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Synthesis and pharmacological evaluation of carboxycoumarins as a new antitumor treatment targeting lactate transport in cancer cells

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ABSTRACT

Under hypoxia, cancer cells consume glucose and release lactate at a high rate. Lactate was recently documented to be recaptured by oxygenated cancer cells to fuel the TCA cycle and thereby to support tumor growth. Monocarboxylate transporters (MCT) are the main lactate carriers and therefore represent potential therapeutic targets to limit cancer progression. In this study, we have developed and implemented a stepwise in vitro screening procedure on human cancer cells to identify new potent MCT inhibitors. Various 7-substituted carboxycoumarins and quinolinone derivatives were synthesized and pharmacologically evaluated. Most active compounds were obtained using a palladium-catalyzed Buchwald–Hartwig type coupling reaction, which proved to be a quick and efficient method to obtain aminocarboxycoumarin derivatives. Inhibition of lactate flux revealed that the most active compound **19** (IC_{50} 11 nM) was three log orders more active than the CHC reference compound. Comparison with warfarin, a conventional anticoagulant coumarin, further showed that compound **19** did not influence the prothrombin time which, together with a good in vitro ADME profile, supports the potential of this new family of compounds to act as anticancer drugs through inhibition of lactate flux.

1. Introduction

Cancer cells consume large amounts of glucose to survive and proliferate.[1] The high turnover of the glycolytic pathway uncoupled from mitochondrial respiration accounts for the high concentration of lactate (up to 10 mM) in tumors,[2,3] that is proposed to be associated with tumor invasiveness and poor patient outcomes.[4–7] Lactate however does not merely represent a waste metabolite or a biomarker of tumor aggressiveness. It can indeed be captured by oxidative tumor cells and reconverted into pyruvate to be used in the TCA cycle.[2] A synergy actually takes place between glycolytic tumor cells exporting lactate, and oxygenated cells importing it to feed their metabolism.[8,9] In addition, lactate shuttle has been reported to occur in tumors between glycolytic tumor cells and endothelial cells[10,11] as well as between tumor-associated fibroblasts and oxidative tumor cells.[12,13] These observations have led investigators to focus on the expression and the regulation of the monocarboxylate transporters (MCT) in tumors since these proteins represent the major path for inward and outward lactate fluxes.[14]

The family of MCT (also named SLC16 solute carrier) is composed of 14 members.[15,16] Among them, only four isoforms (MCT1-4) have been documented to act as proton-linked transporters that can carry short chain monocarboxylates such as lactate and pyruvate across cell membranes.[17,18] In cancer cells, MCT1 and MCT4 are the most widely expressed.[16,19] MCT1 shows a better affinity for L-lactate than MCT4,[16] but MCT4 has a higher turnover rate than MCT1.[20] These differences are consistent with their respective roles in tumors.[18,21] With a high affinity for lactate, MCT1 enables lactate entry into oxidative tumor cells whereas low affinity MCT4 is mainly expressed in glycolytic tumor cells and tumor-associated fibroblasts that export lactate. The

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complementarity between MCT1 and MCT4 to drive lactate shuttle(s) in tumors, represents an attractive target for new anticancer drugs. MCT1 blockade can indeed prevent oxygenated tumor cells to use lactate and therefore force them to consume glucose more avidly.[8] Consequently, hypoxic tumor cells that are essentially dependent on glucose and have limited or no access to replacement fuels die from glucose deprivation.[8,9] As an alternative therapeutic strategy, interference with tumor pH regulation through the inhibition of carbonic anhydrases, anion exchangers but also MCT4 can lead to a cytosolic acidification of glycolytic tumor cells up to a level incompatible with cell survival.[22]

α-Cyano-4-hydroxycinnamate (CHC) was historically reported as the first MCT inhibitor.[23] CHC, however, is usually described to be active in the upper μM range and lacks specificity since in some experimental setups, it can also inhibit the mitochondrial pyruvate carrier.[24] More recently, AR-C155858, a highly potent MCT1/MCT2 inhibitor was disclosed by Astra-Zeneca.[25] This compound was demonstrated to strongly and selectively block MCT1 and MCT2 activity in activated T-lymphocytes, obstructing lactate efflux and thereby acting as a powerful immunosuppressive drug.[26] The therapeutic effects of this compound in tumors is however limited by the compensatory effects of MCT4 that can take the lead to facilitate lactate efflux when the high affinity MCT1 transporter is blocked.[20]

Here, we designed a stepwise screening procedure to identify MCT inhibitors able to prevent lactate influx into tumor cells expressing both MCT1 and MCT4. Rational design led us to identify monocarboxylate-containing coumarins as a potential scaffold endowed with inhibitory effects on lactate transport. The Knoevenagel reaction[27] and Palladium-coupling methods[28–30] were the main routes of synthesis. SAR studies confirmed the ability of synthesized compounds to efficiently block lactate transport in the nanomolar range, leading to anti-proliferative effects.

2. Results

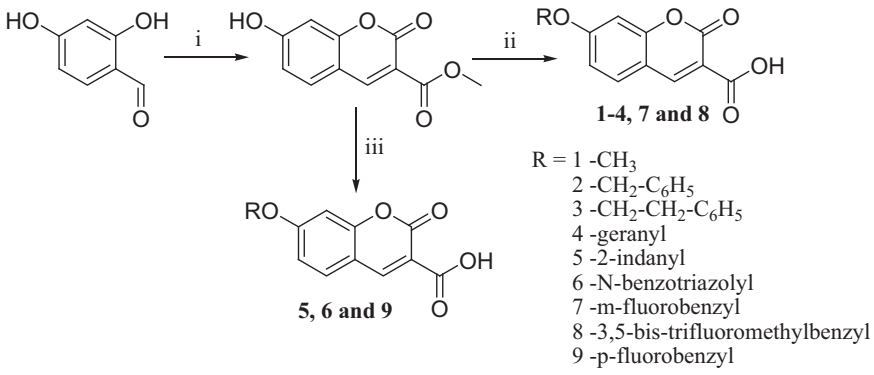
2.1. Chemistry

We used the Knoevenagel condensation to obtain substituted carboxycoumarins for the first in vitro structure–activity relationship studies. We then carried out further modulation on the coumarin scaffold using simple nucleophilic substitutions or Mitsunobu reactions to optimize the activity of hits by varying the substituent on the position 7 (Scheme 1). The various coumarin derivatives **1–9** were easily prepared starting from 2,4-dihydroxy benzaldehyde. Knoevenagel condensation with dimethyl malonate gave the common precursor in excellent yield. Alkylation of the phenol group was then carried out using either the corresponding alkyl or benzylic halide in DMF, or the Mitsunobu etherification with the corresponding alcohol as the alkylating agent. The methyl ester group was then hydrolyzed with lithium hydroxide in THF/water, and the desired carboxylic acids were isolated in good overall yields and suitable purity after acidification and crystallization in ethanol.

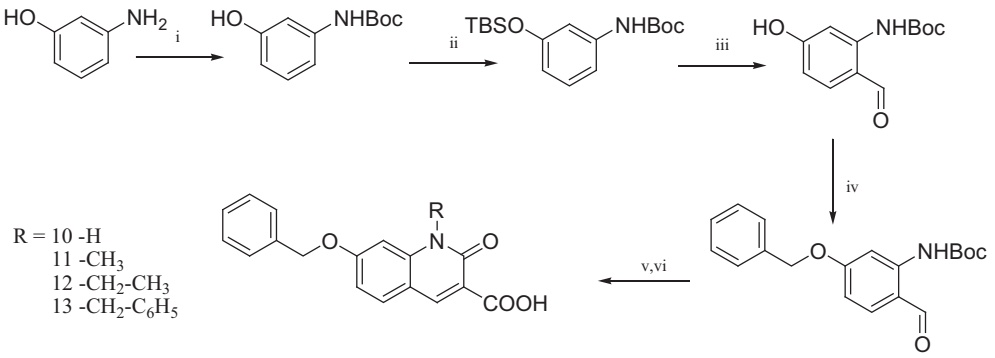
We then investigated the replacement of the oxygen atom of the lactone ring by a substituted or un-substituted nitrogen atom (Scheme 2). This aimed to examine the impact of the hydrogen bond donor –NH– (instead of the H-bond acceptor –O–) but also of diverse nitrogen substitutions on the ability of the compounds to more efficiently inhibit lactate transporters. Protected 3-hydroxy aniline bearing an NH-Boc *ortho*-directing group[31,32] was easily prepared in two-step starting from commercially available 3-amino-phenol. *ortho*-Lithiation was then carried out with *tert*-butyl lithium (2.4 equiv) in diethyl ether at −40 °C. The lithio intermediate was trapped with DMF, and the desired protected amino aldehyde was isolated in a 44% yield after chromatographic purification. Deprotection of the TBS and benzylation of the phenol with benzyl bromide was carried out in a one-pot procedure by using potassium fluoride in DMF. Cyclisation to the corresponding unsubstituted lactam ring with diethyl malonate and followed by alkylation of the nitrogen atom and final saponification of the ester group gave the desired acids **10–13** in good overall yields.

The impact of substitution on the position 4 of the coumarin ring was also investigated (Scheme 3) and we designed the preparation derivatives **14–16** starting from 2,4-dihydroxy acetophenone. Regioselective alkylation occurred on position 4 to yield the mono-alkylated acetophenones. The methyl ketone being much less electrophilic compared to the corresponding aldehyde, no reaction occurred when the ketones were directly reacted with either methyl malonate or Meldrum acid using various conditions. It was found that the desired coumarins could however be obtained by prior activation of the methyl ketone by ammonia which gave the corresponding imine derivatives which were not isolated and directly reacted with Meldrum acid.

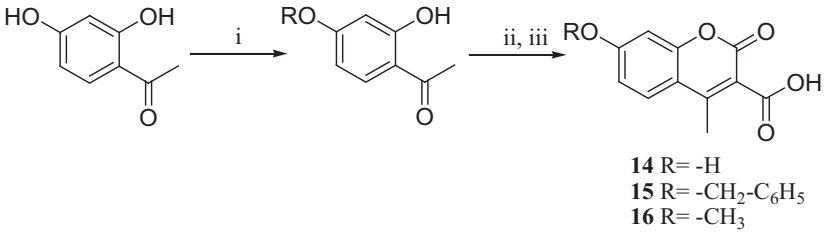
We then focused on the direct introduction of various amino substituents of the position 7 starting from the corresponding 7-triflyl or 7-iodoalkylcarboxycoumarin derivatives. In our hands, all the attempts aiming at the substitution of either the triflate or iodo[35,36] derivatives by an amino group via palladium-catalyzed cross-coupling[33] as well as copper (I)-catalyzed Buchwald amination failed to produce the desired pure aminocarboxycoumarins (Scheme 4). We then adopted and optimized a second route that started by a palladium-catalyzed Buchwald–Hartwig type coupling reaction, which proved to be a quick and efficient method to obtain aminocarboxycoumarin derivatives.[28–30] Indeed, after careful selection of the palladium precatalyst [$Pd(AcO)_2$] associated with



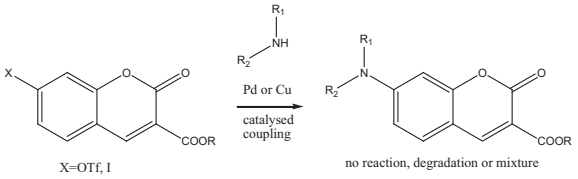
Scheme 1. General synthesis of compounds **1–9**. Reagents and conditions: (i) Dimethyl malonate, (piper/AcOH) cat in EtOH, 3 h reflux. (ii) R-X, Cs_2CO_3 or K_2CO_3 in DMF, O/N reflux followed by LiOH in THF/H_2O, 1 h reflux. (iii) R-OH, DTBAD/PPH3 in THF, O/N at RT followed by LiOH in THF/H_2O, 1 h reflux.



Scheme 2. General synthesis of compounds **10**–**13**. Reagents and conditions: (i) di-*tert*-butyl dicarbonate in THF, O/N reflux. (ii) imidazole, TBSCl in DMF, O/N at RT. (iii) *t*-BuLi, DMF in Et_2O, −40 °C 2 h and 0 °C 1 h. (iv) Benzylbromide, KF in DMF, O/N at RT (v) Diethyl malonate, (piper/AcOH)cat in EtOH, O/N reflux (vi) R-I or R-Br, K_2CO_3 in DMSO, O/N refux followed by LiOH in THF/H_2O, 1 h reflux.



Scheme 3. General synthesis of compounds **14**–**16**. Reagents and conditions: (i) R-X, Cs_2CO_3 in DMF, O/N reflux. (ii) NH_3 7N in MetOH, O/N at RT. (iii) Meldrum acid in EtOH, 5 h reflux.



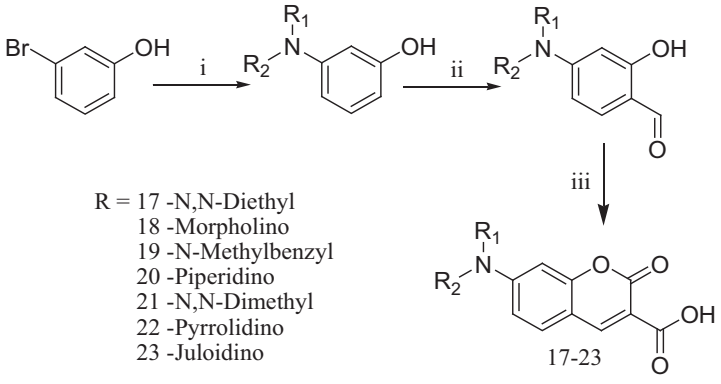
Scheme 4. Palladium- and copper (I)-catalyzed amination reaction.

the strong Verkade base as ligand and LiHMDS as a base in toluene, we successfully substituted 3-bromophenol with various secondary amines to introduce the desired amino group which constituted the main building block. Formylation using classical Vilsmeier–Haack reaction followed by cyclisation with Meldrum acid allowed us to obtain the pure acids **17**–**23** without further purification steps (Scheme 5).

2.2. Pharmacological evaluation

2.2.1. Carboxycoumarin derivatives inhibit the proliferation of lactate-consuming human tumor cells with consistent SAR

The activity of the different compounds was first evaluated by comparing their cytotoxic effects on human cancer cells in either glucose- or lactate-containing medium after 72 h of treatment. This assay was based on preliminary experiments documenting that the human cervix carcinoma cell line SiHa can switch from a glycolytic to an oxidative metabolism when lactate is available as an energy substrate.[8] This primary assay led to the identification of compounds which selectively inhibited tumor cell proliferation in the presence of lactate but failed to exert toxic effects in the presence of glucose (Table 1). Concentration-dependent inhibition of cell proliferation was compared with CHC as a reference compound (see examples in Fig. 1a). Of note, in SiHa cells, lactate uptake primarily depends on the high affinity MCT1 transporter.[8]



Scheme 5. General synthesis of compounds **17**–**23**. Reagents and conditions: (i) secondary amine, $Pd(OAc)_2$, verkade base, LiHMDS in toluene, 24 h reflux. (ii) $POCl_3$ in DMF, 1 h 90 °C. (iii) Meldrum acid, (piper/AcOH)cat in EtOH, 3 h reflux.

Indeed, although SiHa cells express both MCT1 and MCT4,[34] the higher affinity for lactate of MCT1 (K_m: 3–6 mM) versus MCT4 (K_m: 25–30 mM)[16] indicates that in the presence of 10 mM lactate in the culture medium (to mimic lactate concentration reported in tumors),[2] MCT1 functions at maximal rate contrary to MCT4. Thus, the contribution of MCT4 to lactate entry in our experimental model should in theory be minimal and if any, compounds unable to block this compensatory mechanism should not be selected in this primary assay. Furthermore, we found that under hypoxia, the expression of MCT4 was significantly increased in SiHa cells (Supplementary Fig. 1a) and that, as long as glucose was present, our compounds failed to show any cytotoxicty in these conditions (Supplementary Fig. 1b).

Several structure–activity relationships were identified (Table 1). First, *O*-benzyl and secondary amino group on the position 7 of the carboxycoumarin scaffold drastically improved compound toxicity in lactate-containing medium without affecting the survival of SiHa cells in the presence of glucose with or without pyruvate (Fig. 1b). The carboxylic acid function was necessary to keep a potent effect since the ester analogues failed to demonstrate any toxicity (data not shown). However, methyl substitution on the position 4 of the carboxycoumarin led to a complete loss of activity (**1**, **2** vs **14**, **15**, **16**), as well as the replacement of the lactone by a lactam moiety (substituted or not) (**2** vs **10**, **11**, **12**, **13**). These data showed that the oxygen of the carboxycoumarin structure and the freedom of the position 4 played key roles to impair lactate entry or further intracellular metabolism. The primary SAR of these new compounds is summarized in Scheme 6. Compounds **17** and **19** were also used to document the occurrence of apoptosis after 48 h exposure, as indicated by caspase-3 cleavage in tumor cells incubated in lactate-containing medium (Fig. 1c).

2.2.2. Carboxycoumarin derivatives block lactate uptake in human cancer cells

We then developed a secondary assay to test whether our selected compounds functionally blocked lactate entry into cancer cells. Using an enzymatic assay to measure the remaining lactate concentration in culture medium after 24 h of SiHa cell treatment with the compounds, we validated previous SAR conclusions (see Table 1). As shown in Figure 2a, we observed a dose-dependent inhibition of lactate consumption and a shift to the left of the sigmoid dose-response curves of compounds **17** and **19**. To further confirm these observations, we measured [^{14}C]-lactate uptake by SiHa cells on a shorter time frame (12 min). In particular, we validated compounds **17** and **19** as potent inhibitors of lactate uptake with IC_{50} values equal to ~250 and ~10 nM, respectively, in comparison to CHC which exhibited an IC_{50} ~10 μM in this assay (Fig. 2b).

2.2.3. In vitro ADME properties and in vivo PK profile of compounds 17 and 19

We then evaluated the general in vitro ADME profile of compounds **17** and **19**. Both compounds showed a moderate-to-good aqueous solubility, an excellent chemical stability in simulated gastric (SGF) and intestinal (SIF) fluids, a good apparent permeability coefficient (Papp) through Caco-2 monolayer and a high metabolic stability on mouse (MLM) and human liver microsomes (HLM) as well as on human hepatocytes (Table 2). They also showed an excellent stability after 1 h incubation in mouse plasma, and a moderate stability (**17** > **19**) in human plasma. Also, the extent of human plasma protein binding (HPPB) of **17** and **19** supports a good potential for drug distribution. Finally, the intraperitoneal administration of **19** (3 mg/kg) to mice led to a C_{max} of 1246 ng/ml (4 μM) in a very short time (T_{max} = 10 min) associated with a plasma half-life of 4.5 h (Table 2).

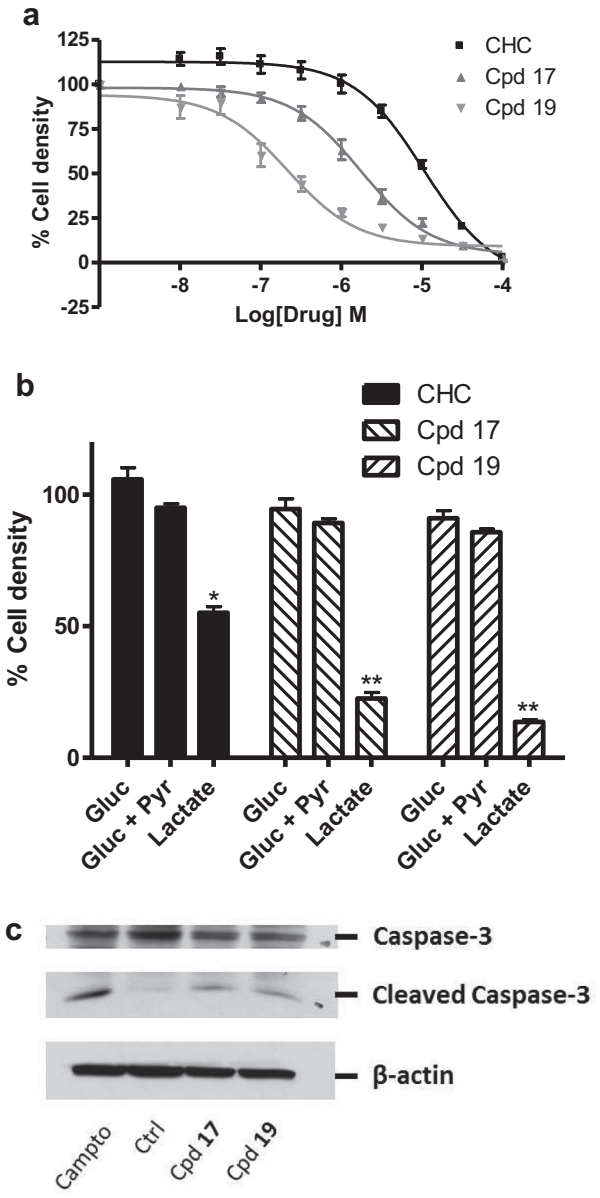
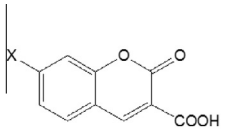


Figure 1. Cytotoxic effects of compounds **17** and **19**. (a) Human SiHa cancer cell proliferation was measured after 72 h of incubation in lactate-containing medium in the presence of increasing concentrations of the indicated compounds (n = 18). (b) Bar graph represents the effect of 10 μM of the indicated compounds on SiHa cell proliferation in glucose-containing medium with or without pyruvate (vs lactate-containing medium); $^{*}P < 0.05$, $^{**}P < 0.01$, n = 18. (c) Caspase-3 activation in SiHa cells treated during 48 h in lactate-containing medium with compounds **17** or **19** (10 μM) and camptothecin (10 μM) used as positive control (n = 3).

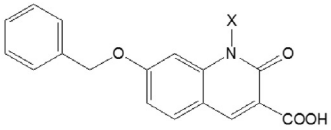
2.2.4. Carboxycoumarins 17 and 19 do not show anticoagulant properties

Since hit compounds had a coumarinic scaffold, we finally aimed to exclude any major anticoagulant side effect. We first tested the effects of compounds **17** and **19** on mouse survival in comparison with Warfarin®, a well-known anticoagulant coumarinic drug. Compounds **17** or **19** did not induce any mortality at the daily dose of 3 mg/kg while Warfarin® induced hemorrhages and mouse death at doses starting from 1.5 mg/kg (Fig. 3a). We next performed a prothrombin time test (PT time) on the plasma of treated mice. We found that while **17** and **19** did not influence the extrinsic pathway of coagulation, Warfarin® dramatically prolonged the clotting time (Fig. 3b).

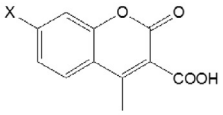
Table 1
Pharmacological evaluation of synthesized compounds



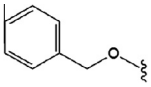
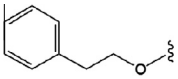
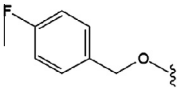
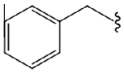
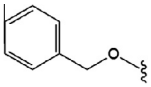
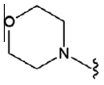
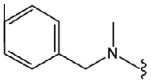
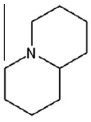
1-3, **9** and **17-23**



10-13



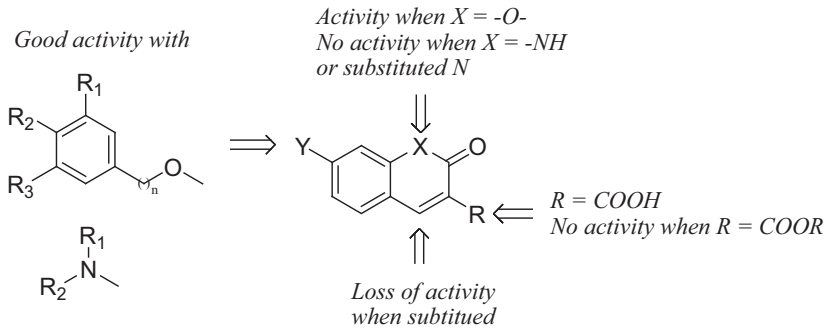
14-16

Compd	Substitutent (X)	Cytotoxicity	Lactate uptake inhibition
		EC_{50} (μM)	IC_{50} (μM)
CHC	Reference compound	10.7	43.5
1		9.8	2.3
2		9.1	1.9
3		5.6	2.4
9		3.9	1.25
10		>30	>30
11		>30	>30
12		>30	>30
13		>30	>30
14		>30	>30
15		>30	>30
16		>30	>30
17		1.8	0.86
18		17.5	7.9
19		0.22	0.059
21		2.9	1.4
22		14.3	>30
23		1.2	0.73

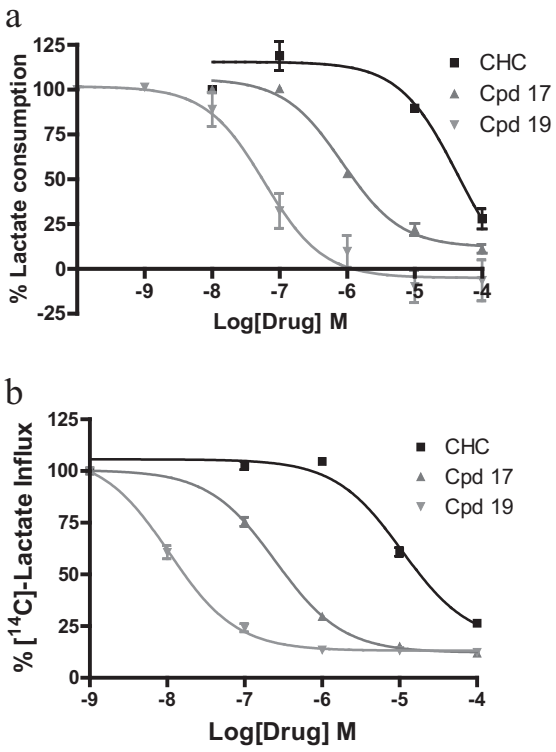
EC_{50}: compound concentration that reduces cell proliferation by 50%.
IC_{50}: compound concentration that reduces lactate uptake by 50%.
Compounds **4**–**8** did not exert any significant cytotoxicity.

3. Discussion

In this study, various 7-substituted carboxycoumarins and quinolinone derivatives were synthesized and pharmacologically evaluated for their capacity (i) to kill lactate-fueled tumor cells and (ii) to spare the same tumor cells when fueled by glucose. This double filter was designed to gain in tumor selectivity of our compounds and thus in the security of use regarding healthy tissues. Our SAR study documented that 7-alkylamino substituents on the 3-carboxycoumarin scaffold significantly improved the anti-proliferative effects of the compounds, with submicromolar EC_{50} values. We previously reported that lactate can support cancer cell proliferation: upon uptake and intracellular oxidation into pyruvate, it supports TCA-cycle-dependent ATP synthesis and cataplerosis.[8,9] To



Scheme 6. SAR study of the synthesized carboxycoumarins as inhibitors of lactate flux.



Compound	[IC_{50}]
CHC	10.7 μM
17	0.25 μM
19	**11.0 nM**

Figure 2. Inhibition of lactate uptake by compounds **17** and **19**. (a) Lactate uptake by human SiHa cancer cells was determined by measuring the remaining lactate concentration in the extracellular medium after a 24 h incubation in the presence of increasing concentrations of compounds **17** and **19** (n = 3–6). (b) Radiolabeled lactate influx was determined by measuring the amount of ^{14}C-lactate captured by SiHa cells after a 12 min incubation with compounds **17** and **19** (n = 3–5); in these experiments, CHC was used as reference compound.

discriminate between compounds interfering with lactate transport or metabolism, we used additional assays evaluating extracellular lactate consumption and intracellular lactate uptake. Thus, the secondary assay measuring lactate clearance from cell supernatant was used to evaluate the most active compounds (i.e., cytotoxic hits in the presence of lactate) using CHC as a reference compound. This assay identified compound **19** as a potent inhibitor of lactate uptake with an IC_{50} = 59 nM, that is, 3 log orders more active than CHC (IC_{50} = 43.5 μM). This was confirmed in the tertiary assay measuring inhibition of [^{14}C]-lactate influx: the IC_{50} value for compound **19** amounted to 11 nM in comparison to 11 μM for CHC. Compounds **17** and **23** showed an intermediary profile with IC_{50} ~50–60 fold more favorable than CHC.

Interestingly, Supuran and colleagues have reported that coumarins could act as inhibitors of carbonic anhydrases, including CAIX and CAXII.[22,35,36] In our experimental model, we could document that CAIX was not expressed in normoxic SiHa cells but could be induced in response to hypoxia while CAXII was expressed independently of the pO_2 levels (Supplementary Fig. 1c). We further found that in the presence of glucose, our compounds failed to exert any cytotoxic effects in normoxia (Fig. 1b) but also under hypoxia (Supplmentary Fig. 1b). These data support that in our experimental model, compounds **17** and **19** exert their antiproliferative activity through an inhibition of lactate uptake and not of CAIX/CAXII. Still, the above observations suggest that some coumarinic compound could have the capacity to inhibit two of the most critical pH regulators in tumors,[22] namely carbonic anhydrases and MCT. Such possibility is supported by the recent work of Bonneau et al who reported that the CA hydrolysis product of 3-cyano-7-hydroxy-coumarin could lead to a CHC derivative.[35]

Finally, compounds **17** and **19** exhibited good in vitro ADME profiles and **19** led to significant systemic exposure following intra-peritoneal mouse administration demonstrating its potential for further in vivo studies. Contrary to Warfarin®, the compounds did not increase the prothrombin time (despite their coumarinic scaffold) and did not lead to fatal hemorrhagic accidents.

4. Conclusions

We have identified a new family of inhibitors of lactate flux through monocarboxylate transporters in human cancer cells. The most active compounds are 7-alkylamino 3-carboxycoumarins, among which compound **19** has an IC_{50} of ~10 nM. The lack of toxicity in cells using glucose (instead of lactate) as a preferential energy fuel (which is the most common situation for healthy tissues) together with the lack of anticoagulant activity allows to anticipate a safe profile and a selective antitumor action of compound **19**.

5. Experimental section

5.1. Chemistry

5.1.1. General procedure for the synthesis of 1–4, 7 and 8 (Scheme 1)

The Knoevenagel condensation allowed us to obtain important coumarin derivatives for our stepwise synthesis. 2,4-dihydroxybenzaldehyde (1.0 equiv) was dissolved in ethanol before the dimethyl malonate (1.2 equiv) was slowly added to the solution.

Table 2
In vitro ADME and PK profiles of compounds **17** and **19**

	Compound **17**	Compound **19**
In vitro		
Aqueous solubility (μM)[a]	199	58
% Remaining after 1 h in SGF[b]	113	105
% Remaining after 4 h in SIF[c]	109	91
MLM[d] ($T_{1/2}$ in min)	50	>60
HLM[e] ($T_{1/2}$ in min)	>60	>60
Human hepatocytes ($T_{1/2}$ in min)	>120	>120
% Remaining after 1H incubation in mouse plasma	108	103
% Remaining after 1H incubation in Human plasma	69	44
In vivo mouse PK (3 mg/kg, intra-peritoneal)[f]		
t_{max} (min)	n.d.	10
C_{max} (ng/ml)	n.d.	1246
$t_{1/2}$ (min)	n.d.	270
AUC_{inf} (min ng/ml)[g]	n.d.	56109

N.d. = nondetermined.
[a] Solubility measured in PBS (pH 7.4) with 2% DMSO.
[b] Simulated gastric fluid.
[c] Simulated intestinal fluid.
[d] Mouse liver microsome.
[e] Human liver microsome.
[f] Injection of 50 μl in pure DMSO. Results are given as average of three mice (serial sampling).
[g] Area under the concentration–time curve from the time of dosing, extrapolated to infinity.

Piperidine and acetic acid were added dropwise to catalyze the reaction (one drop for 3 mmol).The reaction mixture was stirred and heated under reflux 3 h. After cooling, the resulting precipitate was filtered, dried and used for the nucleophilic substitution. The coumarin product (1.0 equiv) was dissolved in DMF (3.5 ml per mmol). The halogenoalkane (bromide or chloride derivative) was then added to the solution (1.1 equiv) before addition of a weak base (1.0 equiv of Cs_2CO_3 or K_2CO_3) to the mixture. The reaction mixture was stirred and heated under reflux overnight. After cooling, an equivalent volume of distilled water was added to the DMF solution before liquid extraction with ethyl acetate. The organic layers were merged and washed with a saturated solution of Li_2SO_4 to ensure residual DMF traces elimination. The final organic layer was dried over Na_2SO_4, filtered and concentrated with the rotary evaporator. The resulting solid was recrystallized from EtOH, followed by hydrolysis with LiOH (10 equiv) in a THF/water solution (1:1) heated 1 h under reflux. After cooling, the mixture was concentrated by rotary evaporation. The resulting precipitate was solubilized in a minimum volume of NH_4OHcc/water (1:4). HCl 36% was carefully added dropwise to the stirred solution until precipitation at low acidic pH (control with pH paper). The resulting precipitate was filtered and recrystallized from EtOH to give the final product.

5.1.1.1. 7-Methoxy-2-oxo-2*H*-chromene-3-carboxylic acid (1). ^{1}H NMR (300 MHz, DMSO): ***δ*** ppm 8.69 (s, 1H), 7.80 (d, ***J*** = 8.4, 1H), 7.00 (s, 1H), 6.98 (d, ***J*** = 9.3, 1H), 3.88 (s, 3H).

^{13}C NMR (75 MHz, DMSO): δ ppm 165.04 (C), 164.69 (C), 157.69 (C), 157.30 (C), 149.29 (CH), 131.96 (CH), 114.60 (C), 113.72 (CH), 112.09 (C), 100.71 (CH), 56.68 (CH_3).

MS (APCI): m/z = 221.09 (MH^+).

HRMS: m/z calcd for [$C_{11}H_9O_5$] 221.04500, measured 221.04433.

Global yield: 56.6%.

5.1.1.2. 7-(Benzyloxy)-2-oxo-2*H*-chromene-3-carboxylic acid (2). ^{1}H NMR (DMSO-d_6) δ ppm 8.72 (s, 1H), 7.84 (d, J = 8.7, 1H), 7.50–7.36 (m, 5H), 7.14 (s, 1H), 7.09 (d, J = 9, 1H), 5.26 (s, 2H) ^{13}C NMR (DMSO-d_6) δ ppm 164.62 (C), 164.06 (C), 157.64 (C), 157.23 (C), 149.40 (CH), 136.45 (C), 132.05 (CH), 129.03 (CH), 128.69 (CH), 128.50 (CH), 114.56 (C), 114.28 (CH), 112.26 (C), 101.61 (CH), 70.64 (CH_2) MS (APCI): m/z = 296.93 (MH^+) HRMS: m/z calcd for [$C_{17}H_{13}O_5$] 297.07629, measured 297.07594.

Global yield: 31.8%.

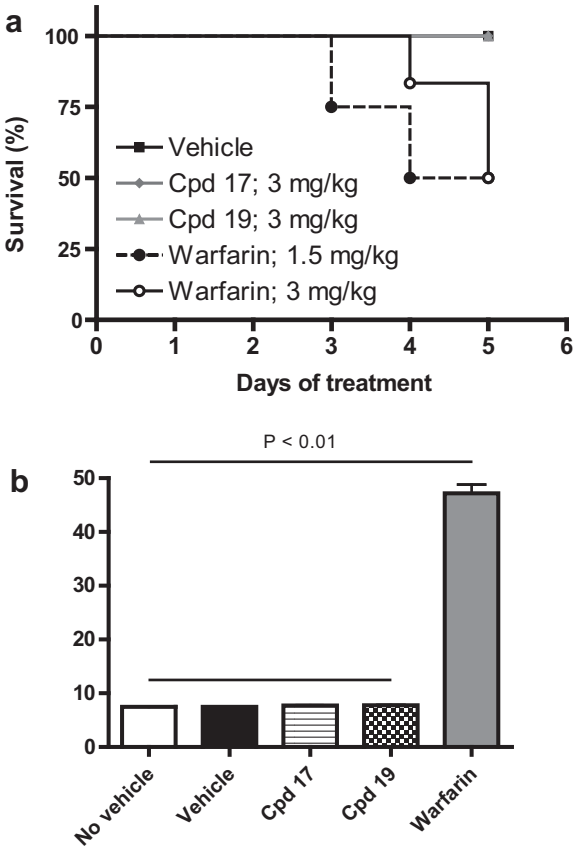


Figure 3. Evaluation of the anti-coagulant effects of compounds **17** and **19** in mice. (a) Survival curves of C57Bl6/J mice daily treated with vehicle, compounds **17** and **19** (3 mg/kg), or Warfarin (1.5 and 3 mg/kg) (10 mice per group). (b) Prothrombin time using mouse plasma collected after 5 days of daily intraperitoneal injection with vehicle (DMSO), **17** (3 mg/kg), **19** (3 mg/kg) or Warfarin (1.5 mg/kg).

5.1.1.3. 2-Oxo-7-phenethoxy-2*H*-chromene-3-carboxylic acid (3). ^{1}H NMR (DMSO-d_6) δ ppm 8.70 (s, 1H), 7.79 (d, J = 8.7, 1H), 7.33–7.22 (m, 5H), 7.02 (s, 1H), 6.98 (d, J = 8.7, 1H), 4.34 (t, J = 6.9, 2H), 3.07 (t, J = 6.6, 2H) ^{13}C NMR (DMSO-d_6) δ ppm 164.62 (C), 164.21 (C), 157.70 (C), 157.29 (C), 149.48 (CH), 138.36 (C), 132.03 (CH), 129.44 (CH), 128.82 (CH), 126.86 (CH), 114.29 (C), 114.01 (CH), 112.09 (C), 101.22 (CH), 69.55 (CH_2), 35.02 (CH_2) MS (APCI): m/z = 310.96 (MH^+) HRMS: m/z calcd for [$C_{18}H_{15}O_5$] 311.0919, measured 311.0925.

Global yield: 9.6%.

5.1.1.4. (*E*)-7-(3,7-Dimethylocta-2,6-dienyloxy)-2-oxo-2*H*-chromene-3-carboxylic acid (4). ^{1}H NMR (DMSO-d_6) δ ppm 12.98 (s, 1H), 8.72 (s, 1H), 7.81 (d, J = 8.7, 1H), 7.01 (s, 1H), 6.98 (d, J = 9, 1H), 5.44 (t, J = 6, 1H), 5.04 (t, J = 5.7, 1H), 4.68 (d, J = 6.6, 2H), 2.09 (s, 4H), 1.72 (s, 3H), 1.59 (s, 3H), 1.55 (s, 3H) ^{13}C NMR (DMSO-d_6) δ ppm 164.61 (C), 164.33 (C), 157.69 (C), 157.29 (C), 149.58 (CH), 142.03 (C), 131.95 (CH), 131.53 (C), 124.14 (CH), 119.10 (CH), 114.26 (CH), 114.07 (C), 111.96 (C), 101.32 (CH), 65.93 (CH_2), 39.68 (CH_2), 26.19 (CH_2), 25.87 (CH_3), 17.99 (CH_3), 16.85 (CH_3) MS (APCI): m/z = 343.16 (MH^+) HRMS: m/z calcd for [$C_{20}H_{23}O_5$] 343.15454, measured 343.15420.

Global yield: 13.9%.

5.1.1.5. 7-(3-Fluorobenzyloxy)-2-oxo-2*H*-chromene-3-carboxylic acid (7). ^{1}H NMR (DMSO-d_6) δ ppm 13.02 (s, 1H), 8.73 (s, 1H), 7.85 (d, J = 8.7, 1H), 7.47 (dd, J = 7.8, 1H), 7.34 (s, 1H), 7.33 (d, J = 7.5, 1H), 7.23–7.09 (m, 3H), 5.29 (s, 2H) ^{13}C NMR (DMSO-d_6) δ ppm 164.59 (C), 164.27 (C), 163.82 (C), 157.58 (C), 157.21 (C), 149.47 (CH), 139.40 (C), 132.11 (CH), 131.10 (CH), 124.33 (CH), 115.32 (CH), 114.89 (CH), 114.55 (C), 114.24 (CH), 112.37 (C), 101.66 (CH), 69.72 (CH_2) MS (APCI): m/z = 314.84 (MH^+) HRMS: m/z calcd for [$C_{17}H_{12}O_5F$] 315.0669, measured 315.0663.

Global yield: 45%.

5.1.1.4. 7-(3,5-Bis(trifluoromethyl)benzyloxy)-2-oxo-2*H*-chromene-3-carboxylic acid (8). ^{1}H NMR (DMSO-d_6) δ ppm 13.04 (s, 1H), 8.73 (s, 1H), 8.22 (s, 2H), 8.13 (s, 1H), 7.87 (d, J = 8.7, 1H), 7.18 (s, 1H), 7.15 (d, J = 8.7, 1H), 5.45 (s, 2H) ^{13}C NMR (DMSO-d_6) δ ppm 164.58 (C), 163.48 (C), 157.16 (C), 157.52 (C), 149.34 (CH), 140.03 (C), 132.16 (CH), 131.13 (C), 130.69 (C), 129.12 (CH), 125.54 (C),122.52 (CH), 122.42 (CH), 121.92 (C), 114.86 (C), 114.13 (CH), 112.58 (C), 101.66 (CH), 68.91 (CH_2) MS (APCI): m/z = 433.01 (MH^+) HRMS: m/z calcd for [$C_{19}H_{11}O_5F_6$] 433.0511, measured 433.0509.

Global yield: 43.2%.

5.1.2. General procedure for the synthesis of 5, 6 and 9 (Scheme 1)

The Knoevenagel condensation allowed us to obtain important coumarin derivatives for our stepwise synthesis. 2,4-dihydroxybenzaldehyde (1.0 equiv) was dissolved in ethanol before the dimethyl malonate (1.2 equiv) was slowly added to the solution. Piperidine and acetic acid were added dropwise to catalyze the reaction (one drop for 3 mmol).The reaction mixture was stirred and heated under reflux 3 h. After cooling, the resulting precipitate was filtered, dried and used for the Mitsunobu reaction. The coumarin derivative (1.0 equiv) was dissolved in THF (8 ml per mmol). The alcohol derivative (1.0 equiv) was added to the mixture. After DTBAD (1.2 equiv) and triphenylphosphine (1.2 equiv) addition, the reaction mixture was stirred overnight at room temperature. We performed a liquid extraction with DCM/HCl 1 M before the organic layer was dried over Na_2SO_4 and concentrated with the rotary evaporator. The resulting powder was recrystallized from EtOH followed by hydrolysis with LiOH (10 equiv) in a THF/water solution (1:1) heated 1 h under reflux. After cooling, the mixture was concentrated by rotary evaporator. The resulting precipitate was solubilized in a minimum volume of NH_4OHcc/water (1:4). HCl 36% was carefully added dropwise to the stirred solution until precipitation at low acidic pH (control with pH paper). The resulting precipitate was filtered and recrystallized from EtOH to give the final product.

5.1.2.1. 7-(2,3-Dihydro-1*H*-inden-2-yloxy)-2-oxo-2*H*-chromene-3-carboxylic acid (5). ^{1}H NMR (DMSO-d_6) δ ppm 13.01 (s, 1H), 8.71 (s, 1H), 7.81 (d, J = 8.7, 1H), 7.27–7.15 (m, 4H), 7.06 (s, 1H), 6.96 (d, J = 8.7, 1H), 5.39 (s, 1H), 3.43 (d, J = 12, 2H), 3.05 (d, J = 15, 2H) ^{13}C NMR (DMSO-d_6) δ ppm 164.61 (C), 163.14 (C), 157.70 (C), 157.30 (C), 149.56 (CH), 140.86 (C), 132.17 (CH), 127.09 (CH), 125.09 (CH), 114.58 (CH), 114.18 (C), 112.05 (C), 101.91 (CH), 79.17 (CH), 39.52 (CH_2) MS (APCI): m/z = 323.20 (MH^+) HRMS: m/z calcd for [$C_{19}H_{15}O_6$] 323.0919, measured 323.0911.

Global yield: 17.7%.

5.1.2.2. 7-((1*H*-Benzo[*d*][1,2,3]triazol-1-yl)methoxy)-2-oxo-2*H*-chromene-3-carboxylic acid (6). ^{1}H NMR (DMSO-d_6) δ ppm 13.11 (s, 1H), 8.72 (s, 1H), 8.13 (d, J = 8.4, 1H), 8.03 (d, J = 8.4, 1H), 7.87 (d, J = 8.7, 1H), 7.67 (dd, J = 8.1, 1H), 7.49 (dd, J = 7.8, 1H), 7.38 (s, 1H), 7.18 (d, J = 8.7, 1H), 6.98 (s, 2H) ^{13}C NMR (DMSO-d_6) δ ppm 164.49 (C), 161.16 (C), 157.27 (C), 156.75 (C), 149.12 (CH), 145.76 (C), 133.19 (C), 132.22 (CH), 129.04 (CH), 125.32 (CH), 119.98 (CH), 115.62 (C), 114.45 (CH), 113.93 (C), 111.15 (CH), 102.92 (CH), 74.04 (CH_2) MS (APCI): m/z = 337.97 (MH^+) HRMS: m/z calcd for [$C_{17}H_{12}N_3O_5$] 338.0777, measured 338.0777.

Global yield: 21.3%.

5.1.2.3. 7-(4-Fluorobenzyloxy)-2-oxo-2*H*-chromene-3-carboxylic acid (9). ^{1}H NMR (DMSO-d_6) δ ppm 13.02 (s, 1H), 8.72 (s, 1H), 7.84 (d, J = 8.7, 1H), 7.56 (d, J = 5.7, 1H), 7.53 (d, J = 5.7, 1H), 7.27–7.06 (m, 4H), 5.23 (s, 2H) ^{13}C NMR (DMSO-d_6) δ ppm 164.59 (C), 163.97 (C), 160.82 (C), 157.62 (C), 157.24 (C), 149.50 (CH), 132.71 (C), 132.07 (CH), 130.92 (CH), 130.81 (CH), 116.01 (CH), 115.73 (CH), 114.42 (C), 114.26 (CH), 112.27 (C), 101.59 (CH), 69.91 (CH_2) MS (APCI): m/z = 315.28 (MH^+) HRMS: m/z calcd for [$C_{17}H_{12}O_5F$] 315.06687, measured 315.06703.

Global yield: 19.9%.

5.1.3. General procedure for the synthesis of 10–13 (Scheme 2)

To a solution of 3-aminophenol (1 g, 9.16 mmol, 1.0 equiv) in THF (35 mL) was added a solution of di-*tert*-butyl dicarbonate (2.41 g, 11.1 mmol, 1.2 equiv) in THF (10 mL). The reaction mixture was refluxed overnight. Then the solvent was evaporated under vacuum to afford a brown residue. The residue was dissolved in EtOAc and washed with H_2O, saturated $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated in vacuo. This procedure provided the carbamate derivative as a grey solid with in a quantitative yield. Imidazole (1.37 g, 20.1 mmol, 2.0 equiv) was slowly added at 0 °C to a solution of the solid previously synthesized (2.11 g, 10.0 mmol, 1.0 equiv) in DMF (15 ml) followed by a solution of TBSCl (1.75 g, 11.6 mmol, 1.15 equiv) in DMF (5 ml). The reaction mixture was stirred at room temperature overnight, partitioned between half saturated $NaHCO_3$ solution and AcOEt. The organic layer was washed successively with half-saturated NH_4Cl solution, saturated $NaHCO_3$ solution, and brine, then dried over $MgSO_4$ and concentrated in vacuo. This procedure provided the diprotected compound as a white solid (2.89 g, 89%) with enough purity for the next step. This compound (500 mg, 1.54 mmol, 1.0 equiv) in solution in freshly distilled diethyl ether (15 ml) was added at −40 °C a solution of *tert*-butyllithium in pentane [1.6 M] (2.31 ml, 3.71 mmol, 2.4 equiv). The reaction mixture was stirred at −40 °C during 2 h. DMF (0.95 ml, 12.36 mmol, 8.0 equiv) was injected to the reaction mixture which was warmed to 0 °C and stirred during 1 h. The reaction mixture was partitioned between water and diethyl ether. The aqueous layer was extracted three times with diethyl ether. The combined organic layers were washed two times with brine, dried over $MgSO_4$ and concentrated in vacuo.

The residue was purified by column chromatography (petroleum ether/diethyl ether, 99:1) to give the desired formylated product as a white powder (239 mg, 44%). Potassium fluoride (140.4 mg, 2.42 mmol, 2.2 equiv) was added to a solution of C (386.2 mg, 1.099 mmol, 1.0 equiv) in DMF (30.9 ml) followed by benzyl bromide (0.17 ml, 1.43 mmol, 1.3 equiv). The reaction mixture was stirred at room temperature overnight then diluted in water and AcOEt. The aqueous layer was extracted two times with AcOEt. The combined organic layer were washed two times with a solution of HCl 0.5 M, one time with water, two times with a solution of LiCl (20%), and brine. The mixture was then dried over $MgSO_4$ and concentrated in vacuo to afford quantitatively a white powder (351.3 mg). To a solution of the previously obtained *O*-benzyl derivative (351.3 mg, 1.073 mmol, 1.0 equiv) in ethanol (4.3 ml) was added diethyl malonate (0.4 ml, 2.68 mmol, 2.5 equiv), piperidine (0.26 ml, 2.7 mmol, 2.5 equiv) and acetic acid

(0.006 ml, 0.107 mmol, 0.1 equiv). The mixture was stirred under reflux overnight. The mixture was then cooled to −5 °C, the resulting precipitate was filtered and washed with cold ethanol (−20 °C) to afford the desired quinolinone product (198 mg, 57%). K_2CO_3 (2 equiv) and LiI (20–25 mol %) were added to a solution of the quinolinone derivative (1 equiv) in DMSO (1.5 ml/mmol) followed by the alkyl halide (1.2 equiv). The reaction mixture was heated under reflux overnight, then partitioned between water and DCM. The aqueous layer was extracted three times with DCM. The combined organic layers were washed with water, dried over $MgSO_4$ and concentrated in vacuo. To a solution of the ester product (1 equiv) in THF (6 ml/mmol) was added a solution of LiOH (10 equiv) in water (6 ml/mmol). The mixture was then vigorously stirred and heated under reflux for 1h40. THF was removed by distillation under vacuum. The pH was then lowered to two by adding the proper amount of HCl (1 M). The white precipitate was diluted in AcOEt. The aqueous layer was extracted three times with AcOEt. The combined organic layers were washed with water, dried over $MgSO_4$ and concentrated in vacuo. The crude product was then recrystallized from ethanol to obtain a sample with enough purity for biological test.

5.1.3.1. 7-(Benzyloxy)-1,2-dihydro-2-oxoquinoline-3-carboxylic acid (10). ^{1}H NMR (DMSO-d_6) δ ppm 13.02 (s, 1H), 8.88 (s, 1H), 7.97 (d, J = 9, 1H), 7.49–7.35 (m, 5H), 7.11 (dd, J = 8.7, 1H), 7.02 (d, J = 2.4, 1H), 5.23 (s, 2H) ^{13}C NMR (DMSO-d_6) δ ppm 165.49 (C), 165.05 (C), 163.62 (C), 146.74 (CH), 142.34 (C), 136.53 (C), 132.84 (CH), 129.17 (CH), 128.84 (CH), 128.63 (CH), 114.91 (CH), 114.50 (C), 99.88 (C), 99.84 (CH), 70.53 (CH_2)HRMS: m/z (MH^+) calcd for [$C_{17}H_{14}NO_4$] 296.09173, measured 296.09176.

Global yield: 29%.

5.1.4.2. 7-(Benzyloxy)-1-methyl-2-oxo-1,2-dihydroquinoline-3-carboxylic acid (11). ^{1}H NMR ($CDCl_3$) δ ppm 14.47 (s, 1H), 8.81 (s, 1H), 7.72 (d, J = 8.8 Hz, 1H), 7.50–7.34 (m, 5H), 7.08 (dd, J = 8.8, 2.2 Hz, 1H), 6.95 (d, J = 2.0 Hz, 1H), 5.25 (s, 2H), 3.77 (s, 3H) ^{13}C NMR ($CDCl_3$) δ ppm 165.58 (C), 164.32 (C), 163.84 (C), 145.58 (CH), 142.80 (C), 135.33 (C), 133.21 (CH), 128.91 (CH), 128.68 (CH), 127.58 (CH), 114.40 (CH), 113.21 (CH), 99.95 (CH), 70.83 (CH_2), 30.22 (CH_3) FTMS (pos, ESI): m/z = 310.1 (MH^+) HRMS: calculated for [$C_{18}H_{16}O_4N_1$], 310.1074, measured 310.1077.

Global yield: 28%.

5.1.3.3. 7-(Benzyloxy)-1-ethyl-2-oxo-1,2-dihydroquinoline-3-carboxylic acid (12). ^{1}H NMR ($CDCl_3$) δ ppm 14.53 (s, 1H), 8.81 (s, 1H), 7.73 (d, J = 8.7 Hz, 1H), 7.56–7.32 (m, 5H), 7.08 (dd, J = 8.7, 2.0 Hz, 1H), 6.95 (s, 1H), 5.26 (s, 2H), 4.37 (q, J = 7.1 Hz, 2H), 1.34 (t, J = 7.1 Hz, 3H) ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.64 (C), 164.73 (C), 163.91 (C), 145.53 (CH), 141.76 (C), 135.43 (C), 133.37 (CH), 128.96 (CH), 128.67 (CH), 127.47 (CH), 114.46 (C), 113.03 (CH), 99.95 (CH), 70.85 (CH_2), 38.57 (CH_2), 12.34 (CH_3) FTMS (pos, ESI): m/z = 324.1 (MH^+) HRMS: calcd for [$C_{19}H_{18}O_4N_1$] 324.1230, measured 324.1233.

Global yield: 15%.

5.1.3.4. 1-Benzyl-7-(benzyloxy)-2-oxo-1,2-dihydroquinoline-3-carboxylic acid (13). ^{1}H NMR ($CDCl_3$) δ 14.37 (s, 1H), 8.89 (s, 1H), 7.72 (d, J = 8.8 Hz, 1H), 7.46–7.28 (m, 10H), 7.19 (d, J = 6.3 Hz, 2H), 7.03 (dd, J = 8.8, 2.2 Hz, 1H), 6.87 (d, J = 2.0 Hz, 1H), 5.55 (s, 2H), 5.06 (s, 2H) ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.54 (C), 164.47 (C), 163.66 (C), 146.11 (CH), 142.35 (C), 135.26 (C), 133.02 (CH), 129.15 (CH), 128.85 (CH), 127.93 (CH), 127.42 (CH), 126.52 (CH), 114.60 (C), 114.32 (C), 113.73 (CH), 100.46 (CH), 70.62 (CH_2), 46.92 (CH_2) FTMS (pos, ESI): m/z = 386.1 (MH^+) HRMS: calcd for [$C_{24}H_{20}O_4N_1$] 386.13868, measured 386.1387.

Global yield: 26%.

5.1.4. General procedure for the synthesis of 14–16 (Scheme 3)

We used the nucleophilic substitution as first step of synthesis. 2,4-dihydroxyacetophenone (1.0 equiv) was dissolved in DMF (1.5 ml per mmol). The halogenoalkane (bromide or iodide derivative) was then added to the solution (1.1 equiv) before addition of the weak base Cs_2CO_3 (1 equiv) to the mixture. The reaction mixture was stirred and heated under reflux overnight. After cooling, an equivalent volume of distilled water was added to the DMF solution before liquid extraction with ethyl acetate. The organic layers were merged and washed with a saturated solution of $LiSO_4$ to ensure residual DMF traces elimination. The final organic layer was dried over Na_2SO_4, filtered and concentrated with the rotary evaporator. The resulting powder was recrystallized from EtOH. The ketone function is then overnight activated into ketimine in a solution of NH_3 7 N in methanol at room temperature before synthesis of the coumarin scaffold by the knoevenagel condensation. The ketimine derivative (1 equiv) was dissolved in ethanol (4 ml per mmol) before addition of Meldrum acid (1.2 equiv). The reaction mixture was stirred 5 h under reflux to obtain the desired final pure coumarinic product.

5.1.4.1. 7-Hydroxy-4-methyl-2-oxo-2*H*-chromene-3-carboxylic acid (14). ^{1}H NMR (DMSO-d_6) δ ppm 8.16 (s, 1H), 7.50 (d, J = 8.7, 1H), 6.79 (d, J = 2.1, 1H), 6.76 (s, 1H), 2.27 (s, 3H) ^{13}C NMR (DMSO-d_6) δ ppm 168.29 (C), 161.02 (C), 159.44 (C), 153.87 (C), 142.52 (C), 126.63 (CH), 126.307 (C), 113.37 (CH), 112.55 (C), 102.45 (CH), 16.09 (CH_3) MS (APCI): m/z = 220.91 (MH^+) HRMS: m/z calcd for [$C_{11}H_9O_5$] 221.04500, measured 221.04522.

Global yield: 3.7%.

5.1.4.2. 7-(Benzyloxy)-4-methyl-2-oxo-2*H*-chromene-3-carboxylic acid (15). ^{1}H NMR (DMSO-d_6) δ ppm 7.62 (d, J = 9.6, 1H), 7.49–7.34 (m, 5H), 7.01 (s, 1H), 6.98 (d, J = 7.5, 1H), 5.20 (s, 2H), 2.29 (s, 3H) ^{13}C NMR (DMSO-d_6) δ ppm 167.88 (C), 160.61 (C), 159.27 (C), 153.65 (C), 141.88 (C), 136.92 (C), 128.97 (CH), 128.51 (CH), 128.36 (CH), 126.68 (CH), 114.36 (C), 112.84 (CH), 101.78 (CH), 70.21 (CH_2), 16.11 (CH_3) MS (APCI): m/z = 311.01 (MH^+) HRMS: m/z calcd for [$C_{18}H_{15}O_5$] 311.0919, measured 311.0916.

Global yield: 10.8%.

5.1.4.3. 7-Methoxy-4-methyl-2-oxo-2*H*-chromene-3-carboxylic acid (16). ^{1}H NMR (DMSO-d_6) δ ppm 7.62 (d, J = 9.3, 1H), 6.92 (d, J = 6, 1H), 6.91 (s, 1H), 3.84 (s, 3H), 2.29 (s, 3H). ^{13}C NMR (DMSO-d_6) δ ppm 167.95 (C), 161.58 (C), 159.3 (C), 153.73 (C), 141.73 (C), 127.75 (C), 126.61 (CH), 114.19 (C), 112.23 (CH), 100.82 (CH), 56.20 (CH_3), 16.12 (CH_3) MS (APCI): m/z = 234.88 (MH^+) HRMS: m/z calcd for [$C_{12}H_{11}O_5$] 235.0606, measured 235.0602.

Global yield: 6.4%.

5.1.5. General procedure for the synthesis of 17–23 (Scheme 5)

The first step is a palladium-catalyzed coupling reaction: 3-bromophenol (1.0 equiv) and substituted secondary amine (1.2 equiv) were stirred together under strictly dry conditions and under inert atmosphere (argon). After 10 min, $Pd(OAc)_2$ (2 mol %) was added to the reaction mixture. The Verkade base was then added dropwise to the reaction (4 mol %). LiHMDS (2.3 equiv, 1 M in THF) was carefully added, followed by freshly distilled toluene (3.5 ml for 1 mmol of A). The reaction mixture was stirred and heated 24 h under reflux at 85 °C. After cooling, the mixture was extracted with a toluene/water solution, the organic phase was finally dried on Na_2SO_4, filtrated and concentrated for column chromatography purification (AcOEt/EP 20:80). This step was used for the compounds **19**, **20** and **22**.

We performed the Vilsmeier–Haack reaction to add the aldehyde function to the 2-aminophenol derivative. The phosphoryloxy chloride $POCl_3$ (1.2 equiv in 3.0 equiv of anhydrous DMF) was carefully added dropwise to the previous synthesis product (also in anhydrous DMF: 200 μl for 1 mmol) under argon at 0 °C. The reaction mixture was stirred 15 min at 0 °C, then 15 min at room temperature, 15 min at 37 °C and finally 30 min at 80–90 °C. After cooling, addition of ice and Na_2CO_3 in the reaction mixture and stirring, we obtain a precipitate (not pure but the cyclisation selects the desired coumarinic end product). This reaction step was used for the compounds **18**–**22**. The previously synthesized salicylaldehyde (1.0 equiv) was dissolved in EtOH (10 ml for 1 mmol). Meldrum acid (1.2 equiv) was added to the stirred solution. Piperidine and acetic acid were added dropwise to catalyse the reaction (six drops for 1 mmol). The solution was stirred and heated 3 h under reflux. After cooling, the yellow to orange precipitate was filtered and obtained pure. This final reaction step was used for all the compounds (the only synthesis step of **17** and **23**).

5.1.5.1. 7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (17). ^{1}H NMR (DMSO-d_6) δ ppm 12.52 (s, 1H), 8.58 (s, 1H), 7.63 (d, J = 9, 1H), 6.78 (d, J = 9.1 1H), 6.56 (s, 1H), 3.48 (d, J = 6.9, 4H), 1.14 (m, 6H) ^{13}C NMR (DMSO-d_6) δ ppm 164.96 (C), 160.00 (C), 158.35 (C), 153.35 (C), 149.9 (CH), 132.31 (CH), 110.50 (CH), 107.84 (C), 107.58 (C), 96.37 (CH), 44.86 (CH_2), 12.78 (CH_3) MS (APCI): m/z = 261.91 (MH^+) HRMS: m/z calcd for [$C_{14}H_{16}NO_4$] 262.1079, measured 262.1079.

Global yield: 59%.

5.1.5.2. 7-Morpholino-2-oxo-2*H*-chromene-3-carboxylic acid (18). ^{1}H NMR (DMSO-d_6) δ ppm 8.59 (s, 1H), 7.68 (d, J = 9, 1H), 7.01 (d, J = 9, 1H), 6.83 (s, 1H), 3.72 (t, J = 4.5, 4H), 3.41 (t, J = 4.8, 4H) ^{13}C NMR (DMSO-d_6) δ ppm 164.88 (C), 159.03 (C), 157.77 (C), 155.72 (C), 149.57 (CH), 131.82 (CH), 111.78 (CH), 110.73 (C), 109.49 (C), 98.96 (CH), 66.17 (CH_2), 47.01 (CH_2) MS (APCI): m/z = 275.93 (MH^+) HRMS: m/z calcd for [$C_{14}H_{14}NO_5$] 276.0872, measured 276.0863.

Global yield: 18.9%.

5.1.5.3. 7-(*N*-Benzyl-*N*-methylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (19). ^{1}H NMR (DMSO-d_6) δ ppm 8.59 (s, 1H), 7.64 (d, J = 9, 1H), 7.35–7.20 (m, 5H), 6.86 (d, J = 9, 1H), 6.62 (s, 1H), 4.78 (s, 2H), 3.20 (s, 3H) ^{13}C NMR (DMSO-d_6) δ ppm 164.92 (C), 159.47 (C), 157.89 (C), 154.74 (C), 149.88 (CH), 137.93 (C), 131.99 (CH), 129.41 (CH), 127.56 (CH), 126.99 (CH), 110.85 (CH), 108.91 (C), 108.33 (C), 97.25 (CH), 56.49 (CH_2), 40.24 (CH_3) MS (APCI): m/z = 309.98 (MH^+) HRMS: m/z calcd for [$C_{18}H_{16}NO_4$] 310.1079, measured 310.1085.

Global yield: 12.2%.

5.1.5.4. 2-Oxo-7-(piperidin-1-yl)-2*H*-chromene-3-carboxylic acid (20). ^{1}H NMR (DMSO-d_6) δ ppm 8.04 (s, 1H), 7.48 (d, J = 9, 1H), 6.92 (d, J = 9, 1H), 6.72 (s, 1H), 3.34 (s, 6H), 1.59 (s, 6H) ^{13}C NMR (DMSO-d_6): DMSO-d_6 saturation at higher cc, no ^{13}C NMR possible MS (APCI): m/z = 273.97 (MH^+) HRMS: m/z calcd for [$C_{15}H_{16}NO_4$] 274.1079, measured 274.1087.

Global yield: 11.6%.

5.1.5.5. 7-(Dimethylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (21). ^{1}H NMR (DMSO-d_6) δ ppm 8.60 (s, 1H), 7.66 (d, J = 9, 1H), 6.81 (d, J = 9, 1H), 6.58 (s, 1H), 3.09 (s, 6H). ^{13}C NMR (DMSO-d_6) δ ppm 164.97 (C), 157.94 (C), 155.41 (C), 149.99 (CH), 149.96 (C), 131.98 (CH), 131.92 (C), 110.71 (C), 108.40 (CH), 108.06 (CH), 96.91 (C), 40.53 (CH_3) MS (APCI): m/z = 233.93 (MH^+) HRMS: m/z calcd for [$C_{12}H_{12}NO_4$] 234.0766, measured 234.0777.

Global yield: 22.7%.

5.1.5.6. 2-Oxo-7-(pyrrolidin-1-yl)-2*H*-chromene-3-carboxylic acid (22). ^{1}H NMR (DMSO-d_6) δ ppm 8.58 (s, 1H), 7.64 (d, J = 9, 1H), 6.66 (d, J = 9, 1H), 6.43 (s, 1H), 3.39 (s, 4H), 1.99 (s, 4H) ^{13}C NMR (DMSO-d_6) δ ppm 165.04 (C), 159.91 (C), 157.96 (C), 152.78 (C), 149.99 (CH), 132.10 (CH), 111.30 (CH), 108.04 (C), 107.81 (C), 96.95 (CH), 48.30 (CH_2), 25.33 (CH_2) MS (APCI): m/z = 259.97 (MH^+) HRMS: m/z calcd for [$C_{14}H_{14}NO_4$] 260.0923, measured 260.0928.

Global yield: 22.1%.

5.1.5.7. 7-(Juloidino)-2-oxo-2*H*-chromene-3-carboxylic acid (23). ^{1}H NMR (DMSO-d_6) δ ppm 8.44 (s, 1H), 7.22 (s, 1H), 3.35 (s, 4H), 2.71 (d, J = 5.7, 4H), 1.88 (d, J = 5.4, 4H). ^{13}C NMR (DMSO-d_6) δ ppm 165.13 (C), 160.97 (C), 156.81 (C), 149.65 (CH), 149.15 (C), 127.95 (CH), 119.97 (C), 107.70 (C), 105.61 (C), 105.18 (C), 50.11 (CH_2), 49.57(CH_2), 27.22 (CH_2), 26.97 (CH_2), 20.93(CH_2), 19.96 (CH_2) MS (APCI): m/z = 285.95 (MH^+) HRMS: m/z calcd for [$C_{16}H_{16}NO_4$] 286.1079, measured 286.1089.

Global yield: 24%.

5.2. Pharmacology

5.2.1. Cytotoxicity assay

SiHa cells (human cervix squamous carcinoma) were routinely cultured in DMEM containing serum and antibiotics as previously described.[8] Two distinct media were used for the cytotoxicity assay, either DMEM containing 25 mM D-glucose (without pyruvate) or DMEM containing 10 mM L-lactate (without glucose). SiHa cells were seeded in flat-bottom 96-well plates in normal DMEM. After 6 h incubation, the culture medium was replaced by 100 μl of glucose- (with or without 1 mM pyruvate) or lactate-containing medium and incubated overnight for metabolic adaptation. SiHa cells were treated with the synthesized compounds at a concentration range of 0.01–100 μM. After a 72 h incubation at 37 °C, cell medium was removed and replaced by freshly prepared MTT in PBS (1 mg/ml, 100 μl/well). After a 3 h incubation at 37 °C, the plates were centrifuged (1000g, 10 min at 4 °C) and the supernatant was removed before addition of DMSO (100 μl/well). Plates were kept for 10 min in the dark before reading at the spectrophotometer (Victor X4).

5.2.2. Lactate consumption assay

SiHa cells were seeded on flat-bottom 24-well plates (500,000 cells/well) in normal DMEM. After 6 h of incubation, the culture medium was replaced by 1 ml of DMEM containing 10 mM lactate and incubated overnight for metabolic adaptation. Cells were then treated for 24 h with increasing concentrations of compounds (0.1, 1, 10 and 100 μM) in lactate-containing medium at 37 °C. The supernatants were then centrifuged using deproteinizing columns (15 min, 10000g at 4 °C) and lactate concentration was determined using the enzymatic assay commercialized by CMA Microdialysis AB on a CMA600 analyzer (Aurora Borealis).

5.2.3. [^{14}C]-lactate uptake assay

SiHa cells were seeded on flat-bottom 24-well plates previously coated with poly-L-lysine (500,000 cells/well). After 6 h of incubation, the culture medium was replaced by 1 ml of DMEM containing 10 mM lactate and incubated overnight for metabolic adaptation. Cells were first rinsed with a modified Krebs solution (containing 10 μM L-lactate, without glucose) and pre-exposed to vehicle or increasing concentrations of compounds (0.1, 1, 10 and 100 μM) at 37 °C in the modified Krebs solution before addition of 2 μM [^{14}C]-lactate for 12 min. Cells were then rinsed with a

ice-cold D-lactate-containing Krebs solution (10 μM D-lactate, without glucose) and lysed with 0.1 M NaOH. Sample aliquots were then incubated with liquid scintillation solution (Microscint 40) into a 96-well plate (Optiplate). After a 1 h agitation, radioactivity was measured (PerkinElmer Topcount); cpm values were normalized per protein amounts.

5.2.4. Western blotting

Caspase-3 antibodies were from Cell Signaling. CAIX and CAXII antibodies were from R&D and Abcam, respectively. MCT antibodies were produced and validated in our lab.[9,10] Immunoblotting was performed as previously described,[9,10] β-actin was used as a loading control.

5.2.5. ADME, pharmacokinetics and prothrombin time assay

In vitro ADME and in vivo pharmacokinetics profiles were performed by Cerep (France). PT time assay was performed using Innovin® reagents according to the manufacturer's instructions; plasma was collected at day 5 on citrated tubes (3.2%) from mice (four mice/group) daily ip injected with vehicle or compounds.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.09.010.

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