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 recaptured by oxidative cancer cells to feed the tricarboxylic acid (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-aminocarboxycoumarins (7ACC) developed to selectively interfere with lactate fluxes in the lactate-rich tumor microenvironment. The pharmacologic properties of two compounds of this family, including their effects on lactate influx and efflux and antitumor 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 orthotopic MCF-7 breast tumors. MCT target engagement was confirmed by the lack of activity of 7ACC on bladder UM-UC-3 carcinoma that does not express functional MCT. 7ACC also inhibited SiHa tumor relapse after 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 (3BP) through MCT1, and contributed to the inhibition of tumor relapse after 3BP treatment. In conclusion, our results indicate that 7ACC selectively affects 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. Mol Cancer Ther; 13(6); 1410–8. ©2014 AACR.

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 (i.e., glucose to lactate conversion) under aerobic conditions (3). Although this paradigm fostered a new impetus in reexploring (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 reoxidized 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; refs. 8, 11). We also recently reported that lactate can stimulate angiogenesis through PHD2 inhibition and the consecutive stimulation of NF-κB and hypoxia-inducible factor-1α-dependent pathways (15–17). Together with studies documenting that in patients with cancer, elevated lactate concentrations are associated with poor prognosis (18–21), these findings

References

place the regulation of lactate flux as a particularly druggable process to impact on tumor progression. The main targets for such pharmacologic intervention are monocarboxylate transporters (MCT; refs. 22, 23).

The MCT family (also solute carriers SLC16) actually comprises 14 members, out of which four are proton-linked short chain MCT (MCT1–4; refs. 23, 24). In cancer, MCT1 (SLC16A1) and MCT4 (SLC16A3) are most often described (25). MCT1 is the most ubiquitous MCT and its expression is increased in p53-deficient tumors (12), whereas MCT4 expression is upregulated under hypoxic conditions (26). These two MCTs also differ by their substrate affinity: MCT1 shows a greater affinity for L-lactate (Km 3–6 mmol/L) and pyruvate (Km 1–2.5 mmol/L) than MCT4 (with Km equal to 25–30 mmol/L and 150 mmol/L, respectively; ref. 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 NADH (27, 28). As mentioned above, the factors 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; refs. 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-aminocarboxycoumarins (7ACC) as a new family of lactate flux inhibitors (synthesis and chemistry described in ref. 38); the most active compound 7ACC2 (see structure in Supplementary Fig. S1) exhibited a low IC₅₀ of 11 nmol/L 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) support 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 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 chemotheraphy but also with 3-bromopyruvate (3BP), 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 3 years from American Type Culture Collection 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, MCF-7) were cultured in Dulbecco’s Modified Eagle Medium (DMEM), and 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 RPMI-1640 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. Nonadherent 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 nonadherent cells.

Mice and in vivo treatments

Eight-week-old NMRI female nude mice (Elevage Janvier) were injected subcutaneously (s.c.) with 2 × 10⁶ SiHa cells, 2 × 10⁶ HCT-116 cells, or 5 × 10⁶ UM-UC-3 cells. An orthotopic breast cancer model was also used with MCF-7 tumor cells injected into the mammary fat pad of mice; a 17β-estradiol pellet had first been subcutaneously 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 intraperitoneally (i.p.); in some experiments, 7ACC treatment was combined with cisplatin (5 mg/kg) injected intraperitoneally at days 0 and 7 (7ACC administered daily except at days 0 and 7) or 3BP (3 mg/kg) injected i.p. from day 0 to 4 and day 7 to 11 (7ACC administered together with 3BP). Cisplatin and 3BP were also administered alone and control mice were injected with vehicle (dimethyl sulfoxide). Tumor sizes were tracked with an electronic calliper and determined using the formula: (length × width²)/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 mmol/L lactate and cells were treated for 24 hours with the compounds. For the lactate release measurements, cells were treated for 16 to 24 hours with the compounds in flat-bottom 24-well plates (500,000 cells/well) in normal DMEM.
DMEM medium (MDA-MB-231) or RPMI-1640 medium (HL-60 and K562). At the end of the lactate uptake or release experiments, cell supernatants were centrifuged using deproteinizing columns (15 minutes, 10,000 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) or rabbit polyclonal antibodies against MCT1 and MCT4 followed by a secondary antibody coupled to Alexa Fluorophores as previously described (12, 15). For immunoblotting, cell extracts were separated on SDS-PAGE and transferred onto polyvinylidene difluoride membranes before incubation with MCT1 and MCT4; gel loading was normalized with a β-actin antibody (Sigma).

**Statistical analysis**

Results are expressed as mean ± SEM. Student 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 (ref. 38; see also Supplementary Fig. S1 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 Supplementary Fig. S1 for structure). We found that in cervix cancer SiHa cells, which express both MCT1 and MCT4 (Fig. 1A), the 7ACC compounds blocked lactate influx, whereas the AR-C155858 compound failed to do so (Fig. 1B); similar results were obtained with another cervix cancer cell line (Hela; data not shown) and also with human pharynx squamous carcinoma FaDu cells (Supplementary Fig. S2). In contrast, we observed that lactate efflux by the highly glycolytic leukemia cells HL60, which express MCT1 but not MCT4 (Fig. 1A), was inhibited by AR-C155858 but not by the 7ACC compounds (Fig. 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 (Fig. 1D). The cytotoxicity resulting from exposure to the different compounds was in adequation with the observed inhibition of lactate influx or efflux, whereas 7ACC compounds inhibited the proliferation of cervix cancer cells but failed to interfere with leukemia cell growth, AR-C155858 exhibited the opposite behavior, being only toxic for the latter (Fig. 1E); MDA-MD-231 cells were resistant to both types of inhibitors (Fig. 1E). We also tested the effects of 7ACC on normal human fibroblasts (hTERT BJ-5a), human umbilical vein endothelial cells, and embryonic kidney cells and failed to observe any significant cytotoxic effects (Fig. 1F).

**Combination of 7ACC compounds with conventional chemotherapy or 3-bromopyruvate leads to significant reduction in posttreatment tumor relapse**

In the next experiments, we further explored the combination of 7ACC compounds with conventional chemotherapy (cisplatin) and 3BP, 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 mono-therapy 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 (Fig. 3). This experiment showed that cisplatin prevented tumor growth and that tumor recurrence could be observed when it was used as monotherapy (Fig. 3). Interestingly, when combined with the 7ACC compounds, tumor relapse was attenuated as shown by a prolonged inhibition of tumor regrowth. 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; Fig. 3).

In a second set of experiments, we examined the possibility to combine MCT inhibitors and 3BP. This compound was indeed recently reported to enter cells through MCT1 (40), a process that 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 Fig. 1. We confirmed that 3BP was cytotoxic in tumor cell lines expressing MCT1 either as a main path to uptake lactate such as SiHa (Fig. 4A) and HeLa (not shown) or instead to release lactate such as HL60 (Fig. 4B) and K562 (not shown). In MDA-MB231 cells, which express MCT4 but not MCT1 (see Fig. 1A), 3BP failed to exert cytotoxic effects (Fig. 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 (Fig. 4A and B). We next examined whether these observations could also be recapitulated in vivo. We therefore treated SiHa tumor-bearing mice either with 3BP alone or together with AR-C155858 or 7ACC2 compound. We found that AR-C155858 abrogated the antitumor effects of 3BP (Fig. 4D) contrary to 7ACC2 compound (Fig. 4E). Of note, 3BP as monotherapy was particularly
efficient to block SiHa tumor growth (see Fig. 4D and E). On the basis of the observations reported in Fig. 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 (Fig. 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 isoforms, 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 expresses MCT4 (and possibly MCT2) but not MCT1, underlying the limitation of MCT isoform-specific inhibitory compounds (see below). The distinct behaviors of 7ACC versus AR-C155858 are summarized in Fig. 5. There are only a few examples of drugs interfering with solute transport that block the flux unidirectionally. One of the best examples is SoRI-20041, a drug that inhibits dopamine uptake, but has no significant effect on dopamine efflux (the so-called reverse transport; ref. 44). Molecular mechanism supporting the pharmacologic profile of SoRI-20041 is currently unknown but is proposed to involve an allosteric regulation subtly 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 patients with cancer 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. Because 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 antitumor effects of 7ACC compounds using mouse xenograft models derived from human cervix cancer SiHa cells and also from the human colorectal cancer cell line HCT116 (Fig. 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 3BP 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 posttreatment 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 antiangiogenic effects that may contribute to tumor vessel normalization (Supplementary Fig. S3).

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 and also with 3BP, another metabolite-mimetic exhibiting a monocarboxylate function (see Supplementary Fig. S4A and S4B). 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 μmol/L 3BP vs. 10 μmol/L 7ACC in Fig. 4A–C). Of note, the mode of action of AR-C155858 is very different because this compound family is proposed to bind the intracellular region of MCT1 (36) and to act as a noncompetitive 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 because 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...
Inhibitors of Lactate Influx but Not Efflux

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 is indicated by a death’s head; all other situations represent a lack of activity.

The pharmacologic 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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