Brief Report

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1,2,3-Triazolyl esterization of PAK1-blocking propolis ingredients, artepillin C (ARC) and caffeic acid (CA), for boosting their anti-cancer/anti-PAK1 activities along with cell-permeability

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Summary **Artepillin C (ARC) and caffeic acid (CA) are among the major anti-cancer ingredients of propolis, and block the oncogenic/melanogenic/ageing kinase PAK1. However, mainly due to their COOH moiety, cell-permeability of these herbal compounds is rather limited. Thus, in this study, in an attempt to increase their cell-permeability without any significant loss of their water-solubility, we have esterized both ARC and CA with the water-soluble 1,2,3-triazolyl alcohol through Click Chemistry. We found that this esterization boosts the anti-cancer activity of ARC and CA by 100 and over 400 folds, respectively, against the PAK-dependent growth of A549 lung cells, but show no effect on the PAK1-independent growth of B16F10 melanoma cells. Confirming this "selective" toxicity, these esters are still capable of blocking the kinase PAK1 strongly in cell culture (with IC_{50} around 5 μM), and the anti-PAK1 activity of 15A (ARC ester) and 15C (CA ester) appears to be 30-fold and 140-fold higher than ARC and CA, respectively. The 15A and 15C are 8-fold and 70-fold more cell-permeable (through the multi-drug resistant cell line EMT6) than ARC and CA, respectively. These data altogether suggest that both 15A and 15C would be far more useful than propolis for the treatment of a wide variety of PAK1-dependent diseases/disorders such as cancers, Alzheimer's diseases (AD), hypertension, diabetes (type 2), and hyper-pigmentation.**

Keywords: PAK1, artepillin C, caffeic acid, click chemistry, triazolyl esters

1. Introduction

The bee product called "propolis", an ethanol extract of bee cumb, has been well known to be among potent antibiotics effective against both bacteria and viruses since the ancient Egyptian era. Around the late 1980s, it was discovered as a potent anti-cancer herb (*1*). The anti-cancer ingredients of propolis depend on the areas where each propolis is harvested. For instance, the major anti-cancer ingredient in Brazilian green propolis is artepillin C (ARC), whereas those in Europian, US, Far-East, and Oceanian propolis are caffeic acid (CA) and its ester called caffeic acid phenethyl ester (CAPE) (*1,2*). Interestingly, both ARC and CA as well as CAPE block the oncogenic/melanogenic/ageing kinase PAK1 (*3,4*). Most interestingly, Okinawa propolis, based on nymphaeols A, B and C that inhibit the kinase PAK1 directly, extends the healthy lifespan of *C. elegans* (*5*).

However, IC_{50} of ARC, CA and CAPE against the growth of A549 lung cancer cells are 25, 100 and 10 μM, respectively. The major reason for the relatively high IC_{50} of ARC and CA is their COOH moiety, their negative charge which prevents their permeability through negatively charged phospholipid-based plasma membranes. In support of this notion, CAPE, a natural ester of CA, is 10 times more potent to inhibit the

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cancer growth than CA. More recently, 1,2,3-triazolyl esterization of PAK1-blocking compounds ursolic acid (UA) and ketorolac boosts their anti-cancer activity by 200-fold and 500-fold, respectively (*6,7*). Accordingly, in this study, through the same Click Chemistry, we have esterized both ARC and CA with 1,2,3-triazolyl alcohol, in an attempt to boost their cell-permeability without loss of their water-solubility.

2. Materials and Methods

2.1. *Cell lines and reagents*

Human lung cancer (A549) and murine melanoma (B16F10) cell lines were obtained from American Type Culture Collection (Rockville, MD, USA), and mouse breast cancer cell line (EMT6) supplied by Dr. Shin-ichiro Masunaga, Kyoto University, Kyoto, Japan. Artepillin C (ARC) was synthesized by us as previously described (*8*). Caffeic Acid (CA) was purchased from Tokyo Chemicals (Tokyo, Japan), while propargyl alcohol and 2-azidoanisole were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. *Synthesis of 15A from artepillin C (ARC) and 15C from caffeic acid by Click Chemistry*

2.2.1. *Preparation of 1,2,3-triazolyl derivative of artepillin C (ARC) (15A or PRC-15A)*

Step a: To solution of compound **1** (ARC, 161 mg, 536 µmol) in propargyl alcohol (6 mL) at 0°C, EDC (100 µL, 567 µmol) and DMAP (17 mg, 139 µmol) were added. The reaction mixture was stirred at room temperature and monitored by TLC till its completion in around a day. The crude mixture was then evaporated. The residue was purified through flash silica gel column chromatography (n-hexane:EtOAc = 3:1) to give pure **2** (100 mg, 295 µmol, 55% yield) as a pale yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.66 (d, *J* = 15.9 Hz, 1H), 7.17 (s, 2H), 6.29 (d, *J* = 15.9 Hz, 1H), 5.30 (t, *J* = 7.30 Hz, 2H), 4.79 (d, *J* = 2.27 Hz, 2H), 3.33 (d, *J* = 7.14 Hz, 4H), 2.49 (t, *J* = 2.44 Hz, 1H), 1.77 (d, *J* = 8.76 Hz, 12H).

Step b: To solution of compound **2** (52 mg, 154 µmol) in t-BuOH:H_2O (2:1, 4 mL), sodium ascorbate (6 mg, 30.3 µmol) and $CuSO_4$ (4 mg, 25.1 µmol) were added at room temperature. To this mixture, 2-azidoanisole (0.5 M solution, 310 µL, 155 µmol) was added and the reaction mixture was sonicated at 42°C till its completion around 7 h, monitored by TLC. The crude mixture was extracted with ethyl acetate (3 × 20 mL) and organic layer was washed with water and brine, then dried over sodium sulfate and purified through flash silica gel column chromatography (n-hexane:EtOAc = 3:1) to give pure **3** (52 mg, 107 µmol, 69% yield) as a pale yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 8.20 (s, 1H), 7.77 (d, *J* = 7.79 Hz, 1H), 7.63 (d, *J* = 16.1, 1H), 7.42 (t, *J* = 7.78Hz, 1H), 7.15 (s, 2H), 7.12-7.07 (m, 2H), 6.30 (d, *J* = 15.6 Hz, 1H), 5.42 (s, 2H), 5.28 (t, *J* = 6.49 Hz, 2H), 3.89(s, 3H), 3.32 (d, *J* = 7.27 Hz, 4H), 1.76 (d, *J* = 7.27, 12H). HRMS calcd for $C_{29}H_{34}N_3O_4$ $(M+H)^+$ *m/z* 488.2549, found *m/z* 488.2586.

2.2.2. *Preparation of 1,2,3-Triazolyl derivative of caffeic acid (CA) (15C or PRC-15C)*

Step c: To solution of compound **4** (CA, 382 mg, 2.12 mmol) in DMF (3 mL) at 0°C, EDC.HCl (423 mg, 2.21 mmol) and DMAP (72 mg, 589 µmol) were added. To this solution, propargyl alcohol (400 µL, 6.87 mmol) was added and the reaction mixture was stirred at room temperature for 17 h. After quenching the reaction with cooled HCl aq. (0.5 M, 20 mL), crude product was extracted with ethyl acetate:n-hexane (1:1) three times. Organic layer was washed with aqueous solution of saturated sodium hydrogen carbonate and brine, and then dried over sodium sulfate. This crude product was purified through flash silica gel column chromatography (n-hexane:EtOAc = 1:1) to give pure **5** (143 mg, 655 µmol, 31% yield) as a milky white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.62 (d, *J* = 16.1 Hz, 1H), 7.07 (d, *J* = 2.08 Hz, 1H), 7.00 (d, *J* = 8.00 Hz, 1H), 6.87 (d, *J* = 8.30 Hz, 1H), 6.27 (d, *J* = 16.1 Hz, 1H), 4.80 (d, *J* = 2.60 Hz, 2H), 2.50 (t, *J* = 2.60 Hz, 1H).

Step d: To solution of compound **5** (130 mg, 595 µmol) in THF:H_2O (1:1, 4 mL), sodium ascorbate (61 mg, 308 µmol) and $CuSO_4$ (17 mg, 107 µmol) were added at room temperature. To this mixture, 2-azidoanisole (0.5 M solution, 1.20 mL, 600 µmol) was added and the reaction mixture was stirred at room temperature till its completion around 5 h. The crude mixture was extracted with ethyl acetate (3 × 20 mL). Organic layer was washed with water and brine, and then dried over magnesium sulfate. Without column chromatography, the pure **6** (185 mg, 504 µmol) was obtained in 85% yield as a milky white powder. ^{1}H-NMR (400 MHz, CD_3OD) δ = 8.37 (s, 1H), 7.64 (d, *J* = 8.04 Hz, 1H), 7.58 (d, *J* = 15.6 Hz, 1H), 7.50 (t, *J* = 7.92 Hz, 1H), 7.23 (d, *J* = 8.30 Hz, 1H), 7.12 (t, *J* = 7.79 Hz, 1H), 7.02 (d, *J* = 2.08 Hz, 1H), 6.93 (d, *J* = 8.04 Hz, 1H), 6.75 (d, *J* = 8.30 Hz, 1H), 6.28 (d, *J* = 15.6 Hz, 1H), 5.35 (s, 2H), 3.89 (s, 3H). HRMS calcd for $C_{19}H_{18}N_3O_5$ $(M+H)^+$ *m/z* 368.1246, found *m/z* 368.1250.

2.3. *Assay for anti-cancer activity in cell culture*

2.3.1. *MTT and trypan blue assays for anti-cancer activity against A549 lung cancer and B16F10 melanoma cells*

The number of viable cells after treatment with ARC or CA was measured by MTT assay as described previously

(*7,9*). The number of viable cells after treatment with "15A" or "15C" was measured by Trypan blue assay in a hemocytometer as described previously (*7,9*), because both "15A" and "15C" themselves interferes with MTT-based colorimetry, as they are converted to colored "azo" dyes as is MTT by mitochondrial reductase. Briefly, A549 or B16F10 cells (2×10^5 cells/well) were seeded for 24 h, and then treated with either ARC, CA or their esters ("15A" or "15C") at the indicated concentrations for 96 h, to measure the number of viable cells by the corresponding protocol. The number of viable cells from non-treated samples was used as the negative control for calculating the anti-cancer activity of each test compound.

2.3.2. *Crystal violet assays for anti-cancer activity against multi-drug resistant cancer cell line (EMT6)*

EMT6 cells (2×10^3 cells/well) were treated with test acids or esters such as "15A" and "15C" at various concentrations for 24 h, and their viability/growth was measured by crystal violet assay as previously described (*10*) at the wavelength 570 nm.

2.4. *Anti-PAK1 (kinase) assay in cell culture*

The kinase activity of PAK1 in A549 cancer cells treated with either "15A" or "15C", in comaparison with those of ARC or CA was assayed according to the procedure ("Macaroni-Western" ATP-Glo kinase assay) previously described (*9*). Briefly, A549 cells (2×10^5 cells/well) were seeded for 24 h, and then treated with "15A" or "15C" at two concentrations (1 and 5 μM), in comaparison with ARC (100 and 300 μM) or CA (400 and 1200 μM), for 24 h. After the drug treatment, cells were lyzed, and PAK1 was immune-precipitated (IP) with anti-PAK1 antibody conjugated with protein A-beads, and after each IP was washed with saline thoroughly, the IP (PAK1) was incubated with ATP and MBP (myelin basic protein), substrates for the kinase, for 60 min with continuous shaking, and the resultant reaction mixture was further incubated with ATP_Glo kinase assay kit for 30 min, to measure the amount of remaining ATP by fluorometry. PAK1 activity from the non-treated cells was used as the negative control for calculating the anti-PAK1 activity of four test compounds.

2.5. *Uptake of herbal acids (ARC or CA) and their esters into cells*

To compare the uptake between acids and their esters into cells, we have chosen the multi-drug resistant (MDR) cancer cell line (EMT6) which could survive 30-100 μM of these PAK1-blockers at least for 12 h. The IC_{50} of ARC and "15A" against the growth of this cell line for 24 h were 169 μM and 22 μM, respectively, indicating that this esterization boosts the toxicity towards EMT6 cells by 8-fold (see Table 1). The IC_{50} of CA and "15C" against the growth of this cell line for 24 h were 1 mM and 8 μM, respectively, indicating that this esterization boosts the toxicity towards EMT6 cells by 125-fold (see Table 1). To compare the uptake between ARC and 15A or between CA and 15C, this cancer cell line was treated with either ARC or 15A at 100 μM for 24 h. However, to compare the uptake between CA and 15C, this cancer cell line was treated with either CA (100 μM) or 15C (30 μM) for 24 h, simply because 100 μM of 15C is too toxic for even 12 h treatment of EMT6 cells. Their uptake into 1×10^5 cells mainly during the indicated hours was quantitated by separation of the original herbal acids and their esters taken by cells through HPLC column chromatography as described previously (*7*), and the amount of each compound was estimated by photometry at the wavelength 322 nm (for detail, see the legend of Figure S1). After 12 h, the cell content of these esters was significantly reduced (see Figure 6), mainly due to both their cytotoxicity and conversion to pigments (detected at wavelength 500-600 nm) by mitochondrial reductase which also converts the tetrazolium MTT to a formazan product(s).

Table 1. Increase in anti-cancer activity of several herbal acids by their esterization

Items	IC50 (μM)		
	A549	B16F10	EMT6
ARC	25	ND	170
15A (ester of ARC)	0.25	> 1	22
CA	100	ND	1,000
15C (ester of CA)	0.225	> 1	8
Ketorolac[a]	13	30	4,000
15K (ester of Ketorlac)[a]	0.024	0.006	450
UA[b]	20	ND	ND
13U (ester of UA)[b]	0.10	ND	ND

[a] reference 7, [b] reference 6, ND: not determined.

2.6. *Statistical analysis*

Data are expressed as mean values with their standard errors. Statistical comparisons were performed by oneway ANOVA followed by Duncan's multiple-range test. Statistical analysis was conducted using SAS (release 9.2; SAS Institute, Cary, NC, USA) and $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. *Preparation of 1,2,3-triazolyl esters from artepillic C (ARC) and caffeic acid (CA) through Click Chemistry*

Both Artepillin C (ARC) and caffeic acid (CA), the

two major anti-cancer and PAK1-blocking ingredients in propolis, have the carboxyl moiety that hampers their cell-permeability. CAPE (caffeic acid phenethyl ester), a natural ester of CA in propolis, is 10 folds more potent than CA for inhibiting cancer cell growth. However, CAPE is less water-soluble than CA or ARC. Thus, in this study, we try to develop a far more cell-permeable and yet water-soluble ester of both ARC and CA. 1,2,3-Triazolyl alcohol is a positively charged water-soluble alcohol, and its esters with two acidic PAK1-blockers called ursolic acid (UA, a triterpene from rosemary leaves) and ketorolac (a synthetic pain-killer) turned out to be both highly cell-permeable and water-soluble, and strongly block PAK1 in cell culture (*6,7*).

Similarly, as summarized in Figures 1 and 2, we have synthesized the 1,2,3-triazolyl esters called 15A and 15C from ARC and CA, respectively, through the "two-step" Click Chemistry, with the relatively high "over-all" yield (around 40% and 25%, respectively), in an attempt to boost the cell-permeability of these two herbal acids abundant in propolis.

3.2. *Increase in the anti-cancer activity of artepillic C (ARC) and caffeic acid (CA) by esterization with 1,2,3-triazolyl alcohol*

As summarized in Figure 3, ARC inhibits the growth of A549 lung cancer cells with the IC_{50} around 25 μM, while its 1,2,3-triazolyl ester called 15A inhibits the growth of the same cancer cells with the IC_{50} around 250 nM. Thus, this esterization boosts the anti-cancer activity of ARC by 100 folds.

CA inhibits the growth of the same cance cells with the IC_{50} around 100 μM as previously published (*11*). As shown in Figure 4, its 1,2,3-triazolyl ester called 15C inhibits their growth with the IC_{50} around 225 nM, indicating that 15C is over 400 folds more potent than CA as the anti-cancer drug. Among the CA esters, CAPE is the best known herbal compound abundant in propolis, and inhibits the growth of cancer cells with the IC_{50} around 10 μM (*11*). Recently a more potent CA ester called 10C was developed, and 10C directly inhibits the enzyme called AKR (aldo-keto reductase) 1B10 with the IC_{50} around 6 nM, while CAPE inhibits AKR1B10 with the IC_{50} around 80 nM (*11*). However, 10C inhibits the growth of AKR-over-expressing brain cancer cell line with the IC_{50} around 1 μM (*11*), suggesting that its cell-permeability is still rather poor. Thus, 15C is so far the most potent cancer-killing CA ester.

As summaried in Table 1, however, either 15A or 15C showed no significant effect on the PAK1-independent growth of B16F10 melanoma cells up to 1 μM. Since this melanoma cell line is clearly 4-fold more sensitive to 15K, the 1,2,3-triazolyl ester of

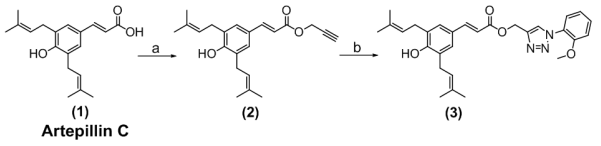

Figure 1. Click chemistry turning artepillin C (ARC) into 15A (or PRC-15A). (A) Propargyl alcohol, EDC•HCl, DMAP, rt, 55% yield; **(B)** 2-Azidoanisole, sodium ascorbate, $CuSO_4$, THF:H_2O (1:1), rt, 69% yield.

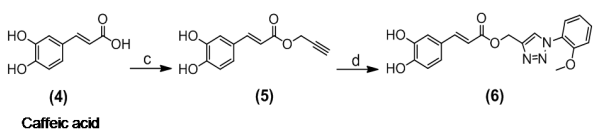

Figure 2. Click chemistry turning caffeic acid (CA) into 15C (or PRC-15C). (C) Propargyl alcohol, EDC•HCl, DMAP, rt, 31% yield; **(D)** 2-Azidoanisole, sodium ascorbate, $CuSO_4$, THF:H_2O (1:1), rt, 85% yield.

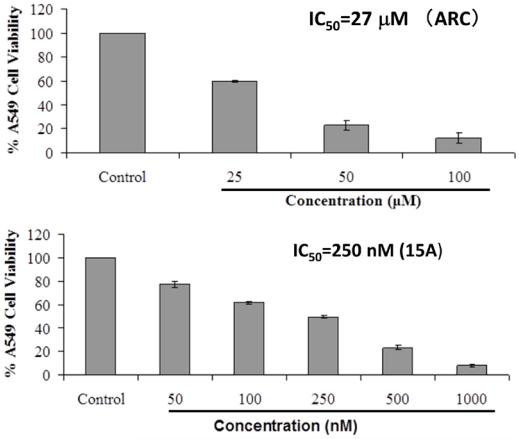

Figure 3. Increase in anti-cancer activity of artepillin C (ARC) against A549 cancer cells by its esterization. A549 lung cancer cells were treated with either ARC or 15A at indicated concentrations for 96 h, stained with trypan blue, and the viable (non-stained) cells were counted. The IC50 of 15A is around 250 nM, indicating that the esterization of ARC boosted its anti-cancer activity by over 100 folds. The results are mean ± SE. Data have the statistical significance at $p \leq 0.01$.

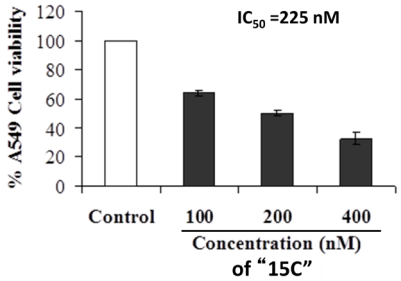

Figure 4. Increase in anti-cancer activity of caffeic acid (CA) against A549 cancer cells by its esterization. A549 lung cancer cells were treated with either CA or 15C at indicated concentrations for 96 h, stained with trypan blue, and the viable (non-stained) cells were counted. The IC_{50} of 15C is around 225 nM, indicating that the esterization of CA boosted its anti-cancer activity by over 400 folds. The results are mean ± SE. Data have the statistical significance at $p \leq 0.01$.

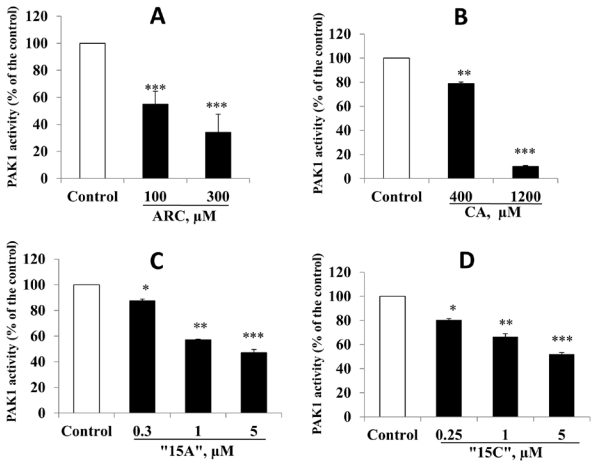

Figure 5. Boosting the anti-PAK1 activity of ARC (A) and CA (B) in cell culture by their esterization. After A549 cancer cells were treated with **(A)** ARC, **(B)** CA, **(C)** "15A" or **(D)** "15C" at the indicated concentrations for 24 h, PAK1 in cell lysates was IPed and its kinase activity was measured by "Macaroni-Western ATP_Glo" kinase assay. IC_{50} of "15A" and "15C" against PAK1 in cell culture is around 4.5 and 5.4 µM, respectively, compared with that of ARC and CA (around 140 and 740 µM, respectively).

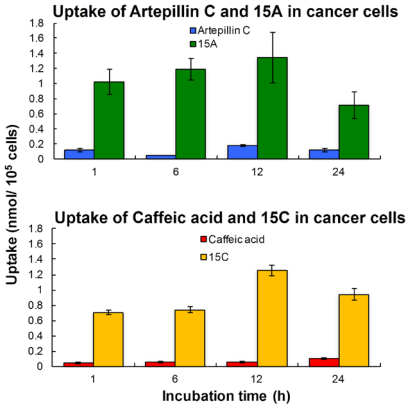

Figure 6. Increase in cell-permeability of ARC and CA by their esterization. The changes in the amount of **(A, top)** ARC and "15A" or **(B, bottom)** CA and "15C" taken into EMT6 cells during the first 12 h was monitored by HPLC analysis. "15A" is 8 fold more cell-permeable than ARC for the first hr, while "15C" is 20 fold more permeable than CA for 12 h. Data are mean ± SEM.

ketorolac, than A549 lung cancer cell line (*7*), these observations altogether suggest that the anti-cancer action of both 15A and 15C is highly specific for the PAK1-dependent cell growth.

3.3. *Anti-PAK1 activity of ARC ester (15A) and CA ester (15C)*

Both ARC and CA are known to inhibit the PAK1-dependent growth of A549 lung cancer cells which carry the oncogenic mutant of Ki-RAS by blocking the oncogenic kinase PAK1 (*12*). Thus, we have examined if their esters also are able to block PAK1 in cell culture. As shown in Figure 5, both 15A and 15C block the PAK1 activity in cell culture in a concentration-dependent manner with IC_{50} around 5 µM, confirming that both 15A and 15C are still potent PAK1-blockers. Furthermore, the anti-PAK1 activity of 15A and 15C appears to be 30 and 140 fold higher than those of ARC and CA (see Figure 5).

3.4. *Uptake of 15A and 15C into cells compared with artepillin C (ARC) and caffeic acid (CA)*

Since esterization of these acids generally abolishes their negative charge, their esters should be far more cell-permeable than the orginal herbal acids. Thus, here we have compared the cell-permeability between ARC and its ester (15A) as well as between CA and its ester (15C) using a drug-resistant breast cancer cell line (EMT6). At the tested concentrations (100 µM of ARC, CA and 15A or 30 µM of 15C), none of these compounds causes any serious effect on the growth or viability of this drug-resistant cell line for at least 12 h. However, 15C at 100 µM is significantly toxic for this cancer cell line for 24 h (IC_{50} = 8 µM, see Table 1). Uptake of each compound into cells was monitored by the HPLC-based analysis described previously (*7*) using the photometry at the wave-length 322 nm. As shown in Figure 6A, uptake of 15A is 8 folds faster than ARC during the first hour. However, the uptake of CA is rather slow, and that of 15C is over 20 fold (20-70 folds) more than CA during 12 h (see Figure 6B). Since both 15A and 15C are converted to a colored azo dye(s) by mito-chondrial reductase, as is the tetrazolium MTT, uptake of both 15A and 15C rapidly reduced shortly after 12 h treatment of cells with these esters.

Nevertheless, it is now clear that esterization of both ARC and CA boosts significantly their cell-permeability, and it is most likely that the dramatic increase in their anti-cancer activity is at least partly due to the increase of their cell-permeability.

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Supllementary Materials

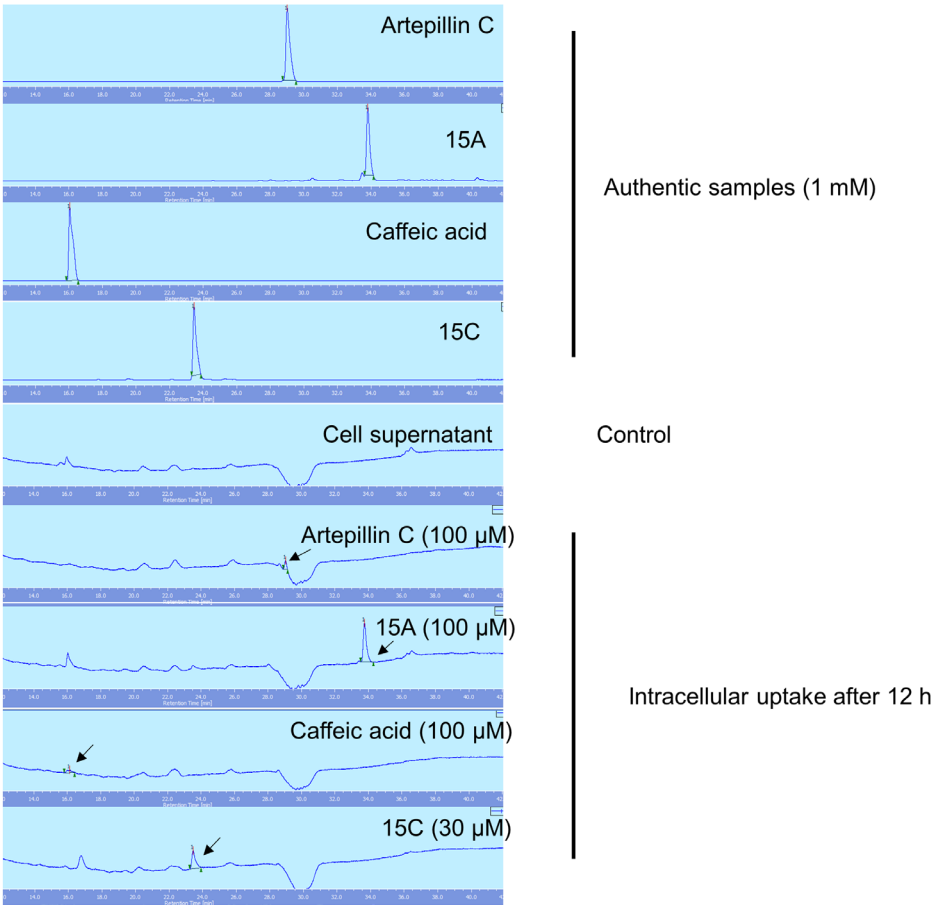

Figure S1. HPLC analysis of "15A" and "15C" compared with ARC and CA uptaken into cells. After treating EMT6 (1×10^5 cells) with either "15A" (100 µM), "15C" (30 µM), ARC (100 µM) or CA (100 µM) for 1, 6, or 12 hrs, cells were washed twice with PBS and then lyzed with 400 µL of 2% Triton. 10 µL of each lysate was injected into an analytical HPLC column (GL-Science Inertsustain C18, 4.6 mm × 150 mm) equilibrated with 0.1% TFA in mQ water **(A)**. The column was eluted with 1% acetonitorile **(B)** for 5 min, then with a linear gradient from 1% to 100% B over 30 min, and finally 100% B for 10 min, at a flow rate 1.0 mL/min. The eluate was monitored at wavelength 322 nm.