Antitumor activity of 7-aminocarboxycoumarin derivatives, 
a new class of potent inhibitors of lactate influx but not efflux.

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ABSTRACT.

High lactate concentration in tumors is associated with bad prognosis. Lactate is released by glycolytic cells in tumors and re-captured by oxidative cancer cells to feed the TCA cycle after conversion into pyruvate. Monocarboxylate transporters (MCT) mediate these fluxes of proton-linked lactate and represent attractive targets to interrupt lactate shuttle and to inhibit tumor growth.

Here, we investigated the properties of 7-amino carboxycoumarins (7ACC) developed to selectively interfere with lactate fluxes in the lactate-rich tumor microenvironment. The pharmacological properties of two compounds of this family, including their effects on lactate influx and efflux and anti-tumor activity, were investigated using human cancer cell lines and mouse xenograft models. Contrary to the reference MCT1 inhibitor AR-C155858, 7ACC unexpectedly inhibited lactate influx but not efflux in tumor cells expressing MCT1 and MCT4 transporters. 7ACC delayed the growth of cervix SiHa tumors, colorectal HCT116 tumors and orthoptopic MCF7 breast tumors. MCT target engagement was confirmed by the lack of activity of 7ACC on bladder UM-UC-3 carcinoma that do not express functional MCT. 7ACC also inhibited SiHa tumor relapse post-treatment with cisplatin. Finally, we found that contrary to AR-C155858, 7ACC did not prevent the cell entry of the substrate-mimetic drug 3-bromopyruvate through MCT1, and contributed to the inhibition of tumor relapse post-3BP treatment.

In conclusion, our results indicate that 7ACC selectively affect a single part of the MCT symporter translocation cycle leading to strict inhibition of lactate influx. This singular activity is associated with antitumor effects less prone to resistance and side effects.
INTRODUCTION.

Metabolic reprogramming of cancer cells is now considered as a hallmark of cancer (1, 2). Changes in the metabolic preferences of tumors are however too often reduced to the sole Warburg effect describing the capacity of tumor cells to exploit glycolysis (ie, glucose to lactate conversion) under aerobic conditions (3). Although this paradigm fostered a new impetus in re-exploring (with the most recent genetic tools) the interest of tumor metabolism characteristics as therapeutic targets, it did introduce some confusion in the understanding of whether mitochondria are functional in cancer cells (4, 5). However, it is clear from several studies that the mitochondrial TCA cycle plays key roles in a large variety of tumor cells to produce biosynthetic intermediates and that other substrates including glutamine and lactate can fuel the TCA cycle and even participate in the production of energy when coupled to oxidative phosphorylations (6-10).

In the last years, we have shown that lactate, the end-product of glycolysis, can actually be recaptured by tumor cells and re-oxidized into pyruvate to feed the TCA cycle (11, 12). This lactate shuttle between cells producing lactate and others using lactate has been shown to also involve tumor-associated fibroblasts (13, 14) and angiogenic endothelial cells (15, 16). This shuttle even becomes a symbiotic process if one considers that the use of lactate can reduce the consumption of glucose by the most oxidative tumor cells and thereby increase its availability for hypoxic tumor cells (particularly dependent on glycolysis) (8, 11). We also recently reported that lactate can stimulate angiogenesis through PHD2 inhibition and the consecutive stimulation of NFκB- an HIF-1α-dependent pathways (15-17). Together with studies documenting that in cancer patients, elevated lactate concentrations are associated with poor prognosis (18-21), these findings place the regulation of lactate flux as a particularly druggable process to impact on tumor progression. The main targets for such pharmacological intervention are monocarboxylate transporters (MCT) (22, 23).
The monocarboxylate transporter (MCT) family (also solute carriers SLC16) actually comprises 14 members, out of which four are proton-linked short chain monocarboxylate transporters (MCT1-4) (23, 24). In cancer, MCT1 (SLC16A1) and MCT4 (SLC16A3) are the most often described (25). MCT1 is the most ubiquitous monocarboxylate transporter and its expression is increased in p53-deficient tumors (12) while MCT4 expression is upregulated under hypoxic conditions (26). These two MCT also differ by their substrate affinity: MCT1 shows a greater affinity for L-lactate (Km 3-6 mM) and pyruvate (Km 1-2.5 mM) than MCT4 (with Km equal to 25-30 mM and 150 mM, respectively) (23). Such differences are consistent with their respective metabolic roles: the high affinity of MCT1 for lactate allows this transporter to take up lactate more easily than MCT4. Also, the low affinity of MCT4 for pyruvate prevents its release from (hypoxic) glycolytic cells and thereby facilitates the cytosolic lactate conversion required to regenerate NADH from NAD⁺ (27, 28). As mentioned above, the actors regulating lactate shuttle in tumors are thus MCT4 for lactate release from hypoxic tumor cells and tumor-associated fibroblasts, and MCT1 for lactate uptake by oxidative tumor cells and angiogenic endothelial cells (11, 12, 15-17).

For several decades, the only MCT inhibitors described like α-cyano-4-hydroxycinnamate (CHC) (29, 30), organomercurials (31) and stilbene disulfonates (32, 33) suffered from a lack of selectivity (34, 35). More recently, a new class of high affinity MCT1/MCT2 inhibitors such as AR-C155858 was developed by Astra-Zeneca (36, 37). From our own drug discovery program, we have recently identified 7-alkylamino 3-carboxycoumarins (7ACC) as a new family of lactate flux inhibitors (synthesis and chemistry described in reference 38); the most active compound 7ACC2 (see structure in Supplementary Figure 1) exhibited a low IC₅₀ of 11 nM when evaluating ¹⁴C-lactate flux inhibition. The lack of toxicity of 7ACC in cells using glucose (instead of lactate) as a preferential energy fuel together with the lack of anticoagulant activity of these non-4-hydroxy-substituted coumarin...
derivatives and a good ADME profile (38) supports the potential of this new family of compounds to act as anticancer drugs through inhibition of lactate flux.

In the current study, we examined how 7ACC compounds could interfere with lactate influx and efflux in a variety of cancer cells expressing MCT1 or MCT4 or both, and whether this could translate \textit{in vivo} in the inhibition of different human tumor xenograft models. We also studied the capacity of 7ACC to delay tumor relapse when combined with conventional chemotherapy but also with 3-bromopyruvate, a substrate-mimetic antitumor drug known to block glycolysis (39) and to enter cells through MCT1 (40).
MATERIALS AND METHODS

Cell models and in vitro treatments. Human tumor cells were acquired in the last three years from ATCC where they are regularly authenticated by short tandem repeat profiling. Cells were stored according to the supplier's instructions and used within 6 months after resuscitation of frozen aliquots. Cervix cancer cells (SiHa and HeLa) and mammary cancer cells (MDA-MB-231, MCF7) were cultured in DMEM, HCT-116 colorectal cancer cells in McCoy’s 5A medium, UM-UC-3 bladder transitional cell carcinoma and pharynx squamous carcinoma FaDu cells in Eagle's MEM, HL-60 acute promyelocytic leukemia cells and K562 chronic myelogenous leukemia cells were cultured in suspension in RPMI1640 medium. For treatments, SiHa, Hela and MDA-MB231 cells were seeded in flat-bottom 96-well plates in DMEM. After overnight incubation, the culture medium was replaced by 100 µl of medium containing 7ACC1, 7ACC2, AR-C155858 or 3BP. Non-adherent HL-60 and K562 cells were directly treated in flat-bottom 96-well plates in RPMI medium. Antiproliferative effects were determined using MTT or Presto Blue assay for adherent cells or cell counting using a Cellometer® Auto T4 for non adherent cells.

Mice and in vivo treatments. 8-week old NMRI female nude mice (Elevage Janvier, LeGenest-St-Isle, France) were injected subcutaneously with 2x10^6 SiHa cells, 2x10^6 HCT-116 cells or 5x10^6 UM-UC-3 cells. An orthotopic breast cancer model was also used with MCF7 tumor cells injected into the mammary fat pad of mice; a 17β-estradiol pellet had first been s.c. implanted in these mice as previously described (41). When tumors reached a mean diameter of 5 mm, 7ACC compounds (3 mg/kg) or AR-C155858 (3 mg/kg) were daily injected i.p.; in some experiments, 7ACC treatment was combined with cisplatin (5 mg/kg) injected i.p. at day 0 and day 7 (7ACC administered daily except at day 0 and 7) or 3-bromopyruvate (3BP) (3 mg/kg) injected i.p. from day 0 to day 4 and day 7 to day 11 (7ACC administered together with 3BP). Cisplatin and 3BP were also administered alone and control mice were injected with
vehicle (DMSO). Tumor sizes were tracked with an electronic calliper and determined using the formula: length x width² x π)/6. Each procedure was approved by the local authorities according to national animal care regulations.

**Lactate assay.** For lactate uptake measurements, tumor cells were seeded on flat-bottom 24-well plates (500 000 cells/well) in normal DMEM. After 6 hours, the culture medium was replaced by 1 ml glucose-free DMEM containing 10 mM lactate and cells were treated for 24 hours with the compounds. For the lactate release measurements, cells were treated for 16-24 hours with the compounds in flat-bottom 24-well plates (500 000 cells/well) in normal DMEM medium (MDA-MB-231) or RPMI1640 medium (HL-60 and K562). At the end of the lactate uptake or release experiments, cell supernatants were centrifuged using deproteinizing columns (15 min, 10000 g at 4°C) and lactate concentration was determined using the enzymatic assay commercialized by CMA Microdialysis AB on a CMA600 analyzer (Aurora Borealis).

**Immunostaining and immunoblotting.** Tumors were cryosliced and sections were probed with a rat monoclonal antibody against CD31 (BD PharMingen, Lexington, KY, USA) or rabbit polyclonal antibodies against MCT1 and MCT4 followed by a secondary antibody coupled to Alexa Fluorophores as previously described (12, 15). For immunoblotting, cells extracts were separated on SDS-PAGE and transferred onto PVDF membranes before incubation with MCT1 and MCT4; gel loading was normalized with a beta-actin antibody (Sigma).

**Statistical analysis.** Results are expressed as mean ± SEM. Student’s t test or ANOVA were used where indicated. *P<0.05, **P<0.01 or ***P<0.001 was considered statistically significant.
RESULTS.

7ACC compounds inhibit the influx but not the efflux of lactate in cancer cells.

We recently reported the chemical synthesis of new MCT inhibitors (38) (see also Suppl. Figure 1 for structures). To get further insights on the profile of lactate flux inhibition by these compounds (named 7ACC1 and 7ACC2 in the current study), we examined the capacity of these molecules to interfere with lactate uptake and lactate efflux in different human tumor cell lines. Leukemia cells are indeed reported to be highly glycolytic and to release lactate in the presence of oxygen (42) whereas oxidative cervix cancer cells have the capacity to take up lactate to fuel TCA cycle after reconversion into pyruvate (11). As a reference compound, we used the recently developed MCT1/MCT2 inhibitor AR-C155858 (see Suppl. Fig. 1 for structure). We found that in cervix cancer SiHa cells which express both MCT1 and MCT4 (Figure 1A), the 7ACC compounds blocked lactate influx whereas the AR-C155858 compound failed to do so (Figure 1B); similar results were obtained with another cervix cancer cell line (Hela) (not shown) but also with human pharynx squamous carcinoma FaDu cells (Suppl. Figure 2). By contrast, we observed that lactate efflux by the highly glycolytic leukemia cells HL60 which express MCT1 but not MCT4 (Figure 1A) was inhibited by AR-C155858 but not by the 7ACC compounds (Figures 1C); similar results were obtained with K562 leukemia cells (not shown). Finally, in the breast cancer cell line MDA-MB-231 which expresses MCT4 but not MCT1, neither drug was able to prevent lactate release (Figure 1D). The cytotoxicity resulting from exposure to the different compounds was in adequation with the observed inhibition of lactate influx or efflux: while 7ACC compounds inhibited the proliferation of cervix cancer cells but failed to interfere with leukemia cell growth, AR-C155858 exhibited the opposite behaviour, being only toxic for the latter (Figure 1E); MDA-MD-231 cells were resistant to both types of inhibitors (Figure 1E). We also tested the effects of 7ACC on normal human fibroblasts (hTERT BJ-5ta), endothelial cells (HUVEC) and
embryonic kidney cells (HEK) and failed to observe any significant cytotoxic effects (Figure 1F).

**7ACC compounds inhibit the growth of MCT-expressing tumors.**

To further determine whether the capacity of the 7ACC compounds to block lactate influx but not efflux was relevant *in vivo*, we next treated mice bearing tumors, including cervix cancer SiHa xenografts but also tumors derived from human colon cancer cells HCT-116 and bladder cancer cells UM-UC3; highly glycolytic tumors such as those derived from leukemia cells and MDA-MB-231 cells were not included in this *in vivo* validation since they do not consume lactate. When tumors reached a mean diameter of 5 mm, compounds 7ACC1 and 7ACC2 were daily injected i.p. and tumor growth was tracked using a caliper. A dose of 3 mg.kg\(^{-1}\).day\(^{-1}\) was used based on pilot pharmacokinetics studies that had shown that this dosage led to a \(C_{\text{max}} > 1 \mu\text{g.ml}^{-1}\) and a plasma half-life of 4.5 hours (38). Both 7ACC compounds led to significant SiHa and HCT116 tumor growth delays (Figures 2A and 2B). However, the two compounds failed to exert any anticancer activity on the human UM-UC3 bladder carcinoma (Figure 2C). A careful profiling of the MCT expression however showed that although UM-UC3 cells express MCT1 (and not MCT4) *in vitro* (not shown), UM-UC-3-derived tumors did not show any positive membrane staining for MCT1 and MCT4 (Figure 2C, right panels); in some UM-UC-3 tumor area, we did find punctate MCT1 staining reflecting immature or vesicle-trapped MCT1 expression (see green staining in Figure 2C inset). Of note, immunostaining of SiHa and HCT116 tumor sections showed that while MCT1 was homogeneously expressed, MCT4 displayed a mirror picture of the CD31-labelled vasculature (Figures 2A and 2B, right panels). Finally, we also used the orthotopic model of human mammary cancer using MCF7 cells implanted in the mammary fat pad and confirmed a net inhibition of tumor growth when 7ACC2 was daily administered (Figure 2D).
Combination of 7ACC compounds with conventional chemotherapy or 3-bromopyruvate leads to significant reduction in post-treatment tumor relapse.

In the next experiments, we further explored the combination of 7ACC compounds with conventional chemotherapy (cisplatin) and 3-bromopyruvate, a drug known to enter tumor cells through MCT1 (40).

Cisplatin generally represents the first option to treat human cervix cancer but even in well-responding tumors, this therapy is often associated with rapid tumor relapse after the end of the treatment (43). In our experimental protocol, cisplatin was injected at Days 0 and 7 as monotherapy or together with the administration each other day of 7ACC compounds up to Day 12 where the treatment was stopped to study tumor growth relapse (Figure 3). This experiment showed that cisplatin prevented tumor growth and that tumor recurrence could be observed when it was used as monotherapy (Figure 3). Interestingly, when combined with the 7ACC compounds, tumor relapse was attenuated as shown by a prolonged inhibition of tumor re-growth. Removal of tumors at Day 27 confirmed a significantly lower size of tumors collected from mice treated with both cisplatin and 7ACC compounds (vs. cisplatin alone) (Figure 3).

In a second set of experiments, we examined the possibility to combine MCT inhibitors and 3-bromopyruvate (3BP). This compound was indeed recently reported to enter cells through MCT1 (40), a process which could therefore lead to resistance if MCT1 is simultaneously blocked. To explore this hypothesis, we first examined the effects of both treatments on the different tumor cell lines described in Figure 1. We confirmed that 3BP was cytotoxic in tumor cell lines expressing MCT1 either as a main path to uptake lactate such as SiHa (Figure 4A) and HeLa (not shown) or instead to release lactate such as HL60 (Figure 4B) and K562 (not shown). In MDA-MB231 cells which express MCT4 but not MCT1 (see Fig. 1A), 3BP failed to exert cytotoxic effects (Figure 4C). Interestingly, we found that the MCT1
inhibitor AR-C155858 prevented 3BP cytotoxicity in leukemia cells and in cervix cancer cells whereas 7ACC compounds did not (Figures 4A and 4B). We next examined whether these observations could also be recapitulated \textit{in vivo}. We therefore treated SiHa tumor-bearing mice either with 3BP alone or together with AR-C155858 or 7ACC2 compound. We found that ARC155858 abrogated the antitumor effects of 3BP (Figure 4D) contrary to 7ACC2 compound (Figure 4E). Of note, 3BP as monotherapy was particularly efficient to block SiHa tumor growth (see Figures 4D and 4E). Based on the observations reported in Figure 3, we also examined whether the combination of 7ACC and 3BP could influence tumor relapse after the end of treatment. We found that when tumors were collected 15 days after the last drug injection, the tumor volumes of mice exposed to both treatments were systematically smaller than those of mice treated with either single compound (Figure 4F).
DISCUSSION.

The major findings of this study are (i) the identification of compounds endowed with the capacity to block lactate influx but not lactate efflux, and (ii) the demonstration of their in vivo antitumor effects as monotherapy and when combined with another therapeutic modality.

We proved the selective effects of the 7ACC compounds on lactate influx using oxidative cancer cells known to maintain in vitro their capacity to take up lactate as an energetic fuel, and the lack of effects on lactate efflux using highly glycolytic cells. Accordingly, in oxidative human cancer cervix cells, SiHa and Hela, which express both MCT1 and MCT4 isforms, a potent inhibition of both lactate influx and cell proliferation was obtained with 7ACC whereas the bona fide MCT1/MCT2 inhibitor AR-C155858 failed to do so. The effects of 7ACC were confirmed in MCT1/4-expressing pharynx squamous FaDu tumor cells. These observations strongly suggest that 7ACC compounds are inhibitors of lactate entry through both MCT1 and MCT4 preventing any compensatory effects when MCT1, the main path for lactate uptake, is inhibited. Conversely, the 7ACC compounds failed to block lactate efflux from leukemia cells HL60 and K562 (which exclusively express MCT1) whereas the AR-C155858 compound prevented lactate release by 50%. The AR-C155858 compound however failed to block lactate efflux from human breast cancer cell MDA-MB231 which express MCT4 (and possibly MCT2) but not MCT1, underlying the limitation of MCT isoform-specific inhibitory compounds (see below). The distinct behaviours of 7ACC vs AR-C155858 are summarized in Figure 5. There are only a few examples of drugs interfering with solute transport that block the flux unidirectionally. One of the best example is SoRI-20041, a drug that inhibits dopamine uptake, but has no significant effect on dopamine efflux (the so-called reverse transport) (44). Molecular mechanism supporting the pharmacological profile of SoRI-20041 are currently unknown but are proposed to involve an allosteric regulation subtlety altering the transporter conformation such that inward transport is impaired, but outward efflux of substrate
is not. Although the demonstration of a similar allosteric modulation of MCT by 7ACC compounds still needs to be done, the profile of such compounds opens new perspectives. First, the lack of activity on lactate efflux is the promise of an absence or at least an attenuation of side effects in all the tissues where lactate release is necessary, including fast-twitch muscle fibers and brain (23, 24). Activated lymphocytes are also reported to be highly glycolytic and therefore dependent on efficient lactate efflux. The inhibition of lymphocyte proliferation was actually at the origin of the discovery of the AR-C155858 compound family (45). Immunosuppressive effects that may be deleterious in the context of cancer patients would therefore be avoided with 7ACC compounds. Second, the capacity to target lactate influx independently of the type of MCT transporter expressed (at least MCT1 and MCT4 in this study) should greatly limit the risk of compensatory mechanism as observed with specific inhibitors such as the AR-C155858 compound. Since most cancers do express these two transporters (25, 46), this property may represent a critical advantage for the 7ACC compound family.

The potential of 7ACC compounds is further supported by a series of in vivo experiments documenting their capacity to inhibit tumor growth and/or tumor relapse. Indeed, we validated the in vivo anti-tumor effects of 7ACC compounds using mouse xenograft models derived from human cervix cancer SiHa cells but also from the human colorectal cancer cell line HCT116 (Figure 2). Although 7ACC compounds failed to exert any antitumor effects in a model of human bladder tumor derived from the UM-UC3 cell line, the immunohistochemical analysis of MCT expression in this tumor revealed a lack of membrane expression of both MCT1 and MCT4 transporters. This result therefore validates tumor MCT as major targets of 7ACC compounds in vivo, and importantly, indicates that the extent of MCT, and in particular MCT1 and MCT4, represents a potential clinical biomarker to anticipate the tumor response to 7ACC compounds. Finally, we did not identify overt side effects with 7ACC compounds but possible
interference with oxidative healthy tissues that uptake monocarboxylates warrants further investigation; for instance, the impact of 7ACC compounds on either lactate uptake by slow-twitch muscle fibers and neurons, or butyrate capture by the colon should be evaluated in long-term studies.

We next found that 7ACC compounds reduced the extent of SiHa tumor relapse after cisplatin and 3-bromopyruvate treatments. Interestingly, during the course of treatment, tumor growth was inhibited to the same extent by 7ACC administration alone or in combination with either cisplatin or 3BP. Possible reasons for the post-treatment 7ACC-mediated reduction in tumor relapse are numerous and warrant further investigation. Cisplatin administration for instance is known to give rise to resistance phenomena in different cancers, in particular through exacerbation of tumor hypoxia. Interestingly, we previously reported a reduction in the hypoxic fraction of tumors treated with CHC, an unspecific MCT inhibitor, or following genetic silencing of MCT1 expression (11, 12). Also, we have recently reported that MCT inhibition could lead to anti-angiogenic effects which may contribute to tumor vessel normalization and consecutive restoration of a more homogenous pO₂ through the entire tumor (15-17). Labelling of SiHa tumor sections with the hypoxia probe pimonidazole confirmed a net reduction in tumor hypoxia following 7ACC2 treatment (Supplementary Figure 3).

Although similar mechanisms could account for the better therapeutic outcomes resulting from the combination 3BP/7ACC on tumor relapse, the observed additive effects of the two drugs is paradoxical considering that 3BP enters tumors cells through MCT1 (40). This means that 7ACC2 compound inhibits lactate entry through MCT but at the same dosage does not interfere with 3BP influx. This observation suggests that 7ACC compounds are lactate-mimetic structures, directly competing with lactate but also with 3-bromopyruvate, another metabolite-mimetic exhibiting a monocarboxylate function (see Suppl. Figure 1). We actually found that 3BP could block [¹⁴C]-lactate uptake in cancer cells to the same extent as 7AAC compounds.
and that increasing extracellular lactate concentration could compete in vitro with 7ACC compounds (Supplementary Figures 4A and 4B). It is therefore very likely that the mass action law governs the competitive interaction of 7ACC and 3BP with the transporter in such a way that 3BP preferentially enters cells when its concentration is higher (e.g. 100 µM 3BP vs. 10 µM 7ACC in Figures 4A, 4B and 4C). Of note, the mode of action of AR-C155858 is very different since this compound family is proposed to bind the intracellular region of MCT1 (36) and to act as a non-competitive inhibitor of lactate. As a result, AR-C155858 can prevent the entry of 3BP and thereby rescue tumor cells exposed to 3BP. Altogether, these data identify an additional advantage of 7ACC compounds since 3BP and 7ACC represent very complementary drugs. By inhibiting lactate influx, 7ACC compounds may indeed prevent tumor cells to use lactate as an energetic fuel, and 3BP by blocking glycolysis reduces the use of glucose to fuel alternate routes to support ATP and biosynthetic intermediates production.

In conclusion, we have identified a new family of compounds that selectively affect a single part of the MCT symporter translocation cycle, leading to inhibition of lactate influx but not lactate efflux. In mice bearing tumors, these compounds exert potent anticancer effects and may also significantly delay tumor relapse following conventional chemotherapy. Importantly, the unique pharmacological profile of 7ACC compounds accounts for critical advantages including a potential reduction in side effects (vs. drugs also interfering with lactate efflux), a lack of resistance due to compensatory mechanisms (vs. drug interfering with MCT1 but not MCT4, or inversely) and a lack of major interference with monocarboxylate-mimetic drugs such as 3BP.
REFERENCES.


FIGURE LEGENDS.

**Figure 1. 7ACC compounds inhibit lactate influx but not lactate efflux.** A. Representative MCT1 and MCT4 immunoblotting of extracts of HL-60, K562, SiHa, Hela and MDA-MB231 cells; gel loading is normalized with β-actin immunoblotting. This experiment was repeated 4 times with similar results. Effects of 7ACC1, 7ACC2 and AR-C155858 on the extent of lactate uptake by (B) SiHa cells and lactate release by (C) HL-60 cells and (D) MDA-MB231 cells. All the cells were exposed to the compounds at the indicated concentrations for 24 hours (16 hours for HL60); **P<0.01, ***P<0.001, n=3-5. Effects of the indicated compounds (10 µM, 72 hours) on the viability of the indicated (E) tumor cells and (F) normal cells, expressed as % of viability of untreated cells. **P<0.01, n=5.

**Figure 2. 7ACC compounds inhibit the growth of cervix SiHa, colorectal HCT116 and breast MCF-7 tumors while bladder UM-UC-3 tumors are resistant.** Left panels show the time course of the growth of SiHa (A), HCT-116 (B), UM-UC-3 (C) and MCF-7 (D) tumors following daily administration of 3 mg.kg⁻¹ 7ACC1 or 7ACC2 (vs. vehicle). ***P<0.001, n=5. Right panels show representative MCT1 and MCT4 immunostaining (green) of sections of SiHa (A), HCT-116 (B), UM-UC-3 (C) and MCF-7 (D) tumors; CD31-immunolabelling (red) and DAPI (blue) counterstaining are also shown. Inset Fig.2C : magnification of a UM-UC-3 area depicting punctate MCT1 staining. These experiments were repeated 3 times with similar results.

**Figure 3. 7ACC compounds inhibit SiHa tumor relapse post-treatment with cisplatin.** Time course of tumor growth (and relapse) following administration of 3 mg/kg 7ACC1 or 7ACC2 (vs. vehicle) each day indicated by an arrowhead (up to day 12) and 50 mg/kg cisplatin at days 0 and 8 (see arrows); mice treated only with vehicle are used as controls ** p<0.01, ***
p<0.001, n=8 mice per group. Representative pictures of tumors collected at day 24 (day 21 for control) are presented to illustrate the inhibition of tumor relapse from day 12 after treatments.

**Figure 4. Anti-proliferative effects of 3-bromopyruvate are prevented in vitro and in vivo by the MCT1/MCT2 inhibitor AR-C-155858 but not by 7ACC compounds.** Effects of 3BP treatment alone or in combination with 7ACC1, 7ACC2 or AR-C155858, on the viability of (A) SiHa, (B) HL60 and (C) MDA-MB231 cells; data are expressed as % of viability after 72 h treatment (16 h for HL60) vs. untreated cells, and the effect of each treatment alone is also presented; **P<0.01; ***P<0.001, n=6-18. Time course of tumor growth following daily administration of 3BP in combination with (D) 3 mg/kg AR-C155858 or (E) 3 mg/kg 7ACC2 (vs. vehicle); the effect of each treatment alone is also presented and mice treated only with vehicle are used as controls. *p<0.05, *** p<0.001, n=8 mice per group. (F) Representative pictures (left) and volume extents (right) of tumors collected at day 27 (15 days after the end of any treatment) are presented to illustrate the inhibition of tumor relapse when both 7ACC2 and 3BP were associated. **P<0.01.

**Figure 5. Distinct antitumor profiles of 7ACC and AR-C155858 compounds.** Scheme depicting the expression of MCT1 and/or MCT4 in the indicated human cancer cell lines and the effects of 7ACC and AR-C155858 compounds on lactate influx and/or efflux. Treatment leading to antiproliferative effects are indicated by a death’s head; all other situations represent a lack of activity.
Figure 1.

A. MCT1 - β-actin - MCT4 - β-actin

B. AR-C155858  7ACC1  7ACC2

C. % Lactate release

D. % Lactate release

E. % Cell viability

F. % Cell viability
Figure 2.

A. SiHa

B. HCT-116

C. UM-UC-3

D. MCF-7

**Control vehicle**

**7ACC1**

**7ACC2**

**Tumor volume** (mm³)

**Days of treatment**

***

n.s.
Figure 3.

- Vehicle
- Cisplatin + vehicle
- Cisplatin + 7ACC1
- Cisplatin + 7ACC2

Tumor volume (mm$^3$)

Days

0 3 6 9 12 15 18 21 24

0 100 200 300 400 500 600

Cisplatin  7ACC1 or 7ACC2

Ctrl

Cis

Cis + 7ACC1

Cis + 7ACC2

*  **
Figure 4.

A. SiHa

B. HL60

C. MDA-MB231

D. SiHa

E. SiHa

F. 3BP  7ACC2  3BP+7ACC2

**Figure 4.**

A. SiHa

B. HL60

C. MDA-MB231

D. SiHa

E. SiHa

F. 3BP  7ACC2  3BP+7ACC2

**Figure 4.**

A. SiHa

B. HL60

C. MDA-MB231

D. SiHa

E. SiHa

F. 3BP  7ACC2  3BP+7ACC2

**Figure 4.**

A. SiHa

B. HL60

C. MDA-MB231

D. SiHa

E. SiHa

F. 3BP  7ACC2  3BP+7ACC2
Figure 5.

7ACC

MCT1

SiHa, Hela, HCT116, FaDu

MCT4

MCT1

Lactate

AR-C155858

MCT1

HL-60 & K562

MCT1

Lactate

MCT4

MDA-MB231

MCT4

Lactate

MCT4

Lactate
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Antitumor activity of 7-aminocarboxycoumarin derivatives, a new class of potent inhibitors of lactate influx but not efflux.

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