinase activation
Dichloroacetate	Pyruvate dehydrogenase kinase 1 inhibition
Dimercaprol	Methylation agent
Farnesol	Phospholipase D inhibition
Genistein	Tyrosine kinase inhibition
Gossypol	Lactacte dehydrogenase inhibition
Hydrazine sulfate	Phosphoenol pyruvate carboxykinase inhibition
Hydroxycitrate	ATP citrate lyase inhibition
Ketoconazole	Cytochrome P450 isoenzymes inhibition
Lipoic acid	Pyruvate dehydrogenase kinase 1 (PDHK1) inhibition
Lithium chloride	Phosphoenol pyruvate carboxykinase inhibition of expression
Lonidamine	Hexokinase inhibition
Metformin	AMP-activated protein kinase (AMPK) activation
Miltefosine	Choline kinase inhibition
Niacin	Lipolysis inhibition
Quinacrine	Phospholipase A2 inhibition
Quinine	Phospholipase A2 inhibition
Silibinin	Insulin Growth Factor Binding Protein (IGFBP) activation
Simvastatine	Lipolysis inhibition
Suramine	Citrate synthase inhibition
Tolbutamide	ATP sensitive potassium channel blocker
Xylitol	Protein Phosphatase 2A (PP2A) activation

list of thirty-five different molecules, selected with the same criteria (Table 3) to determine if the addition of a third molecule could provide improved results. One of these molecules, octreotide, although only moderately active on its own, was found to significantly inhibit tumor growth in combination with METABLOC™ in the three tumor models used. The results are described below.

Table 2 Results of in vitro testing of the first group of drugs. The compounds are arranged as a function of their capacity to reduce viable cell number in the culture wells after the incubation: not effective: no inhibition of proliferation; weakly effective : inhibition after 24 h is <20% and inhibition after 120 h is <70%; effective : inhibition after 24 h is >20% and inhibition after 120 h is >70%

Not effective	Weakly effective	Effective
Xylitol	Suramine	Acetazolamide
Niacin	Amobarbital	Albendazole
Lithium chloride	Quinine	Dichloroacetate
Hydrazine sulfate	Metformin	Dimercaprol
Amrinone	Silibinine	Farnesol
	Ketoconazole	Genistein
	Tolbutamide	Gossypol
	Quinacrine	Hydroxycitrate
	Simvastatine	Lipoic acid
	D-Fructose-1,6-bisphosphate	Lonidamine
	Betaine	Miltefosine

Material and methods

Drugs

All the compounds were purchased from Sigma (St Quentin Fallavier, France) except for the following. Hydroxycitrate (*Garcinia cambogia* extract containing 60% hydroxycitrate as a mixture of 11% calcium and 14.5% potassium salts) was purchased from Indo World Trading Corporation (New Dehli, India), while miltefosine was purchased from Calbiochem. Octreotide (500 μg/ml in lactic acid, sodium bicarbonate pH=4.2, water, mannitol) was obtained from Novartis (Sandostatin™).

For in vivo experiments, the following doses were used: lipoic acid (10 mg/kg, twice a day), hydroxycitrate (250 mg/kg,

Table 3 Molecules used for the screening for a third effective drug and their putative mode of action

Drugs	Mechanism of action
6-Diazo-5-oxo-L-norleucine	Glutaminase inhibition
Agmatine	Polyamine synthesis inhibition
Alpha-ketoglutarate	Citrate synthase inhibition
Amiloride	Na+/H + antiport inhibition
Apigenine	IGFBP3 upregulation
Bicalutamide	IGFBP upregulation
Bromocriptine	Hypothalamic D2 receptor agonist
Butyrate sodium	HDAC inhibition
Chitosan	PK-M2 inhibition
Choline chloride	lipotropic factor
Citrate	Citrate synthase inhibition
Cryogenine	PEP carboxykinase inhibition
Curcumin	AID expression inhibition
D-Alanine	Alanine transaminase inhibition
Epigallocatechin gallate	PK-M1/M2 splicing regulation
Fluoxetine	Serotonine reabsorption inhibition
Ibuprofen	NSAIDS
Indole-3-carbinol	Triglycerides reduction
Ketoconazole	Cytochrome P450 demethylase inhibition
Lactoferine	Oxydative stress reduction
Letrozole	Aromatase inhibition
L-Norvaline	Arginase inhibition
Melatonin	Anti-oxidant, anti-proliferative
Menadione	Tyrosine kinase receptor inhibition
Octreotide	GH and IGF-1 pathways inhibition
Omeprazole	IGFBP expression induction
Oxythiamine	Transketolase inhibition
PEG8000	PK activation
Pegvisomant	GH receptor antagonist
Pralidoxime	Alanine transaminase inhibition
Retinoic acid	Cellular differentiation activation
Sulpiride	GH secretion inhibition
Suramine	Citrate synthase inhibition
Valproate sodium	HDAC inhibition
Vitamin B12	Lipotropic factor

twice a day), octreotide (0.1 mg/kg, twice a day). The tested doses were based on those currently prescribed in humans for conditions other than cancer and extrapolated to the weight of the mice by using the human/mouse conversion table provided by the FDA [21]. The doses of cisplatin used were 1 mg/kg every other day for Figs. 1, 2 and 3 and 6 mg/kg every week for Fig. 4. All drugs were diluted in saline solution (9 g/l) for intraperitoneal administration. We used 0.5% ethanol (lipoic acid solvent) in saline solution as the vehicle control.

Animals

C3H (6 weeks old) and C57BL/6 mice (8 weeks old) were obtained from the Centre d'Elevage Janvier, and the NMRI: nu/nu mice were bred in EPO facilities (original strain from Taconic)**.** The animals were maintained in accordance with the European community's guidelines concerning the care and use of laboratory animals. Mice were euthanized when animals were cachectic, suffering, or when tumor volume reached 2,000 mm^3.

Tumor models

In all syngeneic tumor models, 10^6 cells were inoculated in the back of mice (n=6/group). For the lung carcinoma cancer model (LL/2 in C57BL/6 mice), the tumor was allowed to develop for 13 days (mV=97.9 mm^3) before administration of the treatment (day 14–42). For the bladder

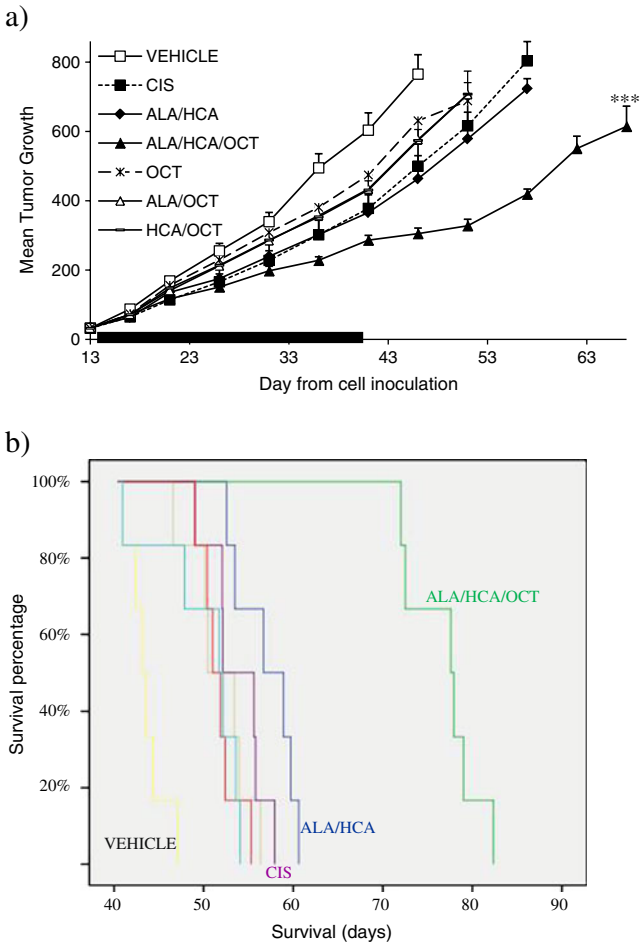

Fig. 1 In vivo anti-tumor activity of different combinations of α-lipoic acid, hydroxycitrate and octreotide on the LL/2 lung cancer model. **a** Mean tumor growth curves (see material and methods for details). The linear time trends of tumor volume for the treated groups differed significantly ($F_{5,182.7}$=22.7, P<0.001). Results are expressed as β (see material and methods for definition) compared to the reference, CIS. (***: P<0.001; ALA: α-lipoic acid; HCA: hydroxycitrate; OCT: octreotide; CIS: cisplatin; dark bar: treatment administration) **b** Kaplan-Meyer representation of the percentage of alive mice along the experiment. Vehicle: yellow; HCA/OCT: red; ALA/OCT: grey; OCT: light blue; ALA/HCA: dark blue; CIS: purple; ALA/HCA/OCT: dark green. Statistical details: see supplemental table 1a and b

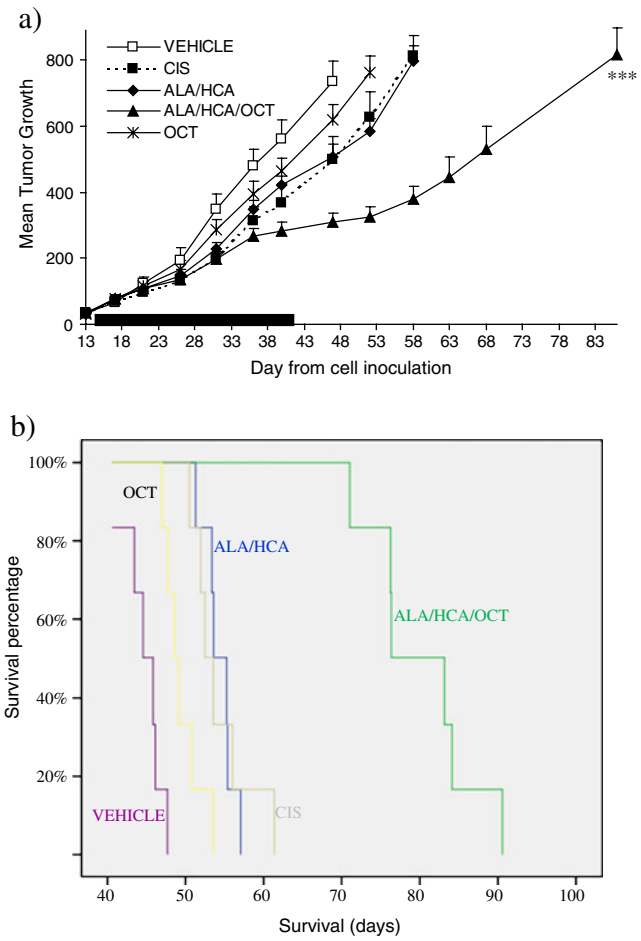

Fig. 2 In vivo anti-tumor activity of the α-lipoic acid, hydroxycitrate and octreotide combination on the MBT-2 bladder cancer model. **a** Mean tumor growth curves. The linear time trends of tumor volume for the treated groups differed significantly (F3,127.3=52.4, P<0.001). Results are expressed as β compared to the reference, CIS. (***: P<0.001; dark bar: treatment administration) **b** Kaplan-Meyer representation of the percentage of alive mice along the experiment. ALA/HCA: blue; ALA/HCA/OCT: green; OCT: yellow; CIS: grey; Vehicle: purple. See supplemental table 2a and b for statistical details

cancer model (MBT-2 cells in C3H mice), the tumor was allowed to develop for 13 days (mV=98.8 mm^3) before treatment (day 14–42). For the melanoma model (B16F10 cells in C57BL/6 mice), the tumor was allowed to develop for 13 days (mV=99.2 mm^3) before treatment (day 13–41). After randomization, treatments were intraperitonealy administered twice a day, either with a single drug or with a combination of two or three substances [18, 22].

The human small cell lung cancer cell line NCI-H69 was used as a xenograft cancer model. Cells were cultivated and subcutaneously transplanted in NMRI: nu/nu mice (4×10^6 cells/mice in 50% Matrigel, 8 mice/group). The tumor was allowed to develop for 15 days (mV=53.5 mm^3) before treatment (day 15–50).

Tumor response

We analyzed in vivo tumor growth and measured response to treatment using several parameters. Animal weight and tumor size were measured twice a week. For Figs. 1, 2 and 3 (experiments performed by NOSCO facilities), tumor volume in mm^3 was calculated from the measurement of two perpendicular diameters using a caliper according to the formula $l \times L \times h \times \pi/6l$, where L and l are the largest and smallest diameters and h the height in mm, respectively [23]. For Fig. 4 (experiments performed by EPO facilities), tumor volume was calculated from the measurement of two perpendicular diameters

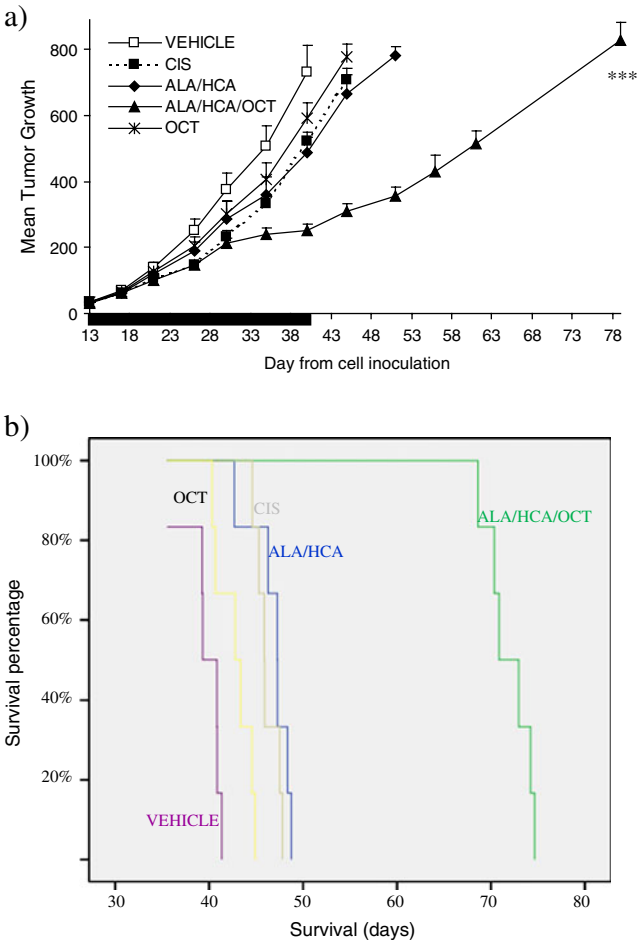

Fig. 3 In vivo anti-tumor activity of the α-lipoic acid, hydroxycitrate and octreotide combination on the B16F10 melanoma model. **a** Mean tumor growth curves. The linear time trends of tumor volume for the treated groups differed significantly (F3,115.6=95.0, $P<0.001$). Results are expressed as β compared to the reference, CIS. (Dark bar: treatment administration) **b** Kaplan-Meyer representation of the percentage of alive mice along the experiment. ALA/HCA: blue; ALA/HCA/OCT: green; OCT: yellow; CIS: grey; Vehicule: purple. See supplemental table 3a and b for statistical details

according to the formula (length×width²)/2. The mean tumor volume was expressed as mean relative tumor volume (mRTV), $mRTV=mV/mV_0$, where mV is the mean tumor volume on a given day and mV_0 is the initial mean tumor volume on day 0 of treatment. Mouse survival was recorded either up to the time of death or when the tumor volume reached 2,000 mm^3. Tumor regression (T/C%) was determined by use of the tumor volume values as follows: T/C%=100×(median tumor volume of treated group)/(median tumor volume of control group).

Data analysis

In every experiment, data on tumor volume were longitudinal and unbalanced. For comparison of the treatments, the interaction between treatment group and time on tumor

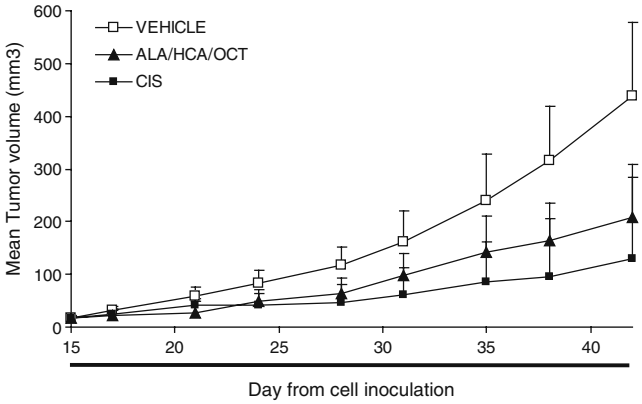

Fig. 4 In vivo anti-tumor activity of the α-lipoic acid, hydroxycitrate and octreotide combination on the NCI-H69 human small cell lung carcinoma model. Mean tumor growth curves. The linear time trends of tumor volume for the treated groups differed significantly ($F_{4,53.8}=5.87$, $P=0.001$). Results are expressed as β compared to the reference, CIS. Dark bar: treatment administration. Statistical details: see supplemental Table 4a

volume was analyzed by a linear mixed model, assuming an unstructured covariance matrix for the random effects and a first-order auto-regression structure for the within-mouse correlation. The slope (β value) is interpretable as the rate of growth for each animal. Treatments were compared using contrasts of fixed effects for the group slopes with inference based on the F-test. Estimation by restricted maximum likelihood (REML) was computed using SPSS v.16.0 (SPSS, Inc., Chicago, IL), and the model fit was assessed by Akaike's Information Criterion. Coefficients, confidence intervals (CI) of coefficients, and two-sided P values are reported for the model. A P value<0.05 is considered significant.

Evaluation of the correlation between raw tumor data and time suggested that tumor growth data were well approximated by a one third power function for each group (Fig. 1a: overall mean $r^2=0.966$; Fig. 2a: overall mean $r^2=0.979$; Fig. 3a: overall mean $r^2=0.983$; Fig. 4: overall mean $r^2=0.955$). Therefore, linear mixed-effects models were fitted to the cubic root value of tumor volume over time. This approach allows a parameter controlling the rate of growth (β value) to be estimated for each of the treatment groups, with the random effects being estimated for each subject in a group.

Tumor failure time was defined as the time (in days) from the inoculation of the cells required by individual tumors to reach a volume of 2,000 mm^3. For comparisons of time to tumor failure for different treatments, survival distributions of each treatment group were compared to the survival distribution of the control group using the log-rank test (Mantel-Cox). Statistical analysis was performed with SPSS v.16.0 software. Two-tailed values of $P<0.05$ were considered statistically significant.

Results

The combination of two compounds, α-lipoic acid and hydroxycitrate (designated as METABLOC™), was found to be effective in inhibiting the growth of three tumor models. In order to search for a third component that might improve the tumor response, since the combination of hydroxycitrate and lipoic acid only slowed tumor development but did not stop it, we established a new list of molecules, also chosen because they interfered with enzymes key to cancer cellular proliferation, although not necessarily focused on cancer metabolism (Table 3). Most of them are readily available and are also used clinically for diseases other than cancer.

This time we screened for anticancer activity in vivo by using mice bearing Lewis Lung carcinoma. Tumor cells were inoculated into the back of syngeneic mice, and tumor growth was measured twice a week. In each group, a third drug from Table 3 was added to the previously identified combination of lipoic acid and hydroxycitrate. Treatments were administered for 4 weeks. All but one drug, octreotide, were either deleterious or ineffective (data not shown).

We then confirmed the efficacy of METABLOC™ (the combination of α- lipoic acid (ALA) and hydroxycitrate (HCA)) and octreotide (OCT) in three syngeneic models, LLC, MBT-2 and B16F10. Results for the analysis of transformed tumor volume data using linear mixed models are shown in Figs. 1, 2 and 3.

In the LLC model the ALA/HCA/OCT combination displayed a more pronounced inhibition of tumor development compared to CIS ($P<0.001$) than did the ALA/HCA combination: when treated, the optimal tumor regression was 34.9% of the vehicle tumor volume (T/C% value) (Fig. 1a and Suppl Table 1a). In comparison, the optimal regressions obtained with CIS or ALA/HCA treatments were respectively 58.0% and 56.9% of the vehicle control group tumor volume. Thus, the ALA/HCA/OCT combination was almost 2-fold more effective than CIS. In order to better characterize the role of each drug, we also analyzed the efficacy of OCT alone, as well as ALA/OCT and HCA/OCT combinations. The combination of ALA and HCA was statistically equivalent to CIS alone ($P=0.3$), as previously reported [22], and OCT alone ($P=0.6$). Surprisingly, the ALA/OCT and HCA/OCT combinations resulted in significantly enhanced tumor growth compared to CIS (respectively $P=0.002$ and $P=0.001$), OCT alone or ALA/HCA.

Analysis of survival data gives similar results (Fig. 1b and Suppl Table 1b); the ALA/HCA/OCT combination displayed the longest survival (77.6 days) compared to CIS (52.1 days, $P=0.001$) or to the ALA/HCA combination (56.7 days, $P=0.001$). OCT administered alone was less efficient (51.7 days, $P=0.125$ compared to CIS) and only prolonged survival by 8.5 days compared to the vehicle treated group (43.2 days). ALA/OCT (50.5 days, $P=0.417$) and HCA/OCT (51.0 days, $P=0.603$) combinations were equivalent to OCT alone and less efficient than CIS.

The second set of experiments assessed the efficacy of the combination of drugs using the MBT-2 bladder carcinoma model. The administration of ALA/HCA/OCT strongly inhibited tumor development (Fig. 2a and Suppl Table 2a) compared to CIS treatment ($P<0.001$). In fact, the optimum tumor volume regression was 32.2% (day 63) of the vehicle group tumor volume (T/C% value), whereas treatment with CIS resulted in only an optimum regression of 59.9% of the vehicle group value, equivalent to ALA/HCA ($P=0.7$) or OCT ($P=0.7$). Thus the ALA/HCA/OCT combination was more than 2-fold more effective than CIS in inhibiting tumor development.

Median survival value of the ALA/HCA/OCT combination was 76.4 days while the CIS treated group value was only 52.5 days ($P=0.001$) (Fig. 2b and Suppl Table 2b). The ALA/HCA (53.6 days, $P=0.950$) combination was equivalent to CIS while OCT alone was less efficient (48.6 days, $P=0.02$).

In the last experiment performed in syngeneic models, we studied the effectiveness of the treatment using the B16F10 melanoma model. As in the two previous models, the ALA/HCA/OCT combination displayed a strong inhibition of tumor development (Fig. 3a and Suppl Table 3a). The tumor regression due to the ALA/HCA/OCT combination reached an optimum regression at day 56 (30.4%), whereas the optimum T/C% of CIS was 61.2%. Thus, the ALA/HCA/OCT combination was 2-fold more efficient than CIS ($P<0.001$).

Identical results were obtained with median survival analysis (Fig. 3b and Suppl Table 3b). The mice treated with the ALA/HCA/OCT combination survived to 70.9 days ($P=0.001$), considerably longer than those treated with CIS (45.9 days), while survival following ALA/HCA treatment (47.3 days, $P=0.247$) was equivalent to CIS. OCT was less effective (42.8 days, $P=0.002$).

The results of the ALA/HCA/OCT combination were similar and consistent in the three animal models studied: strong inhibition of tumor development (2-fold more effective than CIS) and significant survival extension. The efficacy of the treatment takes at least 2 weeks to be visible. After that period, the tumor stops growing. A week after the end of the treatment, the tumor starts again to proliferate strongly suggesting that the treatment is cytostatic and not cytotoxic.

In order to extend these results, we assessed the effectiveness of the ALA/HCA/OCT combination in the NCI-H69 small cell lung cancer model (Fig. 4 and Suppl Table 4). This choice allowed us to validate our results in a human xenograft cancer model and in an independent

laboratory. Here again, the ALA/HCA/OCT combination has a strong inhibitory effect on tumor development being as effective as CIS (P=0.18). In this model, we noticed side effects: two toxic deaths (out of 8 animals, day 37 and 38) with bloody gastrointestinal tracts; convulsions after intraperitoneal injection which disappeared with increasing injection volume (from 200 to 400 μl/20 g body weight); slight body weight loss (- 2.2% compared to initial value). No major secondary effect (convulsions or deaths) had been observed with the three tested syngeneic models.

To summarize, our experimental data demonstrates that the combination of METABLOC™ and OCT (ALA/HCA/OCT) is a very efficient cancer treatment in the four models studied. This treatment strongly reduced tumor development and improved the overall survival of the animals.

Discussion

Recognizing that a single drug designed to target a single enzyme altered in cancer metabolism is not likely to be effective, given the plasticity of a malignant cell, we tested a large number of binary combinations of metabolic inhibitors and found that METABLOC™, a combination of α-lipoic acid and hydroxycitrate, was the most effective of those tested [18]. Although this combination slows down tumor growth in several different cancer models, it does not stop cancer development. A logical extension of this strategy was the inclusion of a third drug that does not necessarily target altered metabolism. The first attempt was to combine lipoic acid and hydroxycitrate with a known and clinically useful cytotoxic anti-cancer drug. Indeed, combining the two metabolic inhibitors with either cisplatin or methotrexate showed a clear improvement compared to either the binary combination or the cytotoxic drug alone [22]. An alternative approach was to identify a third molecule, one that would have minimal side effects and increase the effectiveness of the α-lipoic acid and hydroxycitrate combination. A total of 35 molecules (Table 3) were tested from which only one was found to be effective; namely, octreotide (brand name Sandostatin). The addition of octreotide markedly improved the efficacy of α-lipoic acid and hydroxycitrate. However, the ALA/OCT and HCA/OCT combinations were less effective than OCT alone, CIS or ALA/HCA (Fig. 1a); demonstrating that all three compounds are necessary to obtain an effective result.

In our first publication [18] we indicated that the most likely mechanism of action for α-lipoic acid for its inhibition of tumor growth was inhibition of pyruvate dehydrogenase kinase. This enzyme inhibits the activity of pyruvate dehydrogenase, and is known to be up-regulated in cancer cells expressing the aerobic glycolytic phenotype. Pyruvate dehydrogenase catalyzes the conversion of pyruvate to acetyl CoA, the initial step of the ultimate conversion of glucose to carbon dioxide and water, with the concomitant production of ATP, in the TCA cycle. Therefore, it is reasonable to suggest that blocking the activity of pyruvate dehydrogenase will at least partially restore the activity of pyruvate dehydrogenase, thereby increasing the flux of pyruvate through the TCA cycle in the mitochondria while simultaneously reducing the production of lactic acid. Hydroxycitrate, the other component of METABLOC™ is known to inhibit ATP citrate lyase, another enzyme that is known to be up-regulated in cancer cells expressing the aerobic glycolytic phenotype. Up-regulation of ATP citrate lyase increases cancer cells' ability to synthesize lipids, required for rapid cellular proliferation. Therefore, inhibiting its activity would be expected to reduce cancer cell growth, as was observed in our results. In addition, however, there is also evidence that inhibition of ATP citrate lyase may also reduce the activity of lactate dehydrogenase A, the specific isoform of lactate dehydrogenase most prevalent in cancer cells that overproduce lactate. Therefore both α-lipoic acid and hydroxycitrate may act to reduce lactic acid formation, and there have been a number of findings that suggest that lactic acid production provides cancer cells with a competitive advantage as compared to cells that metabolize pyruvate through the TCA cycle. Clearly, these are currently only hypotheses, and further molecular biological research is required to confirm them.

A key question involves the role that octreotide (OCT) is playing in the ternary combination. OCT is an octapeptide analog of somatostatin initially developed to treat acromegaly It has been in use for years in the treatment of neuroendocrine tumors, even if its use has been primarily for the relief of patient symptoms, while its anticancer activity has been controversial [24, 25]. Its activity with respect to neuroendocrine tumors has clearly been established to be mediated through two of the five known somatostatin receptors (SSTR); namely SSTR2 and SSTR5 [24]. In a recent review, Oberg pointed out that on the basis of a recent clinical trial in which octreotide LAR (long-acting release) significantly lengthened the median time to tumor progression compared with placebo (in functionally active or inactive neuroendocrine tumors of the small intestine), several clinician networks have presented an updated version of their guidelines for the treatment of carcinoid tumors [26]. A number of potential mechanisms for the anti-proliferative activity of OCT have been proposed, one of which is clearly its negative regulation of insulin growth factor 1 (IGF 1) as well as increasing the level of the insulin growth factor binding proteins (IGFBP) [24]. The effect of OCT on IGF 1 led to a phase II trial in which its effectiveness was assessed with respect to non-metastatic castration-resistant prostate cancer. Although

levels of IGF 1 were reduced, whereas the level of IGFBP 1 was increased, there was no observed decrease of PSA levels in any of the patients treated. As a consequence, OCT alone did not appear to be effective with respect to the cancer itself. Nevertheless, the authors recommend that OCT could be useful when combined with other drugs that target the IGF 1 axis [27].

The most likely effect of increased production of IGF 1 with respect to increased cancer cell proliferation involves activation of PI3K, which, in turn, activates AKT. There is published evidence suggesting that AKT activation plays a key role in the shift of the metabolism of glucose from oxidative phosphorylation to aerobic glycolysis, although several mechanisms have been proposed [2]. Of considerable potential importance is the fact that activated AKT is involved in the activation of mTOR, an extremely important master regulator of cellular proliferation. As a consequence of the possibility that octreotide can influence the activity of mTOR, two recent papers have investigated concomitant treatment of three different neuroendocrine tumor cell lines with octreotide with mTOR inhibitors, either rapamycin itself, or the rapalog RAD001. In the earlier study, the effect of either octreotide, RAD001, or their combination was assessed in the rat insulinoma cell line (INSI). Both of these treatments resulted in a significant inhibition of proliferation; however, there was no difference observed with the combined treatment as compared to either drug used individually. Interestingly, no inhibition of the phosphorylation of AKT was observed with either drug; however, both drugs resulted in the inhibition of Ser2448 phosphorylation on mTOR [28]. In a later paper octreotide was tested in an immortalized pituitary tumor cell line (AtT-20) in conjunction with rapamycin. In this cell line, treatment with rapamycin was found to increase the extent of AKT phosphorylation, not a unique observation, but treatment with both rapamycin and octreotide was found to reverse the phosphorylation of AKT [29].

The two papers cited above clearly establish that octreotide can interact with the IGF 1/PI3K/AKT/mTOR axis. However, each study proposes alternative mechanisms with respect to downstream events. Although it is not unreasonable to propose that octreotide can interact synergistically with the ALA/HCA combination through a pathway that has no relationship to aerobic glycolysis, evidence that activation of mTOR in cancer could increase the extent of aerobic glycolysis in these cells would be of considerable interest. As it turns out, a very recent paper provides such evidence. Sun et al. [30] reported that *Tsc1* or *Tsc2* null mouse embryonic fibroblasts (MEF) produced lactic acid, a hallmark for aerobic glycolysis, in culture, which was not the case for WT cells. In that ablation of *Tsc1/2* leads to constitutive activation of mTOR, this is a clear demonstration that mTOR activation plays a key role in the shift from oxidative phosphorylation to aerobic glycolysis. In addition, the authors also showed that this effect was reversed by rapamycin treatment, further demonstrating a key role for mTOR in aerobic glycolysis. Further work demonstrated that the *Tsc* null MEF had an abundance of pyruvate kinase M2 (PKM2), the isoform of pyruvate kinase that is invariably present in cancer cells that exhibit aerobic glycolysis the specific properties of which appear to be a requirement for aerobic glycolysis [31]. The production of PKM2 was also significantly reduced by treatment with rapamycin. Although these authors did not evaluate the activity of octreotide in these MEF cells, as indicated there is ample evidence that octreotide can indeed down-regulate the activity of mTOR. Therefore, it appears highly likely that all three of the effective combination, ALA, HCA, and OCT, are targeting different steps of aerobic glycolysis. It is important to note that Sun et al. indicate that the fact that rapamycin treatment did not completely abolish the expression of PKM2 suggests that other factors may be involved [30]. This is consistent with the fact that octreotide by itself was only moderately effective in the three models we tested.

The results of Sun et al. are particularly interesting in light of published results on a novel somatostatin analogue designated as TT-232. This compound has already been shown to be of interest in a phase II clinical trial under the designation of TLN-232 [32]. Data have been published that this compound interacts directly with pyruvate kinase to translocate it to the nucleus resulting in cell death [33, 34]. Although the work of Sun et al. suggest that the interaction of a somatostatin analogue with PKM2 would be an indirect effect mediated through mTOR, it is not impossible that TT-232 is acting by the same mechanism. Although there is much research that needs to be done to develop a complete picture of the relationship between somatostatin analogues and PKM2, the fact that two such compounds have been found to reduce PKM2 levels strongly suggests that there is indeed a relationship.

One issue that must be resolved is whether or not octreotide is mediating the observed effect on the four cancer models evaluated herein through SSTR2 and SSTR5 or by some other mechanism. There is literature evidence that NCI-H69 cells, the small cell lung cancer cell line used as the xenograft model, express somatostatin receptors that bind octreotide [35, 36]. There is no indication in the published literature, however, that the other three models we evaluated express these receptors. There are data that suggest that octreotide may act through mechanisms independent of its interaction with somatostatin receptors. First of all, octreotide displays a spectrum of activity with respect to SSTR1-5 identical to another somatostatin analogue, lanreotide [37]. However, published clinical data suggest that octreotide and lanreotide

can have very different efficacies in certain cancers [38, 39]. Furthermore, there are both experimental and clinical data that show an anti-cancer activity of octreotide in systems where somatostatin receptors appear not to be expressed [40, 41]. Another possibility, however, is that these cell lines do indeed possess SSTRs. Msaouel et al. [24] point out that the expression of SSTRs has been documented in primary tissue samples in a wide variety of non-neuroendocrine tumors, including breast, prostate, colon pancreatic adenocarcinoma, lung, liver, renal, adrenal cortex and thyroid cancers. However, treatment of these tumors with somatostatin analogues has generally been disappointing. The lack of results is completely consistent with our results, where octreotide was found to have a modest effect at best when used singly, but a significant effect in the ALA/HCA/OCT combination.

Probably the most important result of this work is the observation that different cell lines respond similarly to the combination therapy. In vitro, the five cell lines tested (four human and one murine) responded similarly to the treatment, while the growth of the three murine tumors (bladder and lung cancer as well as melanoma) as well as the human (small cell lung cancer) was similarly controlled in mice. This result significantly supports the logic of targeting a phenotypic aspect of cancer as opposed to a genotoxic event.

Generally speaking, these three molecules have well-established long track records of safety. Both α-lipoic acid and hydroxycitrate are well known to the clinician and are available over the counter [18]. α-Lipoic acid has been widely used as a dietary supplement, a treatment for diabetic neuropathy [42], and for its potential usefulness in the prevention of numerous diseases (among them diabetes, atherosclerosis, neurodegenerative diseases, etc.) [43]. Furthermore, α-lipoic acid has been used in clinical trials to fight cancer-related cachexia and oxidative stress syndrome [42, 44]. Hydroxycitrate, extracted from *Garcinia cambogia* fruit, is used as a weight loss agent, because it presumably limits lipogenesis and reduces food intake [45, 46]. Octreotide has been approved by the FDA for the treatment of acromegaly, the treatment of flushing and diarrhea episodes associated with carcinoid syndrome, and treatment of diarrhea in patients with vasoactive intestinal peptide-secreting tumors (VIPomas). It has been marketed for a long period (first approval in 1988) and has only very limited side effects [47]. In the small cell lung cancer xenograft model, we noted mild toxicity resulting from the combination of the three compounds with convulsions and 2 deaths (out of 8 animals) from gastrointestinal tract inflammation, but no adverse effect was noted in the three syngeneic models. These side effects could be attributed to the intraperitoneal administration or the presence of 0.5% ethanol as a vehicle. They most probably will not be an issue in human treatment following refinement of formulation, doses and administration schedule.

The combination of ALA/HCA with OCT leads to tumor growth inhibition more efficiently than cisplatin, but not regression. This may be because of non-optimized doses for each of the three compounds. However, the therapeutic interest is high because this combination is largely devoid of major sides-effects such as those encountered with standard chemotherapies.

We are now exploring the precise mechanism of action of these molecules. We are also screening for a fourth agent which could lead to further improvements and ultimately lead to actual shrinkage of the tumor mass.

In summary we have identified a combination of inexpensive and non-toxic molecules which appear to significantly slow down tumor growth without noticeable side effects. Moreover, this combination appears to be effective on tumors from diverse sites of origin.

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