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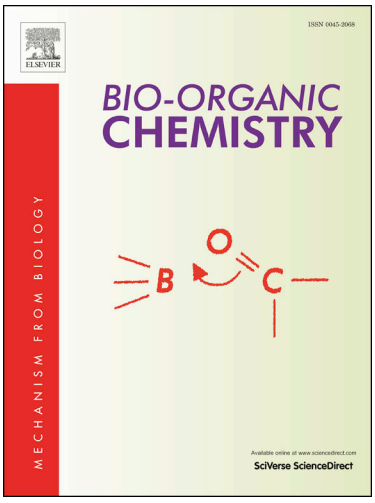

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PII: S0045-2068(19)31937-6
DOI: https://doi.org/10.1016/j.bioorg.2020.103643
Reference: YBIOO 103643

To appear in: *Bioorganic Chemistry*

Received Date: 13 November 2019
Revised Date: 23 January 2020
Accepted Date: 29 January 2020

Please cite this article as: A. Ghinet, X. Thuru, E. Floquet, J. Dubois, A. Farce, B. Rigo, Enhanced antitumor potential induced by chloroacetate-loaded benzophenones acting as fused tubulin-pyruvate dehydrogenase kinase 1 (PDHK1) ligands, *Bioorganic Chemistry* (2020), doi: https://doi.org/10.1016/j.bioorg.2020.103643

Enhanced antitumor potential induced by chloroacetate-loaded benzophenones acting as fused tubulin-pyruvate dehydrogenase kinase 1 (PDHK1) ligands

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Abstract

The majority of cancers detected every year are treated with anti-cancer compounds. Unfortunately, many tumors become resistant to antineoplastic drugs. One option is to use cocktails of compounds acting on different targets to try to overcome the resistant cells. This type of approach can produce good results, but is often accompanied by a sharp increase of associated side effects.

The strategy presented herein focuses on the use of a single compound acting on two different biological targets enhancing potency and lowering the toxicity of the chemotherapy. In this light, the approach presented in the current study involves the dual inhibition of human pyruvate dehydrogenase kinase-1 (PDHK1) and tubulin polymerization using mono-, di- and tri-chloroacetate-loaded benzophenones and benzothiophenones.

Synthesized molecules were evaluated *in vitro* on tubulin polymerization and on pyruvate dehydrogenase kinase 1. The cell cycle distribution after treatment of DA1-3b leukemic cells with active compounds was tested.

Twenty-two benzo(thio)phenones have been selected by the National Cancer Institute (USA) for evaluation of their anti-proliferative potential against NCI-60 cancer cell lines including multidrug-resistant tumor cell lines. Seventeen molecules proved to be very effective in combating the growth of tumor cells exhibiting inhibitory activities up to nanomolar range.

The molecular docking of best antitumor molecules in the study was realized with GOLD in the tubulin and PDHK1 binding sites, and allowed to understand the positioning of active molecules.

Chloroacetate-loaded benzo(thio)phenones are dual targeted tubulin- and pyruvate dehydrogenase kinase 1 (PDHK1)- binding antitumor agents and exhibited superior antitumor activity compared to non-chlorinated congeners particularly on leukemia, colon, melanoma and breast cancer cell lines.

Keywords

DCA, dichloroacetic acid; PDHK1 (h), human pyruvate dehydrogenase kinase 1; SAR, structure-activity relationship; benzophenone; chloroacetate.

1. Introduction

Cancer remains the second leading cause of death in the world and is expected to increase over 70% in the next two decades according to WHO [1]. Nearly one in six deaths worldwide is caused by cancer.

The majority of the new cancer cases detected every year is treated with anti-cancer compounds. Unfortunately, many tumors become resistant to these antineoplastic drugs. One option is to use cocktails of compounds acting on different targets to try to overcome the resistant cells. This type of approach can produce good results, but is often accompanied by a sharp increase of associated side effects.

Another strategy is to use a single compound acting on two different biological targets enhancing potency and lowering the toxicity of the chemotherapy [2]. In this light, the innovative approach presented in the current study involves the simultaneously inhibition of PDHK-1 (h) and tubulin. To the best of our knowledge, this is the very first therapeutic proposal using dual inhibitors acting on these two particular targets involved in cancer development.

The first point of action is the inhibition of the tubulin. This protein is a largely studied biological target in oncology, involved in cell proliferation for its ability to be polymerized and form microtubules, key components of the cytoskeleton. Tubulin is the target of many small molecules that interfere with the

dynamics of polymerization or depolymerization. Most of them bind to the taxol, vinblastine or colchicine binding site. Several tubulin inhibitors are already marketed (*e. g*. colchicine, paclitaxel, vinflunine, vincristine, Halichondrin B, etc.) or in clinical development (*e. g*. combretastatin A-4 derivatives, 2-methoxyestradiol, etc) [3].

Dichloroacetic acid (DCA) and sodium dichloroacetate have been used in human therapy for over thirty years especially in the treatment of mitochondrial disorders. DCA is a non-specific inhibitor of the mitochondrial pyruvate dehydrogenase kinases (PDK1-4 or PDHK1-4) which thus activates pyruvate dehydrogenase complex (PDH) that manage the control between oxidative phosphorylation and glycolysis. The PDH is located in the mitochondrial matrix catalyzing the irreversible decarboxylation of pyruvate to acetyl-CoA and NADH [4].

DCA is currently being investigated as a potential target in oncology and studied in different clinical trials. A completed phase 1 study evaluated the safety and tolerability of oral DCA in the treatment of recurrent malignant brain tumors (RMBTs). DCA was also used in combination with Cisplatin and radiation treatment in patients with stage III-IV squamous cell carcinomas of the head and neck (SCCHN) in a phase 2 clinical trial regrouping fifty subjects. Another active clinical trials explore the benefit of DCA in the vascular remodeling in PAH (early phase 1), in patients with breast and non-small cell lung cancer (phase 2), diabetic hyperglycaemia (phase 1) or in congenital lactic acidosis (phase 3) [3].

Increasing interest is currently being paid for DCA analogues. Multiple DCA-loaded amides [5] and dichloroacetophenones [6] have been recently developed as potent PDHK targeting compounds and displayed cell growth inhibition on leukemic cells [5] and non-small cell lung cancer cells [6].

The poor pharmacokinetic parameters of DCA (low lipophilia, low permeability through cell membranes and short half-time life impose high dosages to achieve therapeutic efficiency) prevent its use for cancer treatment. To overcome this inconvenient and in order to build strong SAR in these series, we have designed and synthesized novel DCA-carrier molecules by associating anti-tubulin benzophenones derived from phenstatin, known potent tubulin polymerization inhibitor, with mono-, di- and tri-chloroacetate groups targeting PDHK1 (Figure 1). Moreover, these hybrid molecules may constitute mutual prodrugs (codrugs). Through hydrolysis in the tumor cell biological system and hydrolytic cleavage promoted by hepatic microsomal esterases or amidases, these potential codrugs can provide both anti-cancer compounds (*e.g*. phenstatin and DCA), without releasing other molecular entities with unknown mode of action and ADMET profile.

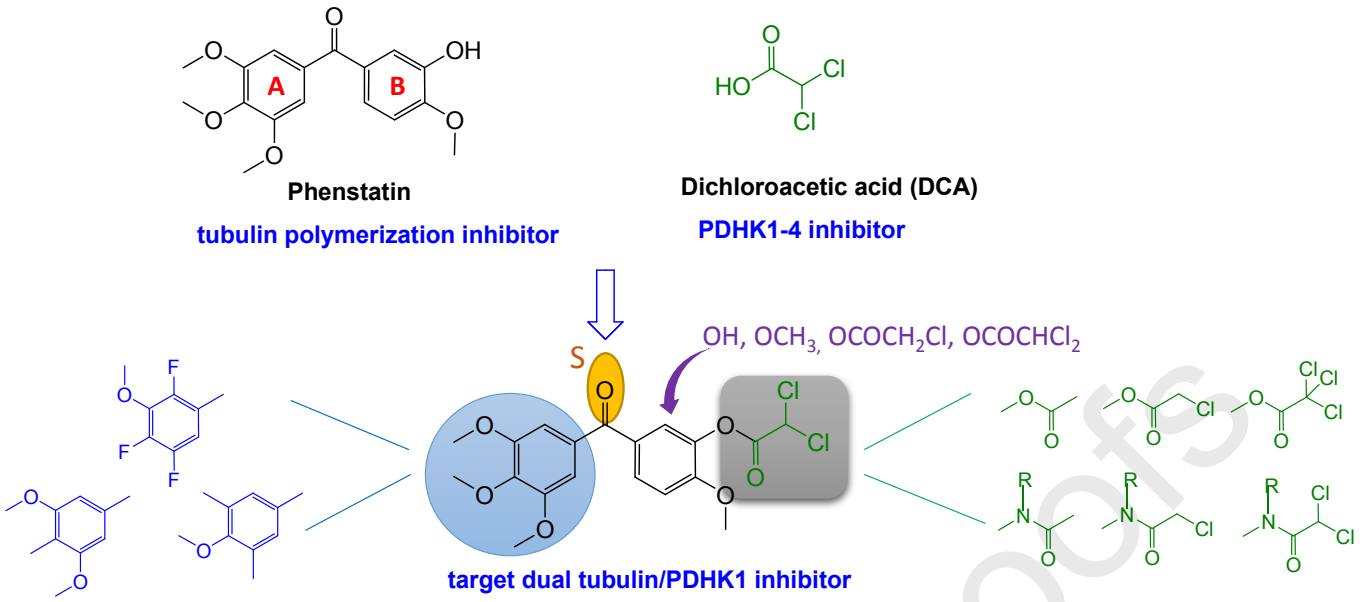


Figure 1. Structure of phenstatin, dichloroacetic acid (DCA) and of target dual inhibitors decorated with acetyl and mono-, di- and trichloroacetyl units

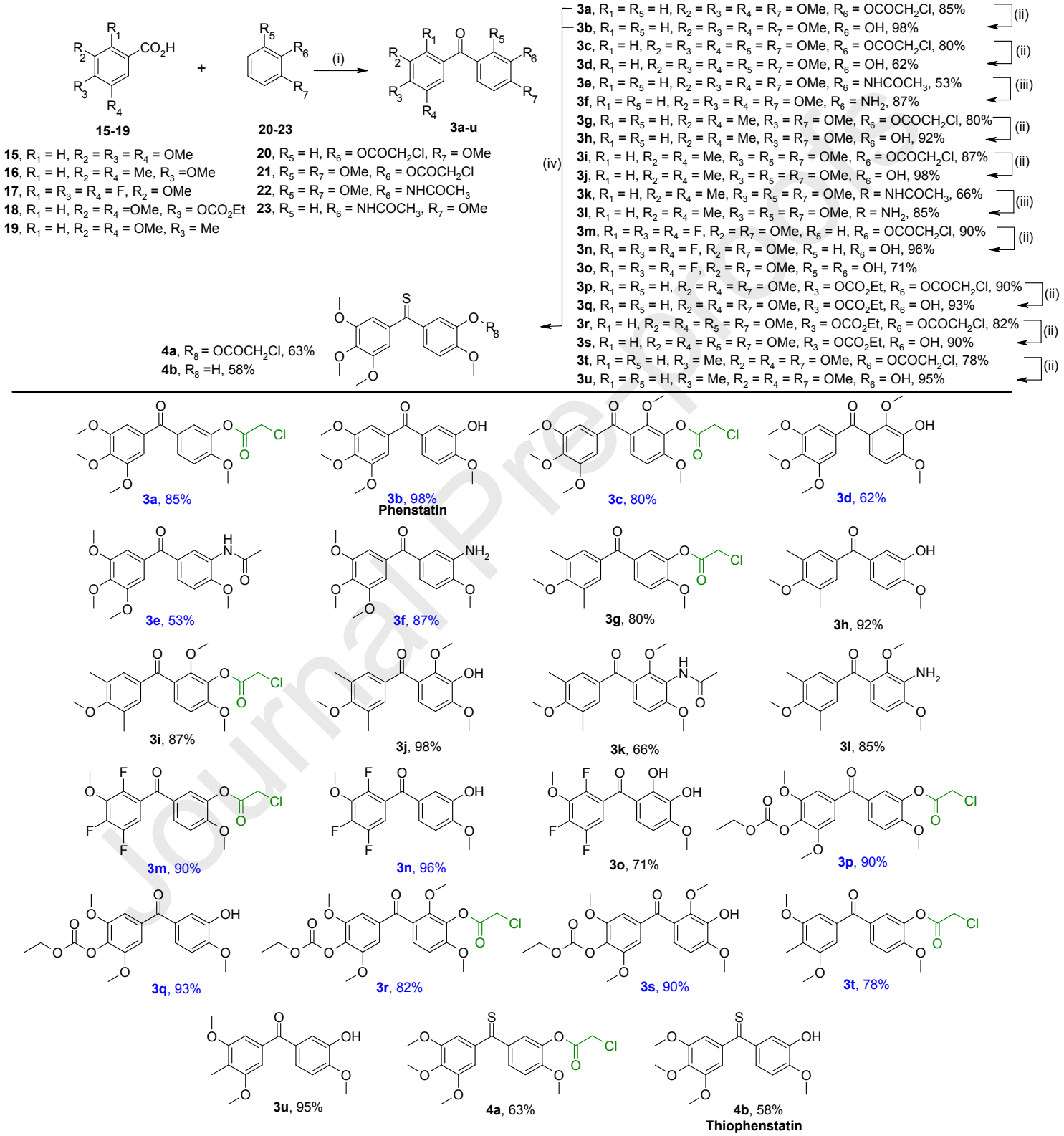
2. Results and discussion

2.1. Chemistry

All benzophenones described in the current manuscript, and also used as starting material for the synthesis of thioketones **4a-b**, were obtained by Eaton's reagent mediated Friedel-Crafts reaction. This procedure has already been described for ketones **3a**,**c**,**e**,**k**,**m**,**o**,**p**,**r**,**t** (Scheme 1) [7-8]. During these syntheses, the phenol groups were protected as monochloroacetic ester (reagents **20** and **21**, Scheme 1) in order to avoid side reactions as previously observed when using acetate protective group for phenols [9], while the amino group was protected as acetamide function (reagents **22** and **23**, Scheme 1). In the sole case of the Friedel-Crafts acylation promoted by Eaton's reagent between the 2,4,5-trifluoro-3-methoxybenzoic acid **17** and 2,6-dimethoxyphenyl chloroacetate **21**, the structure of the final benzophenone isolated after flash liquid chromatography purification of the crude on silica column did not correspond to the expected one. A deprotection of the ester function, and a mono *O*-demethylation occurred, leading to benzophenone **3o** as the unique reaction product. The *O*-demethylated position of this phenol was secured by NOESY study (Scheme 1).

In order to provide an additional chemical modulation on the A ring of parent phenstatin, the classical 3,4,5-trimethoxyphenyl unit (ring A, Figure 1) was replaced by 3,5-dimethyl-4-methoxyphenyl unit by using the same synthetic strategy to yield benzophenones **3g** and **3i**. Chloroacetyl protected ketones **3a**,**c**,**g**,**i**,**m**,**p**,**r**,**t** were next reacted with sodium acetate in refluxing methanol to yield ketones with free phenol group **3b**,**d**,**h**,**j**,**n**,**q**,**s**,**u** while acetamide functions of products **3e** and **3k** were deprotected in

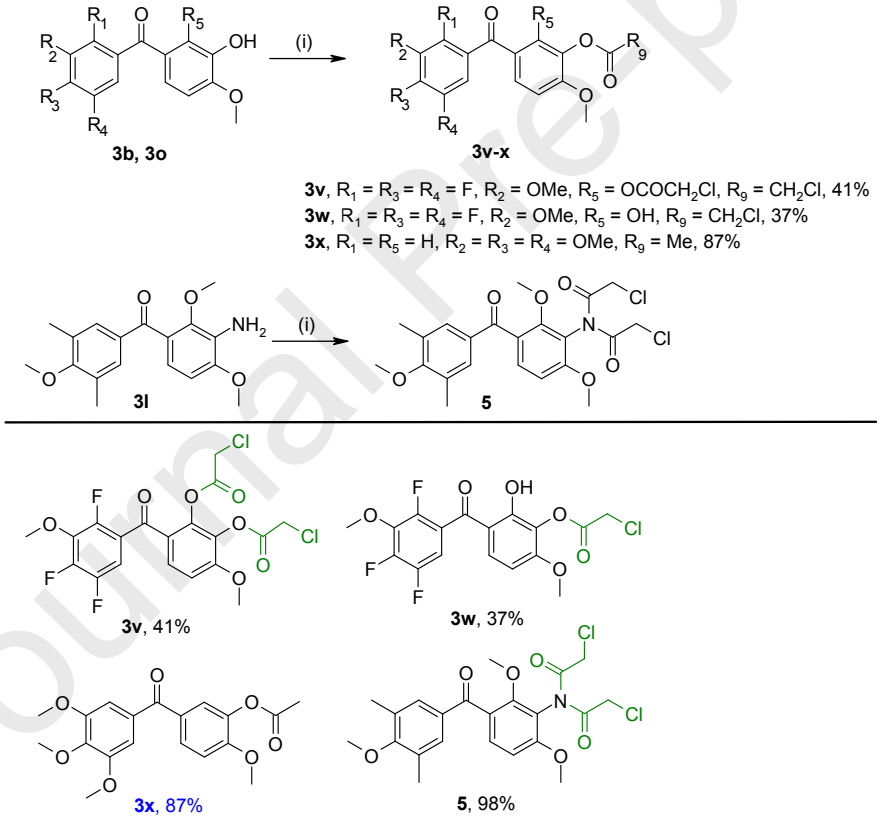
acidic conditions in refluxing methanol to provide free amines **3f** and **3l**. Next, the thionation of phenstatin **3b** and its monochloroacetic ester analogue **3a** with the reagent combination of P_4S_{10} and hexamethyldisiloxane [10] provided the first synthesis of thiophenstatin **4b** and its monochloroacetic derivative **4a** in 58 and 63% yields, respectively (Scheme 1).



Scheme 1. Synthesis of **monochloroacetate carriers**, acetamides **3e** and **3k** and corresponding free phenols and amines. *Reagents and conditions*: (i) 4 equiv. Eaton's reagent, 60°C, 1-24h; (ii) 4.5 equiv.

$CH_3COONa.3H_2O$, MeOH, reflux, 2-10 h; (iii) 1.5 equiv. aq HCl 10%, MeOH, reflux, 24h; (iv) 0.5 equiv. phosphorus pentasulfide (P_4S_{10}), 2 equiv. hexamethyldisiloxane (HMDSO), toluene, reflux, 24 h. Compounds numbered in blue were already described in the literature: benzophenones **3a-e** and **3p-s** have been reported in ref. [7]; benzophenone **3f** was reported in ref. [11]; benzophenones **3m** and **3n** have been reported in ref. [8]; benzophenone **3t** has been reported in ref. [12].

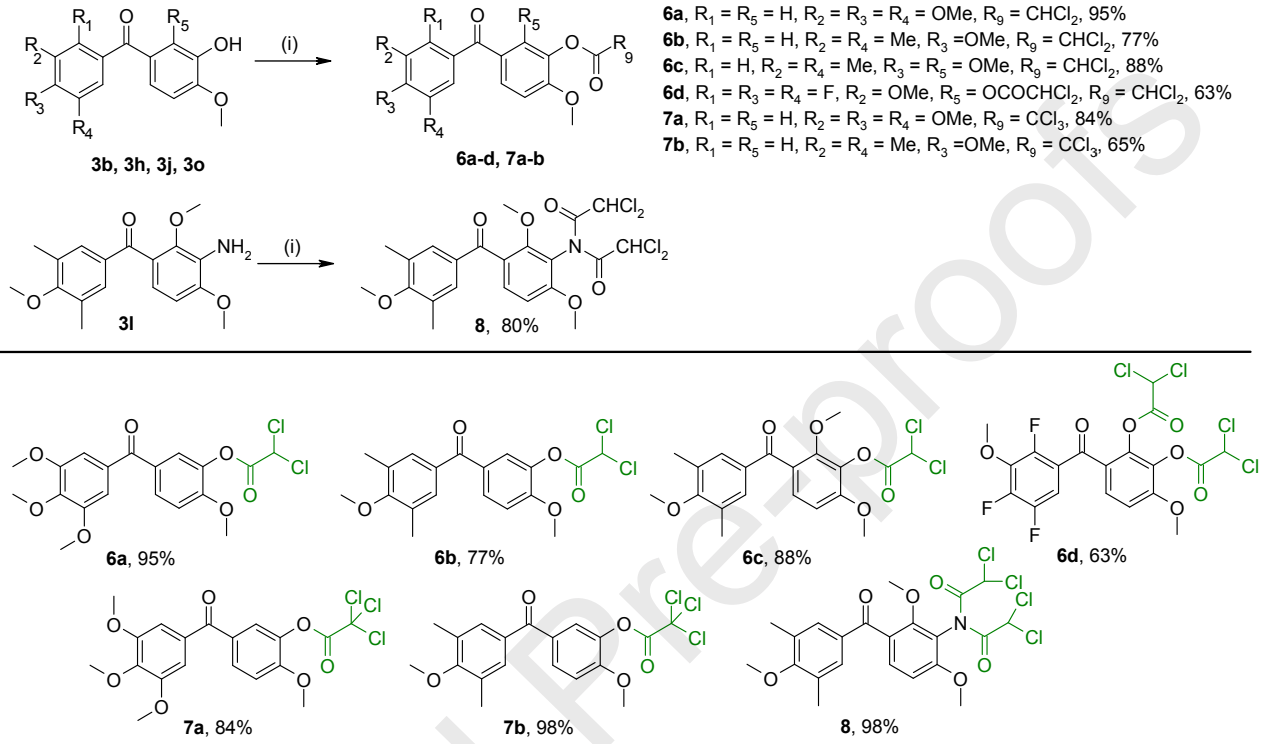
In order to perform the different modulations allowing to enrich structure-activity relationships in this series, and to conclude on the importance of the chloroacetic ester unit, the replacement by an acetic ester was first envisaged by synthesizing acetylphenstatin **3x** in 87% yield upon treatment of phenstatin **3b** with acetic acid chloride at reflux for 1 hour (Scheme 2). The bisphenol **3o** was reacted with 6 equivalents of monochloroacetic acid chloride to obtain a bis-monochloroacetate carrier which possibly could improve the activity against human PDHK1. The reaction provided the target diester **3v** in 41% yield as the major product along with the monoester **3w** in similar yield (Scheme 2). Next, the treatment of free amine **3l** with 4 equivalents of monochloroacetic acid chloride at reflux for 4 hours resulted in the synthesis of bis-chloroacetyl amide carrier **5** in excellent 98% yield (Scheme 2). The ^{1}H NMR monitoring of the reaction progress indicated the presence of the diacylated product **5** very quickly thus preventing the isolation of the monoacylated product in these operatory conditions.



Scheme 2. Synthesis of additional **monochloroacetate carriers 3v, 3w**, acetate **3x** and **bis-monochloroacetamide 5**. *Reagents and conditions*: (i) 2-6 equiv. monochloroacetic acid chloride or acetic acid chloride, reflux, 1-4h. Benzophenone **3x** numbered in blue has been previously reported in ref. [13].

Afterward, the chemical efforts turned on the same benzophenones but decorated with dichloroacetate groups first, and then with trichloroacetate groups in a second time (Scheme 3). All these synthetic strategies were deployed to obtain a clear trend in antitumor activity related to the nature of the chloroacetate (monochloro, dichloro or trichloroacetic group). Phenstatin **3b** and benzophenones **3h, 3j** and **3o** were treated with dichloroacetic acid chloride (2 equivalents for monoacylated benzophenones **6a-c** and 6

equivalents for diacylated benzophenone **6d**) in the absence of base and provided corresponding dichloroacetates **6a**-**d** in good to excellent yields (63-95%) (Scheme 3). Similar synthetic strategy was applied to easily obtain trichloroacetates **7a** and **7b** by reacting phenstatin **3b** and benzophenone **3h** with 2 equivalents of trichloroacetic acid chloride (Scheme 3). The treatment of benzophenone **3l** bearing free amino group with dichloroacetic chloride provided bisacylated derivative **8** in excellent 98% yield (Scheme 3). In the same way as during the synthesis of the bismonochloroacetylated congener **5**, the monoacylated compound has not been observed in the crude.



Scheme 3. Synthesis of **dichloroacetate and trichloroacetate carriers**. *Reagents and conditions*: (i) 2-6 equiv. dichloroacetic acid chloride or trichloroacetic acid chloride, reflux, 1-6h.

2.2. *In Vitro* Pharmacology:

2.2.1. Human pyruvate dehydrogenase kinase (PDHK1, 2 and 4) enzymatic radiometric assay [k_m ATP], KinaseProfiler

The newly synthesized benzophenones and benzothiophenones and reference chloroacetic acids (monochloroacetic acid, dichloroacetic acid and trichloroacetic acid) and sodium dichloroacetate were submitted to biological evaluation on human PDHK1. The selectivity of reference dichloroacetic acid was studied on PDHK1, 2 and 4. Compounds were tested at 100 and 1000 µM concentration. Compound PDHK (*h*) inhibition effect was calculated as a % inhibition of control kinase activity. Results showing an inhibition higher than 50% are considered to represent significant effects of the test compounds. The PDHK assay of molecules was realized by Eurofins Cerep SA, Celle L'Evescault, France.

2.2.1.1. *Selectivity of DCA on PDHK1, PDHK2 and PDHK4 (h)*

Many papers describe DCA as a non-specific inhibitor of the mitochondrial pyruvate dehydrogenase kinases PDHK1-4. The selectivity of DCA was also evaluated in this study on PDHK-1, 2 and 4 via the enzymatic radiometric assay [km ATP] KinaseProfiler. DCA was tested at 1mM concentration. It is interesting to note that no inhibitory effect was registered on PDHK4 at this dose while similar low activities were measured on PDHK1 and PDHK2 (14% and 16% inhibition, respectively) (Figure 2). There is a lot of confusion in the literature concerning the DCA abbreviation. It refers to both dichloroacetic acid or sodium dichloroacetate and sometimes to the chloroacetate anion without specifying the counter ion [14]. This confusion may

explain the differences in inhibitory activity among these chemical entities, which are different from one another. For instance, the behavior of dichloroacetate on PDHK1 and PDHK2 was already reported at 0.1-1 mM concentration by Makita *et al*. 2017 [14]. There was no important difference in inhibitory activities for concentrations of dichloroacetate below 1mM. However, at concentrations above 1 mM, the dichloroacetate behavior is reversed inhibiting PDHK1 two to three more times than PDHK2 at increasing concentrations (*e.g*. 10 mM) [14].

In this study, we used exclusively dichloroacetic acid as the reference molecule and abbreviated DCA.

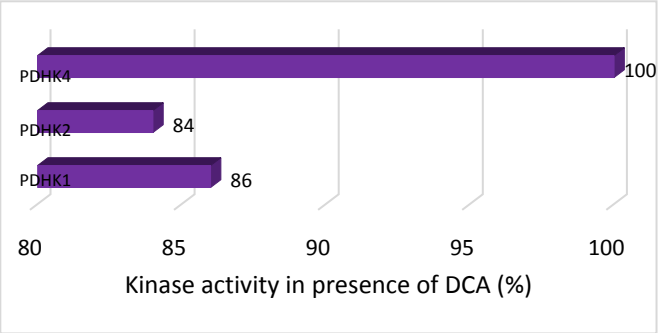


Figure 2. *Selectivity of DCA on PDHK1, PDHK2 and PDHK4 (h)*: DCA slightly inhibits PDHK1 and PDHK2 and has no impact on PDHK4 at 1mM concentration.

2.2.1.2. Effects of selected molecules on human PDHK1

Fifteen mono-, di- and tri-chloroacetate carriers were tested for their ability to inhibit human PDHK1 *in vitro* at two different concentrations (1 mM and 100 µM). DCA and sodium dichloroacetate were used as reference compounds in the same assay. Monochloroacetic acid (MCA) and trichloroacetic acid (TCA) were also included in order to compare their activity with that of known PDHK1 inhibitor DCA and to serve as references for mono- and trichloroacetate-loaded molecules, respectively. Results are provided in Table 1. Dichloroacetic acid exhibited enhanced inhibitory potential compared to sodium dichloroacetate. Esters **3g**, **3v**, **4a** and **6d** showed improved inhibitory potential compared to DCA. Monochloroacetate esters **3p** and **3w** and bis-monochloroacetamide **5** displayed equivalent activity to that of reference DCA. It is interesting to note that the best PDHK1 inhibitor **3v** is decorated by two monochloroacetate units and is much more active than related analogue **6d** bearing two dichloroacetate units. Contrary to what was expected, the monochloroacetate constituted the best pharmacomodulation in the current study for targeting human PDHK1 among designed mono-, di- and tri- chloroacetates. Compounds **3v** and **6d** were also found to weakly inhibit tubulin polymerization (48% and 42% inhibition at 100 µM, respectively). Interestingly, the benzophenone **3g** and benzothiophenone **4a**, potent tubulin polymerization inhibitors (IC_{50} of 1.30 µM and 1.17 µM, respectively), also exhibited additional inhibitory activity against PDHK1 (h) 2.3 times greater than dichloroacetic acid and constituted the best potential codrugs from the series.

2.2.2. Effects of synthesized molecules on tubulin polymerization

The potential of synthesized benzophenones and benzothiophenones to inhibit tubulin polymerization *in vitro* was first tested at a single concentration of 100 µM (Table 1). Phenstatin **3b** and desoxypodophyllotoxin have been used as positive references and DMSO as negative control. Molecules that resulted in more than 50% inhibition were selected for IC_{50} calculation. Eighteen molecules proved to be excellent tubulin polymerization inhibitors exhibiting IC_{50} values in the micromolar and submicromolar range. The replacement of the carbonyl unit of phenstatin **3b** with a thiocarbonyl (thiophenstatin **4b**) improved the antitubulin efficiency. The classical A ring (3,4,5-trimethoxyphenyl unit) of phenstatin has been successfully replaced by a 3,5-dimethyl-4-methoxyphenyl ring in compound **3h**, slightly improving the

inhibitory effect. On the other hand, all other modifications of the classical A ring (the replacement by 3,5-dimethoxy-4-methylphenyl in molecule **3u**, 2,4,5-trifluoro-3-methoxyphenyl in molecule **3n** or 3,5-dimethoxy-4-ethylcarbonatephenyl unit in molecule **3s**) conserved or slightly diminished the antitubulin potential compared to parent phenstatin.

Table 1. Evaluation of synthesized molecules on tubulin polymerization and on pyruvate dehydrogenase kinase 1 *in vitro*

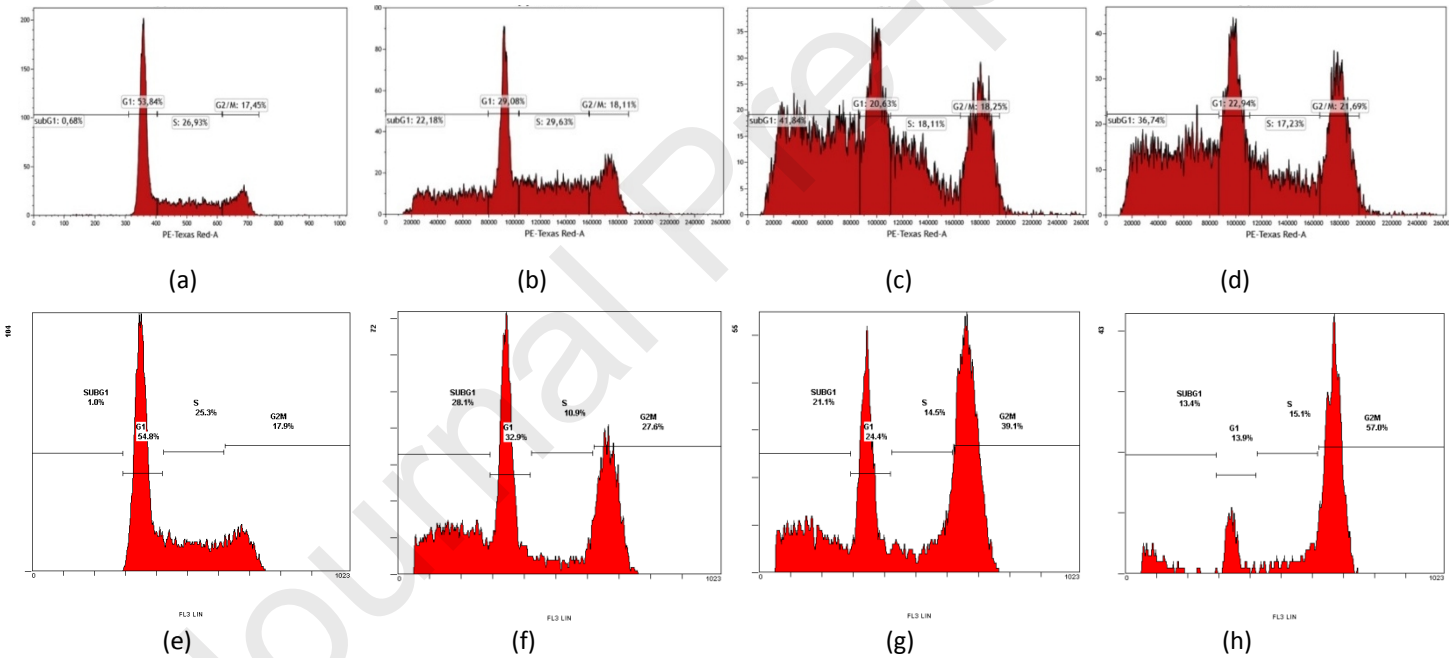
Molecule	% TPI[a,b]	IC_{50} (tubulin) (μM)[b]	R^2[c]	% PDHK1 (h)[e]	
				1 mM	0.1 mM
3a	98	1.21	0.9299	-	0
3c	91	1.34	0.9264		
3d	98	1.22	0.9625		
3e	0	-[d]	-		
3g	95	1.30	0.8871	32	0
3h	93	0.96	0.8702		
3j	96	0.69	0.8920		
3k	0	-	-		
3l	95	1.69	0.9208		
3m	5	-	-		
3n	88	28.23	0.9676		
3p	57	-	-	15	-
3r	97	0.88	0.9415	-	1.2
3s	97	1.60	0.9355		
3t	72	5.30	0.9831		
3u	88	5.07	0.9410		
3v	48	-	-	93	55
3w	32	-	-	15	-
3x	89	5.99	0.9802	-	0
4a	96	1.17	0.9312	33	6
Thiophenstatin **4b**	100	0.75	0.9259	-	-
5	18	-	-	12	2
6a	97	1.17	0.9393	0	0
6b	-	-	-	-	0
6c	94	0.26	0.9103	-	0
6d	42	-	-	78	10
7a	93	0.79	0.9272	0	0
8	3	-	-	0	0
Phenstatin **3b**	96	3.42	0.9378	-	-
Desoxypodophyllotoxin	100	1.76	0.9740	-	-
Monochloroacetic acid (MCA)	-	-	-	12	-
Dichloroacetic acid (DCA)	0	-	-	14	9
Sodium dichloroacetate	0	-	-	3	-
Trichloroacetic acid (TCA)	-	-	-	16	-

[a] Inhibition of tubulin polymerization at a 100 μM concentration
[b] Values represent mean of two experiments

[c] Standard deviation
[d] Not determined
[e] Inhibition of human pyruvate dehydrogenase kinase 1 (PDHK1); values represent mean of two experiments

2.2.3. Cell cycle analysis on DA1-3b cells

Cell cycle analyses of newly synthesized chloroacetyl-thiophenstatin **4a** and thiophenstatin **4b** were performed by flow cytometry (Figure 3). The two thiophenstatins acted differently on cell cycle arrest. Thiophenstatin **4b** strongly induced G2/M arrest in DA1-3b cells [15]. The effect was observed in a dose-independent manner after treatment for 24 h with increased concentrations of this compound (Figure 3). These results confirmed that thiophenstatin is a potent antitubulin agent and acts as a classic phenstatin on the cell cycle. Treated murine leukemia DA1-3b cells showed decreased G1 population (22.9%) compared with 53.8% in the control when treated with 1 µM of chloroacetyl-thiophenstatin **4a** (Figure 3). Whereas, the percentages of sub-G1 phase (apoptotic cells) were significantly increased after cells were treated with 1, 10 and 100 µM of **4a** up to 41.8% respectively compared with 0.7% in the control (Figure 3). This may be related to the dual inhibition of both tubulin polymerization and PDHK1 which induces increased apoptotic effect. The reference DCA had no effect on DA1-3b cells at low concentration (10 nM to 10 mM) and was highly cytotoxic at high concentration (50 mM, 100 mM or 150 mM) (see supplementary section for full results).



	SubG1	G1	S	G2/M	>4N
(a) DMSO	**0.7**	**53.8**	**26.9**	**17.5**	**1.1**
(b) **4a** 100 µM	**22.2**	**29.1**	**29.6**	**18.1**	**1.0**
(c) **4a** 10 µM	**41.8**	**20.6**	**18.1**	**18.3**	**1.2**
(d) **4a** 1 µM	**36.7**	**22.9**	**17.2**	**21.7**	**1.5**
(e) DMSO	**1.0**	**54.8**	**25.3**	**17.9**	**1.0**
(f) **4b** 100 µM	**28.1**	**32.9**	**10.9**	**27.6**	**0.5**
(g) **4b** 10 µM	**21.1**	**24.4**	**14.5**	**39.1**	**0.8**
(h) **4b** 1 µM	**13.4**	**13.9**	**15.1**	**57.0**	**0.6**

Figure 3. Cell cycle distribution after treatment of DA1-3b cells with chloroacetyl-thiophenstatin **4a** and thiophenstatin **4b**. DA1-3b cells were incubated for 24h with DMSO (control) and chloroacetyl-thiophenstatin **4a**

or thiophenstatin **4b**. Cell cycle parameters were analyzed by flow cytometry, as described in Methods. Values (lower table) are expressed as percentage of the total cell population.

2.2.4. Effects of synthesized molecules on cancer cell growth

Twenty-two synthesized benzo(thio)phenones **3a**, **3d**, **3g-h**, **3j-o**, **3r-t**, **3v**, **3x**, **4a**, **5**, **6a**, **6c**, **6d**, **7a** and **8** have been selected by the National Cancer Institute (NCI) for evaluation of their anti-proliferative potential against the NCI-60 cancer cell lines, including multidrug-resistant (MDR) tumor cell lines (HCT-15: colorectal adenocarcinoma; NCI/ADR-RES: human ovary adenocarcinoma; RXF 393: human kidney poorly differentiated hypernephroma; MCF7: human breast adenocarcinoma and SF-539: human CNS glioblastoma). Resumed representative biological efficacy is described in Table 2. All selected molecules underwent first the NCI 60 cell one-dose screen at 10 µM concentration. Only compounds which satisfied pre-determined threshold inhibition criteria in a minimum number of cell lines have progressed to the full 5-dose assay. Seventeen molecules exhibited significant cell growth inhibition in the one-dose screen and were evaluated against the 60 cell panel at five concentration levels (100 µM, 10 µM, 1 µM, 100 nM and 10 nM) in order to calculate the GI_{50} value on each cell line (Table 2). The molecules proved to be very effective in combating the growth of tumor cells exhibiting average activities over all NCI-60 cancer cell lines (MG-MID values) ranging from 2884 to 39 nM (Table 2).

The monohloroacetylated phenstatin **3a** displayed the most important anti-proliferative activity on leukemia, colon, melanoma and SNC cancer cells (GI_{50} < 10 nM). Interestingly, the thio analogue **4a** of benzophenone **3a** exhibited improved anti-proliferative activity with reinforced inhibition particularly on non-small cell lung cancer cells, A498 renal cancer and MCF-7 breast cancer cells (Table 2). The replacement of the monochloroacetate in compound **3a** by a trichloroacetate unit in compound **7a** was tolerated and provided excellent cell growth inhibition predominantly against leukemic, colon cancer, melanoma, ovarian and CNS cancer cells (GI_{50} < 10 nM). The acetylated benzophenone **3x** displayed similar potential to monochloroacetylated **3a** but diminished activity compared to trichloroacetylated phenstatin **7a** (compare **3x** to **7a**, Table 2). The replacement of the classical 3,4,5-trimethoxyphenyl unit of compound **3a** by a 3,5-dimethyl-4-methoxyphenyl unit in compound **3g** constituted a well-tolerated pharmacomodulation providing the most cytotoxic agent in the study (GI_{50} < 10 nM on the most part of tested cell lines) (Table 2). The suppression of the monochloroacetate group of **3g** in benzophenone **3h** resulted in a small decrease of the anti-proliferative activity and suppressed the activity on some cell strains (compare **3g** to **3h**, Table 2), underlining the importance of the chloroacetate unit. The addition of methoxy unit in the 2' position on the B-ring in compound **3j** slightly diminished the cellular activity (compare **3h** and **3j**, Table 2). The same modulation realized on phenstatin provided compound **3d** that showed greater anti-proliferative potential compared to analogue compound **3j** particularly on K-562, MDR NCI/ADR-RES, HS 578T and BT-549 cell lines (Table 2). Acetamide analogue **3k** displayed diminished cell growth inhibition compared to related analogue **3l** bearing a free amine group on the B ring (compare **3k** and **3l**, Table 2). The least cytotoxic derivative in the NCI evaluation was benzophenone **3n** bearing an original 2,4,5-trifluoro-3-methoxy phenyl unit as the A ring displaying cell growth inhibition in the micromolar range. The benzophenone **3r** decorated with a monochloroacetate displayed equivalent potential as analogue benzophenone **3s**. The replacement of the classical A ring of benzophenone **3a** by a 3,5-dimethoxy-4-methylphenyl unit in compound **3t** was less tolerated and diminished the anti-proliferative activity. This modulation was also less interesting than the replacement of the A ring by a 3,5-dimethyl-4-methoxyphenyl unit in compound **3g**.

Benzophenone **6c** decorated with a dichloroacetate unit displayed high anti-melanoma potential strongly inhibiting the MDA-MB-435 cells (GI_{50} < 10 nM) but also CNS cancer cells. Interestingly, the replacement of the acetyl unit by two monochloroacetyl groups in benzophenone **5** was tolerated and improved the antitumor activity (Table 2). The same trend was registered when changing the acetyl unit of compound **3k** by two dichloroacetyl groups in compound **8**.

Table 2 Results of the *in vitro* human cancer cell growth inhibition for selected compounds **3a**, **3d**, **3g-h**, **3j-l**, **3n**, **3r-t**, **3x**, **4a**, **5**, **6c**, **7a** and **8**[a,b]

	Compound	3a	3d	3g	3h	3j	3k	3l	3n	3r	3s	3t	3x	4a	5	6c	7a	8
Cell type	**Cell line**	**GI_{50} (nM)[a,b]**																
Leukemia	HL-60(TB)	**<10**	23.5	**<10**	**<10**	27.6	-[c]	-	2020	-	-	19.0	19.9	**<10**	31.6	25.1	**<10**	30.6
	K-562	**<10**	**<10**	**<10**	**<10**	31.1	1090	14.5	186	457	416	35.4	**<10**	**<10**	52.6	31.9	**<10**	43.1
	CCRF-CEM	23.5	28.8	**<10**	**<10**	34.8	1830	30.4	3310	662	410	293	38.6	**<10**	204	38.2	33.6	71.3
Non-Small Cell Lung Cancer	A549/ATCC	363	41.3	40.4	1200	399	284	34.8	4390	3390	3960	2150	55.2	174	97.3	330	54.7	94.8
	NCI-H522	15.8	**<10**	**<10**	-	35.9	54.8	17	1340	264	311	217	24.7	10.4	56.1	41.7	14	48.7
	NCI-H322M	75.1	**<10**	10.4	-	59.5	98.8	42.1	4470	3410	2200	580	209	47.0	67.2	69.9	93.2	57.4
Colon Cancer	KM12	**<10**	**<10**	**<10**	**<10**	28.9	141	19.6	1030	543	520	90.2	17.7	**<10**	40.1	29.3	**<10**	40.1
	SW-620	**<10**	24.6	**<10**	**<10**	34.7	167	32.9	703	455	489	123	33.8	**<10**	43.3	37.4	**<10**	43.7
	HCT-15	**<10**	10.6	**<10**	**<10**	36.5	397	26.3	1760	415	420	51.1	18.4	**<10**	56.2	34.9	**<10**	58.5
Melanoma	M14	**<10**	**<10**	**<10**	**<10**	21.5	86.9	23.8	804	491	319	54.0	20.5	**<10**	43.7	26.1	**<10**	39.7
	MDA-MB-435	**<10**	**<10**	**<10**	**<10**	**<10**	37.8	**<10**	285	239	288	21.9	**<10**	**<10**	18.1	**<10**	**<10**	17.6
	LOX IMVI	**<10**	26.9	**<10**	**<10**	45.1	549	30.7	5240	677	557	238	32.5	**<10**	70.6	39.7	**<10**	95.5
Ovarian Cancer	OVCAR-3	24.0	22.2	**<10**	**<10**	39.3	533	29.4	1500	414	414	281	29.2	21.6	34.6	29.1	27.8	28.7
	NCI/ADR-RES	**<10**	**<10**	**<10**	**<10**	25.8	131	12.9	789	497	378	1600	23.5	**<10**	32.3	31.0	**<10**	31.4
	OVCAR-8	39.0	29	**<10**	**<10**	43.4	375	38.6	3490	2650	3360	403	54.7	34.9	63.6	43.7	47.5	56.2
Renal Cancer	A498	1720	2720	1590	4110	1630	28.6	19.1	2140	3000	3190	1800	1720	136	20.0	1260	2340	**<10**
	RXF 393	18.8	15.9	**<10**	**<10**	208	29.4	25.3	2470	1540	1120	246	39.1	15.0	27.2	187	33.7	22.4
	SN12C	35.3	46.9	**<10**	**<10**	93.2	273	72.9	5160	3390	2790	656	58.3	14.0	93.3	123	59.8	88.7
Prostate Cancer	PC-3	**<10**	26.4	**<10**	**<10**	41.9	45.2	37.1	3720	2830	562	275	36.1	**<10**	44.8	41.7	11.3	46.3
	DU-145	27.6	20.9	**<10**	**<10**	38.2	48.6	33.8	2080	1830	885	283	38.9	25.7	38.8	37.7	26.4	38.9
CNS Cancer	SF-539	**<10**	15.1	**<10**	**<10**	20.6	268	35.5	2470	805	637	153	24.0	**<10**	27.2	23.6	**<10**	28.5
	SNB-75	**<10**	-	**<10**	**<10**	27.5	42.5	31.5	1740	528	534	30.9	21.0	**<10**	21.7	36.2	**<10**	20.4
	SF-295	38.9	19.9	**<10**	46.3	944	46.4	16.6	2620	886	524	1230	54.7	31.3	31.6	512	39.8	29.8
Breast Cancer	HS 578T	17.3	**<10**	**<10**	>10000	25.3	80.3	60.1	1600	556	524	253	31.7	20.8	27.2	26.2	16.7	26.0
	BT-549	**<10**	**<10**	**<10**	**<10**	18.2	60.1	74.5	3130	996	465	303	39.5	**<10**	63.1	34.7	12.3	70.8
	MCF7	18.8	26.4	**<10**	**<10**	206	445	19.8	3040	880	516	385	35.5	**<10**	59.8	148	24.4	73.0
$Log_{10}GI_{50}$-MID[d]		-7.25	-7.17	-7.41	-7.28	-6.79	-6.61	-7.35	-5.54	-5.77	-5.86	-6.34	-6.81	-7.42	-7.14	-6.87	-7.13	-7.12

[a] Data obtained from NCI's *in vitro* 60 cell 5-dose screen.

[b] GI_{50} is the molar concentration of synthetic compound causing 50% growth inhibition of tumor cells.

[c] Not tested.

[d] Average activity parameter over all NCI 60-cancer cell lines for the tested compounds.

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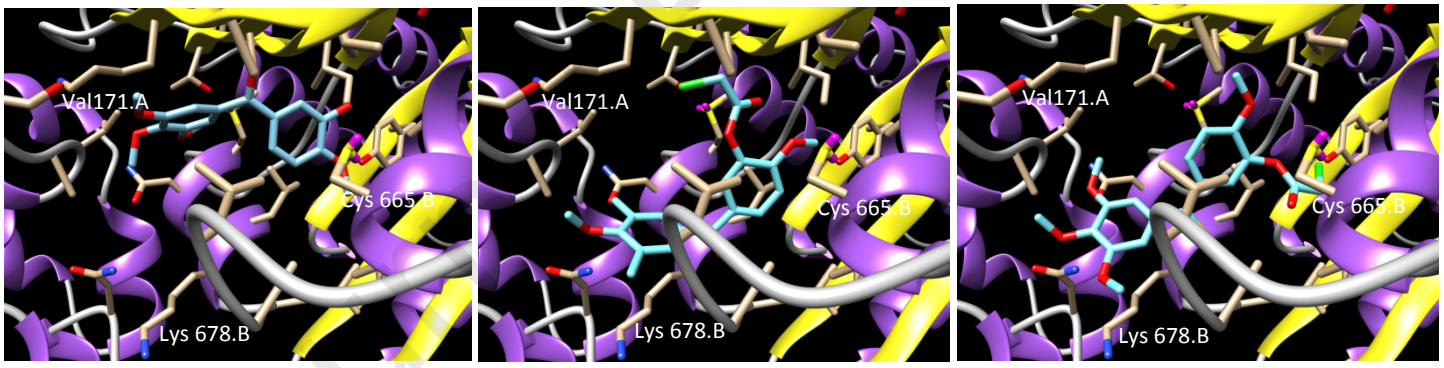
2.3. Molecular docking

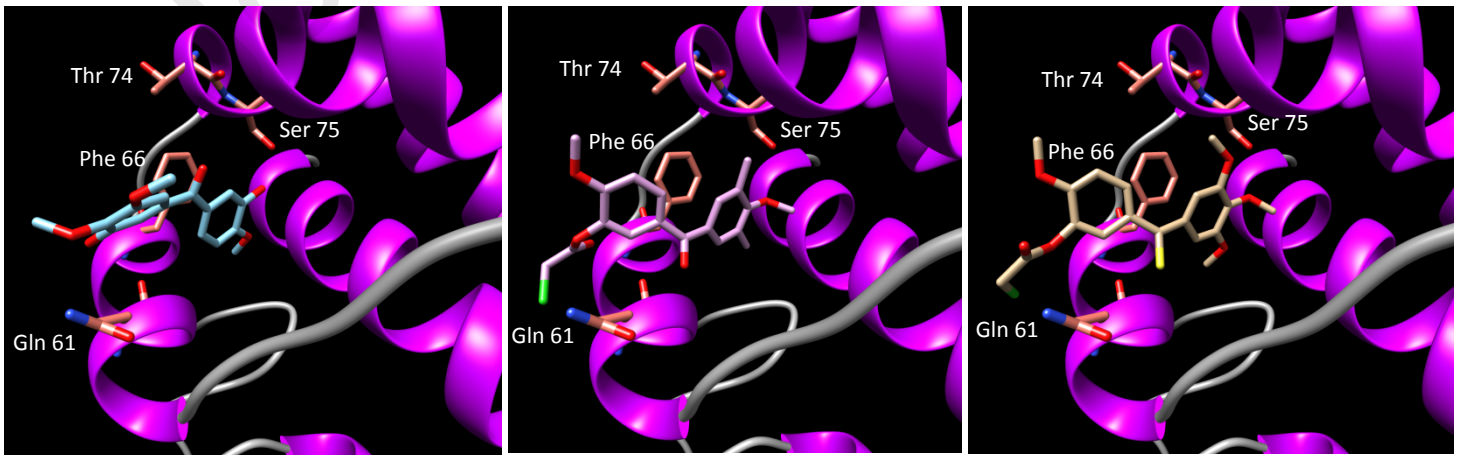
The docking studies were realized with GOLD (Figure 4). The protocol has already been described for tubulin [16] and we chose 2Q8G pdb entry for PDHK1 due to its good resolution [17]. The binding site was defined as a sphere of 10 Å around the extracted co-crystalized ligand. To validate the docking, this ligand was redocked in its own binding site and the docked and crystallographic conformations were closely superimposed.

Reference phenstatin **3b** (Figure 4a) and potent antitubulin agents **3g** and **4a** have been selected for docking in the tubulin binding site. Compounds **3g** and **4a** display a binding mode in tubulin that is an inverted image of phenstatin **3b**, with the linker between the two rings pointing toward the inside of the pocket (Figure 4b and 4c). Compound **3g** is nearly superimposable to compound **4a** (Figure 4c).

In the PDHK1 binding site, phenstatin **3b** has a near unique pose, with the B cycle in the depth of the binding site where the free phenol forms a hydrogen bond with Ser 75 (Figure 4d). As for the other cycle, a hydrogen bond with Gln 61 is found consistently, most often with the middle methoxy and less commonly with the upper one. Compound **3g** places differently, with slightly more than two thirds of the poses arranged with the ester toward the outside of the binding site and the methoxy on the other cycle (A ring) forming a hydrogen bond with Ser 75. The ester binds to Gln 61 very rarely. It is nonetheless sound to guess at a hydrogen between them, not seen here due to the orientation of the residue. This may be due to the rigidity of its side chain as we have not taken its flexibility into account (Figure 4e). Chloroacetyl-thiophenstatin **4a** has a full half of the 30 poses gathered in a single solution placed in reverse when compared with phenstatin **3b** but very similar to chloroacetylbenzophenone **3g** (Figure 4f). The three-methoxy cycle is positioned in the depth of the binding site, with one or two of the oxygens forming a hydrogen bond with Ser 75. The ester carbonyle is sometimes binding to Gln 61. The DCA is very far placed from other inhibitors in another pocket.



(a) (b) (c)



(d) (e) (f)

Figure 4. Docking of selected molecules in the tubulin binding site (colchicine site) (a) phenstatin **3b**; (b) chloroacetyl-benzophenone **3g**; (c) chloroacetyl-thiophenstatin **4a**; Docking of selected molecules in the PDHK1 binding site: (d) phenstatin **3b**; (e) chloroacetyl-benzophenone **3g**; (f) chloroacetyl-thiophenstatin **4a**.

3. Conclusions

The objective of this work was the design, synthesis and pharmacological evaluation of dual-targeted anticancer agents inhibiting both tubulin polymerization and pyruvate dehydrogenase kinase 1 (PDHK1). Among the differences between normal and cancer cells are important expressions of PDHK observed in various cancers including non-small cell lung cancer (NSCLC), breast and colon cancer. Therefore, intensive efforts are being made to discover effective PDHK inhibitors [4], [5], [18].

The use of PDHK inhibitors has been disappointing in clinical trials so far. Proving the efficacy of dual tubulin/PDHK1 inhibitors can be crucial, leading to an innovative strategy for the design of new anti-cancer compounds.

New molecules with improved anticancer activity were thus developed in the current study. Three families of compounds (mono, di and trichloroacetyl-loaded benzo(thio)phenones) regrouping more than 30 molecules could be obtained (Schemes 1-3). Their biological evaluation confirmed the obtaining of: i) inhibitors of the tubulin polymerization, classic cytotoxic agents, ii) inhibitors of pyruvate dehydrogenase kinase 1, rather cytostatic, iii) dual inhibitors tubulin/PDHK1, and iv) highly cytotoxic compounds.

Concerning the specific tubulin polymerization inhibitors discovered in the current study, new SAR have been highlighted: 1) a new A ring (3,5-dimethyl-4-methoxyphenyl) has been identified and puts an end to the dogma that the classical 3,4,5-trimethoxyphenyl ring is considered intangible in maintaining antimitotic activity and consequently, methyl substituents on the A ring can be tolerated in place of methoxy groups in positions 3 and 5 (compare **3a** and **3g**, Table 2); 2) the replacement of the methoxy in position 4 of the A ring by a methyl while conserving the methoxy substituents in positions 3 and 5 slightly decreased antimitotic activity (compare benzophenones **3a** and **3t**, Table 1); 3) The carbonyl bridge between the two rings A and B of parent phenstatin may be successfully replaced by a thiocarbonyl providing unprecedented thiophenstatin (compare **3a**/**4a** and **3b**/**4b**, Tables 1 and 2); 4) the phenol group of the parent phenstatin may be effectively replaced with mono-, di- and trichloroacetates (compare antitubulin activities of benzophenones **3a**, **6a**, **7a** and phenstatin, Table 1); 5) the substitution by trichloroacetate conferring enhanced antimitotic effect compared to free phenol analogs.

The inhibitors of PDHK1 discovered in this study and which are not active on tubulin polymerization have been identified as monocloroacetate-loaded benzophenones **3p** (Scheme 1), **3v** and **3w** (Scheme 2) and also a dichloroacetate-loaded derivative **6d** (Scheme 3). The last three molecules have as common structural feature the same benzophenone scaffold with a 2,4,5-trifluoro-3-methoxyphenyl as A ring. These PDHK1 inhibitors showed cytostatic effect and did not underwent GI_{50} calculation on the NCI-60 cancer cell panel (see Supplementary section for complete one-dose reports). Interestingly, bis-monochloroacetyl (**5**) or dichloroacetyl (**8**) amides resulted in diminished or complete loss of activity against PDHK1, esters being consequently more favorable than amides for preserving activity against PDHK1 in this study. As expected, non-chlorinated benzophenone **3x** (Scheme 2) bearing an acetyl substituent had no effect on the target underlining the importance of the halogen.

Monochloroacetate-loaded benzophenone **3g** and thiobenzophenone **4a** are dual tubulin/PDHK1 anti-cancer agents. Dichloroacetate-analogue **6b** was completely inactive on both targets. In the same way trichloroacetate **7a** was not able to inhibit PDHK1 while conserving a strong antimitotic effect. Monochloroacetates were the only tolerated to preserve both activities on tubulin and on PDHK1.

The impact of the chloroacetyl-thiophenstatin **4a** (dual inhibitor) and thiophenstatin **4b** (specific antitubulin agent) has been evaluated on the cell cycle of DA1-3b cells. Their effect on the cell cycle was different. The

thiophenstatin **4b** acted as an antitubulin agent and arrested DA1-3b cells in the G2/M phase while the dual inhibitor **4a** mainly increased the sub-G1 phase population and maintained a diminished G2/M effect compared to thiophenstatin **4b**. This may be related to the different mechanisms of action of studied molecules. It is interesting to note that dichloroacetate, without specified positive counter ion of the tested molecule, was also reported to arrest bladder cancer cells in G0–G1 phase [19]. Arresting cancer cells in subG1 phase is interesting for anticancer drugs, but we do not know if the cells in the subG1 are not in G0 and therefore in quiescence. Quiescence is also encouraging for stopping tumor growth. Indeed, an inhibition of the production of cyclin D1 may be caused by the dual inhibitor **4a** since a strong expression of cyclin D1 is necessary to pass the control point in phase G1 and start the phase S, but this is an assumption which deserves further biological efforts in due course.

Excellent cell growth inhibition on the NCI-60 cancer cell lines panel has been registered for our antimitotic agents and dual tubulin/PDHK1 inhibitors. The most sensitive cells to these molecules proved to be melanoma (in particular MDA-MB-435 and M14), colon cancer (KM12 and HCT-15) and leukemia (HL-60(TB) and K-562) cell lines. It is also interesting to note that dual tubulin/PDHK1 inhibitor **4a** exhibited improved cancer cell growth inhibition compared to analogue molecule **3a** showing only antitubulin efficacy on non-small cell lung cancer (A549/ATCC, NCI-H522 and NCI-H322M) and renal A498 cancer cells. This may be explained by the significantly up-regulated expression of PDHK1 in NSCLC cells including A549 cells [20]. The same tendency has been observed for benzophenones **3g** and **3h**. The monochloroacetylated benzophenone **3g**, dual inhibitor tubulin/PDHK1, showed improved anticancer potential on A549/ATCC NSCLC cells and on A498 renal cancer cells compared to benzophenone **3h** bearing only a free phenol substituent on the B ring.

To the best of our knowledge this is the first time when a potential codrug acting on tubulin polymerization and on PDHK1 is envisaged. This duality of actions offers new perspectives and supports the importance of such inhibitors in the design of new anticancer drugs.

4. Experimental section

4.1. Materials and methods

Starting materials are commercially available and were used without further purification (suppliers: Carlo Erba Reagents S.A.S., Tokyo Chemical Industry Co. Ltd. and Acros Organics). Melting points were measured on a MPA 100 OptiMelt® apparatus and are uncorrected. Nuclear Resonance Magnetic (NMR) were acquired at 400 MHz for ^{1}H NMR, at 376 MHz for ^{19}F NMR and at 100 MHz for ^{13}C NMR, on a Varian 400-MR spectrometer with tetramethylsilane (TMS) as internal standard, at 25°C. Chemical shifts (δ) are expressed in ppm relative to TMS. Splitting patterns are designed: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; m, multiplet; quint, quintuplet; br s, broaden singlet; br t, broaden triplet. Coupling constants (*J*) are reported in Hertz (Hz). Thin layer chromatography (TLC) was realized on Macherey Nagel silica gel plates with fluorescent indicator and were visualized under a UV-lamp at 254 nm and 365 nm. Column chromatography was performed with a CombiFlash R*f* Companion (Teledyne-Isco System) using RediSep packed columns. IR spectra were recorded on a Varian 640-IR FT-IR Spectrometer. Elemental analyses (C, H, N) of new compounds were determined on a Thermo Electron apparatus by "Pôle Chimie Moléculaire-Welience", Faculté des Sciences Mirande, Dijon, France.

4.1.1. General procedure A for Friedel–Crafts reactions in the presence of Eaton's reagent

Eaton's reagent was prepared from phosphorus pentoxide (P_2O_5) and methanesulfonic acid (CH_3SO_3H) (weight ratio P_2O_5/CH_3SO_3H 1:10). The mixture was heated at 40 °C under nitrogen atmosphere until complete homogeneity. Benzoic acid (1.15–1.5 equiv) and aromatic derivative (1.0 equiv) were then added to Eaton's reagent. The mixture was heated at 60°C under inert atmosphere for 3–24 h. After cooling to room temperature, the reaction medium was diluted with dichloromethane and carefully poured into a separatory funnel containing sodium bicarbonate aqueous solution (50% $NaHCO_3$) (neutralization to pH 7). The aqueous solution was extracted with dichloromethane, and the combined organic layers were dried ($MgSO_4$). Solvent was removed under reduced pressure to produce a brownish oil. The crude product was

purified by Flash chromatography on RediSep packed columns (eluent *n*-heptane/ethyl acetate) to provide pure benzophenones **3g**, **3i**, **3k** and **3o**.

Benzophenones **3a**, **3b**, **3c**, **3e**, **3m**, **3p**, **3q**, **3r**, **3t** and **3x** have been previously reported and re-synthesized accordingly [7], [8], [11], [12], [13].

*4.1.1.1. 2-Methoxy-5-(4-methoxy-3,5-dimethylbenzoyl)phenyl chloroacetate (**3g**).* General procedure A was followed by using 4-methoxy-3,5-dimethylbenzoic acid **16** (1.45 g, 8.05 mmol), 2-methoxyphenyl chloroacetate **20** (1.08 g, 5.38 mmol) and Eaton's reagent (0.53 g P_2O_5 in 3.6 mL CH_3SO_3H). The crude obtained after work-up was purified on silica gel column (eluent: gradient EtOAc/*n*-heptane 3/7 → 100% EtOAc) to provide pure benzophenone **3g** as a beige solid; 80% yield (1.56 g); mp (EtOAc) 76-78 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.33 (s, 6H, 2ArC*H*$_3$), 3.78 (s, 3H, OC*H*$_3$), 3.92 (s, 3H, OC*H*$_3$), 4.35 (s, 2H, OCOC*H*$_2$Cl), 7.04 (d, *J* = 8.8 Hz, 1H, Ar*H*), 7.45 (s, 2H, Ar*H*), 7.60 (d, *J* = 2.1 Hz, 1H, Ar*H*), 7.75 (dd, *J* = 8.8, 2.1 Hz, 1H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.1 (2CH_3), 40.5 (CH_2), 56.1 (CH_3), 59.6 (CH_3), 111.4 (CH), 111.6 (CH), 124.3 (2CH), 124.4 (2C), 133.0 (CH), 133.2 (C), 138.8 (C), 139.5 (C), 154.2 (C), 160.5 (C), 165.2 (C), 193.8 (C). IR ν (cm^{-1}): 1736, 1630, 1593, 1506, 1441, 1265, 1223, 1141, 1116, 1003, 882, 752. Elem. Analysis calcd. for $C_{19}H_{19}ClO_5$: C, 62.90; H, 5.28. Found: C, 62.81; H, 5.13%.

*4.1.1.2. 2,6-Dimethoxy-3-(4-methoxy-3,5-dimethylbenzoyl)phenyl chloroacetate (**3i**).* General procedure A was followed by using 4-methoxy-3,5-dimethylbenzoic acid **16** (1.00 g, 5.55 mmol), 2,6-dimethoxyphenyl chloroacetate **21** (0.85 g, 3.7 mmol) and Eaton's reagent (0.63 g P_2O_5 in 2.46 mL CH_3SO_3H). The crude obtained after work-up was purified on silica gel column (eluent: gradient EtOAc/*n*-heptane 3/7 → 100% EtOAc) to provide pure benzophenone **3i** as a white solid; 87% yield (1.26 g); mp (EtOAc) 104-107 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.33 (s, 6H, 2ArC*H*$_3$), 3.82 (s, 3H, OC*H*$_3$), 3.95 (s, 3H, OC*H*$_3$), 4.36 (s, 2H, OCOC*H*$_2$Cl), 7.06 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.42 (s, 2H, Ar*H*), 7.62 (d, *J* = 8.6 Hz, 1H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.2 (2CH_3), 40.4 (CH_2), 56.3 (CH_3), 60.0 (CH_3), 62.5 (CH_3), 110.9 (CH), 111.1 (CH), 124.2 (2CH), 124.5 (2C), 132.9 (CH), 133.1 (C), 138.9 (C), 139.6 (C), 154.3 (C), 160.6 (C), 165.3 (C), 193.7 (C). Elem. Analysis calcd. for $C_{20}H_{21}ClO_6$: C, 61.15; H, 5.39. Found: C, 61.40; H, 5.62%.

*4.1.1.3. N-[2,6-Dimethoxy-3-(4-methoxy-3,5-dimethylbenzoyl)phenyl]acetamide (**3k**).* General procedure A was followed by using 4-methoxy-3,5-dimethylbenzoic acid **16** (1.62 g, 8.99 mmol), *N*-(2,6-dimethoxyphenyl)acetamide **22** (1.17 g, 5.99 mmol) and Eaton's reagent (0.59 g P_2O_5 in 3.98 mL CH_3SO_3H). The crude obtained after work-up was purified on silica gel column (eluent: EtOAc/*n*-heptane 6/4) to provide pure benzophenone **3k** as a white solid in 66% yield (1.41 g); mp (EtOAc/*n*-heptane) 188-190 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.18 (s large, 3H, NHCOC*H*$_3$), 2.31 (s, 6H, 2ArC*H*$_3$), 3.65 (s, 3H, OC*H*$_3$), 3.77 (s, 3H, OC*H*$_3$), 3.91 (s, 3H, OC*H*$_3$), 6.68 (s large, 1H, ArN*H*), 6.75 (d, *J* = 8.9 Hz, 1H, Ar*H*), 7.30 (d, *J* = 8.9 Hz, 1H, Ar*H*), 7.52 (s, 2H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.2 (2CH_3), 23.3 (CH_3), 56.1 (CH_3), 60.0 (CH_3), 62.1 (CH_3), 106.1 (C), 119.1 (C), 125.7 (C), 129.4 (CH), 131.0 (CH), 131.1 (2CH), 133.3 (2C), 155.5 (C), 157.4 (C), 161.3 (C), 173.9 (C), 194.5 (C). Elem. Analysis calcd. for $C_{20}H_{23}O_5N$: C, 67.21; H, 6.49; N, 3.92. Found: C, 67.01; H, 6.70; N, 3.92%.

*4.1.1.4. (2,3-Dihydroxy-4-methoxyphenyl)(2,4,5-trifluoro-3-methoxyphenyl)methanone (**3o**).* General procedure A was followed by using 2,4,5-trifluoro-3-methoxybenzoic acid **17** (2.5 g, 12.1 mmol), 2,6-dimethoxyphenyl chloroacetate **21** (1.87 g, 8.09 mmol) and Eaton's reagent (0.91 g P_2O_5 in 6.14 mL CH_3SO_3H). The crude obtained after work-up was purified on silica gel column (eluent: EtOAc/*n*-heptane 3/7) to provide pure benzophenone **3o** as a yellow solid; 71% yield (1.89 g); mp (EtOAc) 120-123 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm 3.98 (s, 3H, OC*H*$_3$), 4.09 (s, 3H, OC*H*$_3$), 5.54 (s, 1H, ArO*H*), 6.51 (d, *J* = 9.2 Hz, 1H, Ar*H*), 6.97 (sm, 1H, Ar*H*), 6.98 (d, *J* = 9.2 Hz, 1H, Ar*H*), 11.89 (s, 1H, ArO*H*). ^{19}F NMR ($CDCl_3$, 376 MHz) δ (ppm) -146.37 (ddd, *J* = 20.5, 12.4, 9.6 Hz, 1F, Ar*F*), -138.92 (dddd, *J* = 22.4, 20.5, 13.5, 9.5 Hz, 1F, Ar*F*), -132.42 (sm, 1F, Ar*F*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 56.4 (CH_3), 62.2 (t, *J* = 3.1 Hz, CH_3), 103.3 (CH), 109.9 (dd, *J* = 20.2, 3.1 Hz, CH), 114.4 (C), 121.9 (sm, C), 125.5 (d, *J* = 2.4 Hz, CH), 133.5 (C), 138.3 (m, C), 145.9 (ddd, *J* = 255.0, 15.0, 5.3 Hz, C), 147.4 (ddd, *J* = 248.5, 11.7, 3.1 Hz, C), 148.5 (dt, *J* = 249.6, 3.3 Hz, C), 150.8 (C), 153.1 (C),

194.3 (C). IR ν (cm^{-1}): 3467, 1633, 1597, 1508, 1463, 1429, 1330, 1278, 1095, 1021, 777, 675, 499. Elem. Analysis calcd. for $C_{15}H_{11}O_5F_3$: C, 54.89; H, 3.38. Found: C, 54.77; H, 3.17%.

4.1.2. General procedure B: synthesis of phenols from monochloroacetic esters
Monochloroacetic ester **3g** and **3i** (1.0 equiv) and sodium acetate ($AcONa{\cdot}3H_2O$; 4.5 equiv) were dissolved in MeOH. The solution was heated to reflux for 5 h. After cooling to RT, the mixture was concentrated under reduced pressure and the residue was taken into distilled water. The resulting precipitate was filtered, washed with water several times to remove remaining sodium acetate, and recrystallized from absolute EtOH or purified by flash chromatography to obtain pure benzophenones bearing free phenols **3h** and **3j**.

*4.1.2.1. (3-Hydroxy-4-methoxyphenyl)(4-methoxy-3,5-dimethylphenyl)methanone (**3h**).* General procedure B was followed by using monochloroacetate **3g** (0.86 g, 2.37 mmol) and sodium acetate ($AcONa{\cdot}3H_2O$; 1.45 g, 10.7 mmol) in MeOH (25 mL). After evaporation of the methanol in vacuo, the residue was poured in distilled water. The residue was purified by column chromatography (EtOAc/*n*-heptane, 15:85) and recrystallized from EtOH to obtain benzophenone **3h** as a white solid; 92% yield (0.62 g); mp (EtOAc) 118-119 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.33 (s, 6H, 2ArC*H*$_3$), 3.78 (s, 3H, OC*H*$_3$), 3.98 (s, 3H, OC*H*$_3$), 5.67 (s, 1H, ArO*H*), 6.92 (d, *J* = 8.4 Hz, 1H, Ar*H*), 7.38 (dd, *J* = 8.3, 2.2 Hz, 1H, Ar*H*), 7.41 (d, *J* = 2.1 Hz, 1H, Ar*H*), 7.45 (s, 2H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.2 ($2CH_3$), 56.1 (CH_3), 59.7 (CH_3), 109.7 (CH), 116.3 (CH), 123.6 (CH), 130.8 (C), 131.2 (2C), 131.4 (2CH), 139.7 (C), 145.2 (C), 150.0 (C), 160.6 (C), 195.2 (C). IR ν (cm^{-1}): 3226, 1631, 1603, 1507, 1442, 1313, 1224, 1174, 1141, 1117, 1005, 753. Elem. Analysis calcd. for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found: C, 71.61; H, 5.93%.

*4.1.2.2. (3-Hydroxy-2,4-dimethoxyphenyl)(4-methoxy-3,5-dimethylphenyl)methanone (**3j**).* General procedure B was followed by using monochloroacetate **3i** (0.98 g, 2.5 mmol) and sodium acetate ($AcONa{\cdot}3H_2O$; 1.45 g, 35.7 mmol) in MeOH (35 mL). After evaporation of the methanol in vacuo, the residue was poured in distilled water. The resulting solid was collected by filtration and recrystallized from absolute EtOH to obtain pure benzophenone **3j** as a sparkling white solid; 98% yield (0.77 g); mp (EtOAc) 110-112 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.30 (s, 6H, 2ArC*H*$_3$), 3.76 (s, 3H, OC*H*$_3$), 3.77 (s, 3H, OC*H*$_3$), 3.95 (s, 3H, OC*H*$_3$), 5.79 (s large, 1H, ArO*H*), 6.70 (d, *J* = 8.6 Hz, 1H, Ar*H*), 6.90 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.50 (s, 2H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.2 ($2CH_3$), 56.3 (CH_3), 59.7 (CH_3), 61.8 (CH_3), 105.9 (CH), 120.8 (CH), 126.1 (C), 130.9 (2C), 131.0 (2CH), 133.6 (C), 138.3 (C), 145.8 (C), 149.7 (C), 161.2 (C), 194.9 (C). Elem. Analysis calcd. for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37. Found: C, 67.96; H, 6.09%.

*4.1.3. (3-Amino-2,4-dimethoxyphenyl)(4-methoxy-3,5-dimethylphenyl)methanone (**3l**).* A mixture of acetamide **3k** (1.52 g, 4.25 mmol) and a 10% hydrochloric acid solution (30 mL) in methanol (20 mL) was stirred at reflux for 24 h. The final suspension was neutralized to pH 7 by slow addition of an ammoniac solution. The aqueous solution was extracted with dichloromethane. The organic phase was dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by liquid chromatography on silica column (dichloromethane) to afford pure aniline **3l** as a yellow cotton (1.14 g, 85% yield); mp (CH_2Cl_2) 139 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.29 (s, 6H, 2ArC*H*$_3$), 3.69 (s, 3H, OC*H*$_3$), 3.77 (s, 3H, OC*H*$_3$), 3.92 (s, 3H, OC*H*$_3$), 3.99 (s large, 2H, ArN*H*$_2$), 6.63 (d, *J* = 8.5 Hz, 1H, Ar*H*), 6.75 (d, *J* = 8.5 Hz, 1H, Ar*H*), 7.52 (s, 2H, Ar*H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.2 ($2CH_3$), 55.8 (CH_3), 59.6 (CH_3), 61.2 (CH_3), 105.2 (C), 119.0 (CH), 125.7 (C), 129.6 (C), 130.8 (2C), 131.1 (2CH), 133.7 (C), 145.7 (C), 149.9 (C), 161.1 (C), 195.4 (C). Elem. Analysis calcd. for $C_{18}H_{21}O_4N$: C, 68.55; H, 6. 71; N, 4.44. Found: C, 68.46; H, 6.93; N, 4.34%.

*4.1.4. 2-Methoxy-5-[(3,4,5-trimethoxyphenyl)carbonothioyl]phenyl chloroacetate (**4a**).* Phosphorous pentasulfide (0.92 g, 4.05 mmol) and hexamethyledisiloxane (0.92 mL, 8.1 mmol) were added to a solution of chloroacetic-phenstatine **3a** (1.6 g, 4.05 mmol) in 25 mL toluene and the mixture was stirred at 110 °C under inert atmosphere for 24 hours and then concentrated under vacuum. The residue was purified by silica gel column chromatography (*n*-heptane:ethyl acetate from 100:0 to 0:100) to give **4a** as a blue-green solid (1.05 g, 63% yield); mp (EtOAc) 90-91 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 3.89 (s, 9H, 3OC*H*$_3$), 3.94 (s, 3H, OC*H*$_3$), 4.37 (s, 2H, OCOC*H*$_2$Cl), 7.05 (s, 2H, 2Ar*H*), 7.09 (d, *J* = 7.6 Hz, 1H, Ar*H*), 7.60 (d, *J* = 1.5 Hz, 1H, Ar*H*), 7.86 (dd, *J* = 7.6, 1.5 Hz, 1H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 40.5 (CH_2), 56.2 (CH_3), 56.3 ($2CH_3$), 60.9

(CH_3), 107.6 (2CH), 111.4 (CH), 124.7 (CH), 130.1 (CH), 138.5 (C), 140.2 (C), 141.9 (C), 142.5 (C), 152.4 (2C), 154.5 (C), 165.4 (C), 232.6 (C). IR ν (cm^{-1}): 1737, 1601, 1577, 1505, 1488, 1407, 1329, 1287, 1183, 1123, 989, 829, 791. Elem. Analysis calcd. for $C_{19}H_{19}O_6SCl$: C, 55.54; H, 4.66; S, 7.80. Found: C, 55.59; H, 4.68; S, 7.54%.

*4.1.5. (3-Hydroxy-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanethione (**4b**).* Phosphorous pentasulfide (1.0 g, 4.4 mmol) and hexamethyledisiloxane (1.1 mL, 8.8 mmol) were added to a solution of phenstatine **3b** (1.39 g, 4.4 mmol) in 25 mL toluene and the mixture was stirred at 110°C under inert atmosphere for 24 hours and then concentrated under vacuum. The residue was purified by silica gel column chromatography (*n*-heptane:ethyl acetate from 100:0 to 100:0) to give **4b** as a blue solid (769 mg, 43% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 3.86 (s, 6H, 2OC*H*$_3$), 3.94 (s, 3H, OC*H*$_3$), 3.99 (s, 3H, OC*H*$_3$), 5.62 (br s, 1H, O*H*), 6.84 (d, *J* = 8.7 Hz, 1H, Ar*H*), 6.96 (s, 2H, Ar*H*), 7.34 (dd, *J* = 8.4, 2.1 Hz, 1H, Ar*H*), 7.44 (d, *J* = 2.1 Hz, 1H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm) 56.1 (CH_3), 56.3 ($2CH_3$), 60.9 (CH_3), 107.7 (2CH), 109.3 (CH), 116.6 (CH), 123.5 (CH), 141.2 (C), 141.7 (C), 143.0 (C), 145.0 (C), 150.5 (C), 152.3 (2C), 234.2 (C). IR ν (cm^{-1}): 3448, 1572, 1499, 1451, 1320, 1276, 1123, 1026, 983, 849. Elem. Analysis calcd. for $C_{17}H_{18}O_5S$: C, 61.06; H, 5.43; S, 9.59. Found: C, 61.31; H, 5.49; S, 9.28%.

4.1.6. General procedure C for the synthesis of mono-, di- and trichloroacetic esters and amides from phenols and anilines

A mixture of phenol (**3b**, **3h**, **3j** or **3o**) or aniline **3l** (1 equiv) and monochloroacetic acid chloride, dichloroacetic acid chloride or trichloroacetic acid chloride (2-6 equiv) was placed under nitrogen atmosphere and heated at reflux for 1-6 h. Once the reaction started, a vigorous release of gaseous hydrochloric acid was observed. After reaction completion, the mixture was cooled to room temperature, treated with a saturated solution $NaHCO_3$ and extracted several times with dichloromethane. Combined organic phases were dried over magnesium sulfate and then concentrated. The oily resulting residues were crystallized from absolute ethanol or purified by column chromatography to afford pure protected phenols **3v**, **3w**, **6a**, **6b**, **6c**, **6d**, **7a** or **7b** or amides **5** or **8**.

*4.1.6.1. 3-Methoxy-6-(2,4,5-trifluoro-3-methoxybenzoyl)-1,2-phenylene bis(2-chloroacetate) (**3v**).* General procedure C was followed by using benzophenone **3o** (0.1 g, 3.08 mmol) and monochloroacetic acid chloride (1.47 mL, 18.48 mmol). The crude obtained after final work-up was purified by flash chromatography (eluent: EtOAc/*n*-heptane 6/4) to provide pure bischloroacetate **3v** as a white solid in 41% yield (0.06 g); mp (EtOAc/*n*-heptane) 103-105 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 3.94 (s, 3H, OC*H*$_3$), 4.06 (s, 3H, OC*H*$_3$), 4.26 (s, 2H, OCOC*H*$_2$Cl), 4.34 (s, 2H, OCOC*H*$_2$Cl), 6.93 (d, *J* = 8.9 Hz, 1H, Ar*H*), 7.10 (sym m, 1H, Ar*H*), 7.49 (dd, *J* = 9.0, 1.9 Hz, 1H, Ar*H*). ^{19}F NMR ($CDCl_3$, 376 MHz) – 144.40 (sym m, 1F, Ar*F*), - 138.71 (sym m, 1F, Ar*F*), -130.69 (sym m, 1F, Ar*F*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 40.1 (CH_2), 40.3 (CH_2), 56.7 (CH_3), 62.2 (CH_3), 109.2 (CH), 110.8 (d, CH), 123.3 (C), 130.7 (CH), 132.4 (C), 142.7 (C), 156.2 (C), 164.1 (C), 164.7 (C), 187.0 (C). IR ν (cm^{-1}): 1795, 1661, 1608, 1505, 1452, 1407, 1240, 1121, 1095, 1025, 929, 820, 782, 747, 631. Elem. Analysis calcd. for $C_{19}H_{13}Cl_2F_3O_7$: C, 47.42; H, 2.72. Found: C, 47.78; H, 2.93%.

*4.1.6.2. 2-Hydroxy-6-methoxy-3-(2,4,5-trifluoro-3-methoxybenzoyl)phenyl 2-chloroacetate (**3w**).* By-product from the synthesis of dichloroacetate **3v**. White solid; 37% yield (0.046 g); mp (EtOAc/*n*-heptane) 108-110 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 3.92 (s, 3H, OC*H*$_3$), 4.10 (s, 3H, OC*H*$_3$), 4.43 (s, 2H, OCOC*H*$_2$Cl), 6.54 (d, *J* = 9.0 Hz, 1H, Ar*H*), 6.98 (sym m, 1H, Ar*H*), 7.32 (dd, *J* = 9.0, 2.8 Hz, 1H, Ar*H*), 12.10 (s, 1H, ArO*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 40.4 (CH_2), 56.5 (CH_3), 61.6 (CH_3), 103.3 (CH), 100.0 (CH), 109.8 (dd, C), 114.6 (C), 122.1 (C), 125.5 (C), 127.0 (C), 132.4 (CH), 144.8 (dd, C), 147.3 (d, C), 156.3 (C), 158.2 (C), 164.9 (C), 194.1 (C). IR ν (cm^{-1}): 3062, 1785, 1639, 1505, 1471, 1411, 1370, 1354, 1322, 1281, 1254, 1127, 1011, 945, 926, 811, 782.

*4.1.6.3. 2-Chloro-N-(2-chloro-acetyl)-N-[2,6-dimethoxy-3-(4-methoxy-3,5-dimethyl-benzoyl)-phenyl]-acetamide (**5**).* General procedure C was followed by using benzophenone **3l** (0.3 g, 0.95 mmol) and monochloroacetic acid chloride (0.45 mL, 5.7 mmol). The oily resulting residue was crystallized from

absolute ethanol to provide bis-monochloroacetamide **5** as a white solid; 98% yield (0.44 g); mp (EtOH) 152-155 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.32 (s, 6H, 2C*H*$_3$), 3.57 (s, 3H, OC*H*$_3$), 3.78 (s, 3H, OC*H*$_3$), 3.90 (s, 3H, OC*H*$_3$), 4.47 (q, *J* = 23.1, 16.1 Hz, 4H, 2COC*H*Cl_2), 6.80 (d, *J* = 9.0 Hz, 1H, Ar*H*), 7.49 (s, 2H, Ar*H*), 7.50 (d, *J* = 9.0 Hz, 1H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.3 ($2CH_3$), 45.2 ($2CH_2$), 56.6 (CH_3), 59.7 (CH_3), 62.4 (CH_3), 106.2 (CH), 118.0 (C), 125.0 (C), 131.0 (2CH), 131.4 (2C), 132.8 (CH), 133.2 (C), 156.4 (C), 157.6 (C), 161.7 (C), 168.6 (2C), 193.7 (C). IR ν (cm^{-1}): 1713, 1653, 1594, 1462, 1415, 1317, 1292, 1206, 1125, 1097, 1007, 793. Elem. Analysis calcd. for $C_{22}H_{23}Cl_2O_6N$: C, 56.42; H, 4.95; N, 2.99. Found: C, 56.21; H, 5.07; N, 3.14%.

*4.1.6.4. 2-Methoxy-5-(3,4,5-trimethoxybenzoyl)phenyl 2,2-dichloroacetate (**6a**).* General procedure C was followed by using phenstatine **3b** (0.5 g, 1.57 mmol) and dichloroacetic acid chloride (0.29 mL, 3.14 mmol). The oily resulting residue was crystallized from absolute ethanol to provide dichloroacetate **6a** as a white solid; 95% yield (0.64 g); mp (EtOH) 104-107 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 3.89 (s, 6H, 2OC*H*$_3$), 3.94 (s, 3H, OC*H*$_3$), 3.95 (s, 3H, OC*H*$_3$), 6.21 (s, 1H, OCOC*H*Cl_2), 7.03 (s, 2H, Ar*H*), 7.08 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.66 (d, *J* = 1.96 Hz, 1H, Ar*H*), 7.81 (dd, *J* = 8.6, 1.96 Hz, 1H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 56.2 (CH_3), 56.3 ($2CH_3$), 61.0 (CH_3), 63.8 (CH), 107.4 (2CH), 111.8 (CH), 124.3 (CH), 130.4 (C), 130.6 (CH), 132.5 (C), 138.5 (C), 141.9 (C), 152.9 (2C), 154.3 (C), 162.5 (C), 193.4 (C). IR ν (cm^{-1}): 1780, 1641, 1607, 1580, 1502, 1425, 1330, 1269, 1228, 1125, 997, 834, 760. Elem. Analysis calcd. for $C_{19}H_{18}Cl_2O_7$: C, 53.16; H, 4.23. Found: C, 53.31; H, 4.27%.

*4.1.6.5. 2-Methoxy-5-(4-methoxy-3,5-dimethylbenzoyl)phenyl 2,2-dichloroacetate (**6b**).* General procedure C was followed by using benzophenone **3h** (0.5 g, 1.75 mmol) and dichloroacetic acid chloride (0.34 mL, 3.5 mmol). The oily resulting residue was crystallized from absolute ethanol to provide dichloroacetate **6b** as a beige solid; 77% yield (0.53 g); mp (EtOAc/*n*-heptane) 113-116 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.34 (s, 6H, 2C*H*$_3$), 3.79 (s, 3H, OC*H*$_3$), 3.94 (s, 3H, OC*H*$_3$), 6.21 (s, 1H, OCOC*H*Cl_2), 7.06 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.45 (s, 2H, Ar*H*), 7.65 (d, *J* = 2.0 Hz, 1H, Ar*H*), 7.77 (dd, *J* = 8.6, 2.0 Hz, 1H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.2 ($2CH_3$), 56.3 (CH_3), 59.7 (CH_3), 63.8 (CH), 111.6 (CH), 124.1 (CH), 130.6 (C), 130.8 (CH), 130.9 (2CH), 131.1 (2C), 133.0 (C), 138.6 (C), 154.2 (C), 160.7 (C), 162.4 (C), 194.1 (C). IR ν (cm^{-1}): 1743, 1603, 1574, 1506, 1454, 1438, 1350, 1314, 1264, 1217, 1194, 1169, 1135, 1002, 890, 804, 774, 754, 624. Elem. Analysis calcd. for $C_{19}H_{18}Cl_2O_5$: C, 57.45; H, 4.57. Found: C, 57.80; H, 4.92%.

*4.1.6.6. 2,6-Dimethoxy-3-(4-methoxy-3,5-dimethylbenzoyl)phenyl 2,2-dichloroacetate (**6c**).* General procedure C was followed by using benzophenone **3j** (0.1 g, 0.32 mmol) and dichloroacetic acid chloride (0.06 mL, 0.64 mmol). The crude obtained after final work-up was purified by flash chromatography (eluent: EtOAc/*n*-heptane 6/4) to provide pure dichloroacetate **6c** as a beige oil in 88% yield (0.12 g); ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.31 (s, 6H, 2ArC*H*$_3$), 3.76 (s, 3H, OC*H*$_3$), 3.77 (s, 3H, OC*H*$_3$), 3.95 (s, 3H, OC*H*$_3$), 5.96 (s, 1H, OCOC*H*Cl_2), 6.80 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.32 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.50 (s, 2H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.2 ($2CH_3$), 56.3 (CH_3), 59.7 (CH_3), 61.8 (CH_3), 64.2 (CH), 105.8 (CH), 120.9 (CH), 125.9 (C), 130.9 (2C), 131.1 (2CH), 133.4 (C), 138.3 (C), 145.8 (C), 149.8 (C), 154.8 (C), 161.2 (C), 195.2 (C). Elem. Analysis calcd. for $C_{20}H_{20}Cl_2O_6$: C, 56.22; H, 4.72. Found: C, 56.45; H, 5.00%.

*4.1.6.7. 3-Methoxy-6-(2,4,5-trifluoro-3-methoxybenzoyl)1,2-phenylene bis(2,2-dichloroacetate) (**6d**).* General procedure C was followed by using benzophenone **3o** (0.5 g, 1.52 mmol) and monochloroacetic acid chloride (0.88 mL, 9.14 mmol). The crude obtained after final work-up was purified by flash chromatography (eluent: EtOAc/*n*-heptane 6/4) to provide pure bisdichloroacetate **6d** as a beige solid in 63% yield (0.53 g); mp (EtOAc/*n*-heptane) 107-109 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 3.97 (s, 3H, OC*H*$_3$), 4.05 (t, *J* = 1.1 Hz, 3H, OC*H*$_3$), 6.15 (s, 1H, OCOC*H*Cl_2), 6.20 (s, 1H, OCOC*H*Cl_2), 6.99 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.12 (sym m, *J* = 8.6, 6.2 Hz, 1H, Ar*H*), 7.54 (dd, *J* = 9.0, 2.0 Hz, 1H, Ar*H*). ^{19}F NMR ($CDCl_3$, 376 MHz) – 143.84 (sym m, 1F, Ar*F*), - 138.63 (sym m, 1F, Ar*F*), -130.22 (sym m, 1F, Ar*F*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 56.8 (CH_3), 62.2 (CH_3), 63.3 (CH), 63.5 (CH), 109.8 (CH), 110.9 (dd, *J* = 19.8, 2.8 Hz, CH), 122.1 (sym m, C), 123.5 (C), 130.9 (CH), 131.8 (C), 142.1 (C), 146.0 (sym m, C), 148.5 (sym m, C), 148.8 (sym m, C), 151.4 (sym m, C), 156.0 (C),

161.1 (C), 161.5 (C), 186.6 (C). IR ν (cm^{-1}): 1799, 1645, 1611, 1506, 1466, 1354, 1292, 1255, 1118, 1090, 1022, 935, 829, 771. Elem. Analysis calcd. for $C_{19}H_{11}Cl_4F_3O_7$: C, 41.49; H, 2.02. Found: C, 41.81; H, 2.29%.

*4.1.6.8. 2-Methoxy-5-(3,4,5-trimethoxybenzoyl)phenyl 2,2,2-trichloroacetate (**7a**).* General procedure C was followed by using phenstatine **3b** (0.5 g, 1.57 mmol) and trichloroacetic acid chloride (0.29 mL, 3.14 mmol). The oily resulting residue was crystallized from absolute ethanol to provide trichloroacetate **7a** as a grey solid; 84% yield (0.61 g); mp (Et_2O) 139-142 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 3.89 (s, 6H, 2OCH_3), 3.95 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 7.03 (s, 2H, Ar*H*), 7.10 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.71 (d, *J* = 1.9 Hz, 1H, Ar*H*), 7.82 (dd, *J* = 8.6, 1.9 Hz, 1H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 56.3 ($2CH_3$), 56.4 (CH_3), 61.0 (CH_3), 89.4 (C), 107.4 (2CH), 111.9 (CH), 124.1 (CH), 130.5 (C), 130.9 (CH), 132.5 (C), 138.9 (C), 141.9 (C), 153.0 (2C), 154.3 (C), 160.1 (C), 193.4 (C). IR ν (cm^{-1}): 1782, 1650, 1607, 1583, 1503, 1414, 1332, 1276, 1130, 1178, 1016, 970, 843, 763, 679. Elem. Analysis calcd. for $C_{19}H_{17}Cl_3O_7$: C, 49.22; H, 3.70. Found: C, 49.63; H, 4.01%.

*4.1.6.9. 2-Methoxy-5-(4-methoxy-3,5-dimethylbenzoyl)phenyl 2,2,2-trichloroacetate (**7b**).* General procedure C was followed by using benzophenone **3h** (0.5 g, 1.75 mmol) and trichloroacetic acid chloride (0.39 mL, 3.50 mmol). The crude obtained after final work-up was purified by flash chromatography (eluent: EtOAc/*n*-heptane 6/4) to provide trichloroacetate **7b** as a beige oil; 98% yield (0.74 g); ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.32 (s, 6H, 2CH_3), 3.80 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 7.17 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.46 (s, 2H, Ar*H*), 7.72 (d, *J* = 2.0 Hz, 1H, Ar*H*), 7.80 (dd, *J* = 8.6, 2.0 Hz, 1H, Ar*H*). Elem. Analysis calcd. for $C_{19}H_{17}Cl_3O_5$: C, 52.86; H, 3.97. Found: C, 53.09; H, 4.27%.

*4.1.6.10. 2,2-Dichloro-N-(2,2-dichloro-acetyl)-N-[2,6-dimethoxy-3-(4-methoxy-3,5-dimethyl-benzoyl)-phenyl]-acetamide (**8**).* General procedure C was followed by using benzophenone **3l** (0.3 g, 0.95 mmol) and dichloroacetic acid chloride (0.55 mL, 5.7 mmol). The oily resulting residue was crystallized from absolute ethanol to provide benzophenone **8** as a white solid; 98% yield (0.50 g); mp (EtOAc/*n*-heptane) 129-131 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.32 (s, 6H, 2CH_3), 3.57 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.62 (s, 2H, 2COC*H*Cl_2), 6.83 (d, *J* = 9.0 Hz, 1H, Ar*H*), 7.50 (s, 2H, Ar*H*), 7.55 (d, *J* = 9.0 Hz, 1H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.3 ($2CH_3$), 56.6 (CH_3), 59.7 (CH_3), 62.4 (CH_3), 65.8 (CH), 106.4 (CH), 117.1 (C), 125.0 (C), 131.0 (2CH), 131.5 (2C), 132.6 (C), 133.7 (CH), 156.0 (C), 157.5 (C), 161.9 (C), 166.0 (C), 193.5 (C). Elem. Analysis calcd. for $C_{22}H_{21}Cl_4O_6N$: C, 49.19; H, 3.94. Found: C, 49.50; H, 4.15%.

*4.1.7. 2-Methoxy-5-(3,4,5-trimethoxybenzoyl)phenyl acetate (**3x**).* A mixture of phenstatin **3b** and acetyl chloride was heated at reflux during 3h. After cooling to RT, the reaction medium was diluted with dichloromethane and carefully poured into a separatory funnel containing aqueous sodium bicarbonate solution (10% $NaHCO_3$). The aqueous solution was extracted with dichloromethane and the combined organic layers were dried ($MgSO_4$). Solvent was removed under reduced pressure to produce a brownish oil. The crude product was purified by column chromatography on silica gel (EtOAc/n-heptane 7/3) to provide pure benzophenone **3x** as a white solid; 87% yield (0.52 g); mp (Et_2O) 160-162 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ (ppm) 2.34 (s, 3H, OCOCH_3), 3.89 (s, 6H, 2OCH_3), 3.93 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 7.04 (s, 2H, Ar*H*), 7.05 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.58 (d, *J* = 2.0 Hz, 1H, Ar*H*), 7.76 (dd, *J* = 8.6, 2.0 Hz, 1H, Ar*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.7 (CH_3), 56.1 (CH_3), 56.3 ($2CH_3$), 61.0 (CH_3), 107.4 (2CH), 111.5 (CH), 125.3 (CH), 129.8 (CH), 130.3 (C), 132.8 (C), 139.2 (C), 141.7 (C), 152.9 (2C), 154.8 (C), 168.9 (C), 193.8 (C). IR ν (cm^{-1}): 1769, 1643, 1603, 1579, 1501, 1411, 1321, 1271, 1205, 1113, 1012, 910, 869, 760, 638.

4.2. Human pyruvate dehydrogenase kinase (PDHK1, 2 and 4) enzymatic radiometric assay [k_m ATP], KinaseProfiler

PDHK1, 2 or 4 (h) is incubated with 8 mM MOPS pH 7.0, 0.2 mM EDTA, 250 uM KKKYHGHSMSDPGVSYRT, 10 mM Mg Acetate and [gamma-^{33}P-ATP] (specific activity and concentration as required). The reaction is initiated by the addition of the Mg/ATP mix. After incubation for 40 minutes at room temperature, the

reaction is stopped by the addition of phosphoric acid to a concentration of 0.5%. 10 µL of the reaction is then spotted onto a P30 filtermat and washed four times for 4 minutes in 0.425% phosphoric acid and once in methanol prior to drying and scintillation counting. (*source*: human recombinant; *substrate*: 250 µM KKKYHGHSMSDPG; *incubation*: 40 min at rt; *tracer*: ^{33}P; *detection method*: radiometric; *measured response*: scintillation).

4.3. Tubulin polymerization assay

Sheep brain tubulin was purified according to the method of Shelanski [21] by two cycles assembly–disassembly and then dissolved in the assembly buffer containing 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, and 1 mM of GTP (pH 6.6) to give a tubulin concentration of about 2–3 mg/mL. Tubulin assembly was monitored by fluorescence according to reported procedure [22] using DAPI as fluorescent molecule. Assays were realized on 96-well plates prepared with Biomek NKMC and Biomek 3000 from Beckman coulter and read at 37 °C on Wallac Victor fluorimeter from Perkin–Elmer. The IC_{50} value of each compound was determined as tubulin polymerization inhibition by 50% compared to the rate in the absence of compound. The IC_{50} values for all compounds were compared to the IC_{50} of phenstatin and desoxypodophyllotoxin and measured the same day under the same conditions.

4.4. Analysis of cell cycle

The impact of selected molecules on cell cycle of murine leukemia DA1-3b cells was evaluated after fixation overnight at 4°C with 70% ice-cold ethanol/phosphate-buffered saline (PBS) followed by staining with a solution containing PI (50 µg/mL) and RNase A (0.5 mg/mL), and analyzed with a Becton Dickinson FACScan cytofluorometer. DA1-3b cells were incubated for 24 h in presence of chloroacetyl-thiophenstatin **4a** and thiophenstatin **4b**.

4.5. Cell proliferation assay

The compounds were tested against a panel of 60 human cancer cell lines at the National Cancer Institute, Germantown, MD, USA [23]. The cytotoxicity studies were conducted using a 48 h exposure protocol using the sulforhodamine B assay [24].

Acknowledgments

The authors gratefully acknowledge the National Cancer Institute (NCI) for the biological evaluation of compounds on their 60-cell panel: the testing was performed by the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis (the URL to the Program's website: http://dtp.cancer.gov).

Conflict of interest

The authors state no conflict of interest.

Supplementary data

Full description and graphs from NCI 60 cell one-dose and 5-dose screen and results of cell cycle distribution for dichloroacetic acid (DCA) on DA1-3b cells are available in the 'Supplementary information' section.

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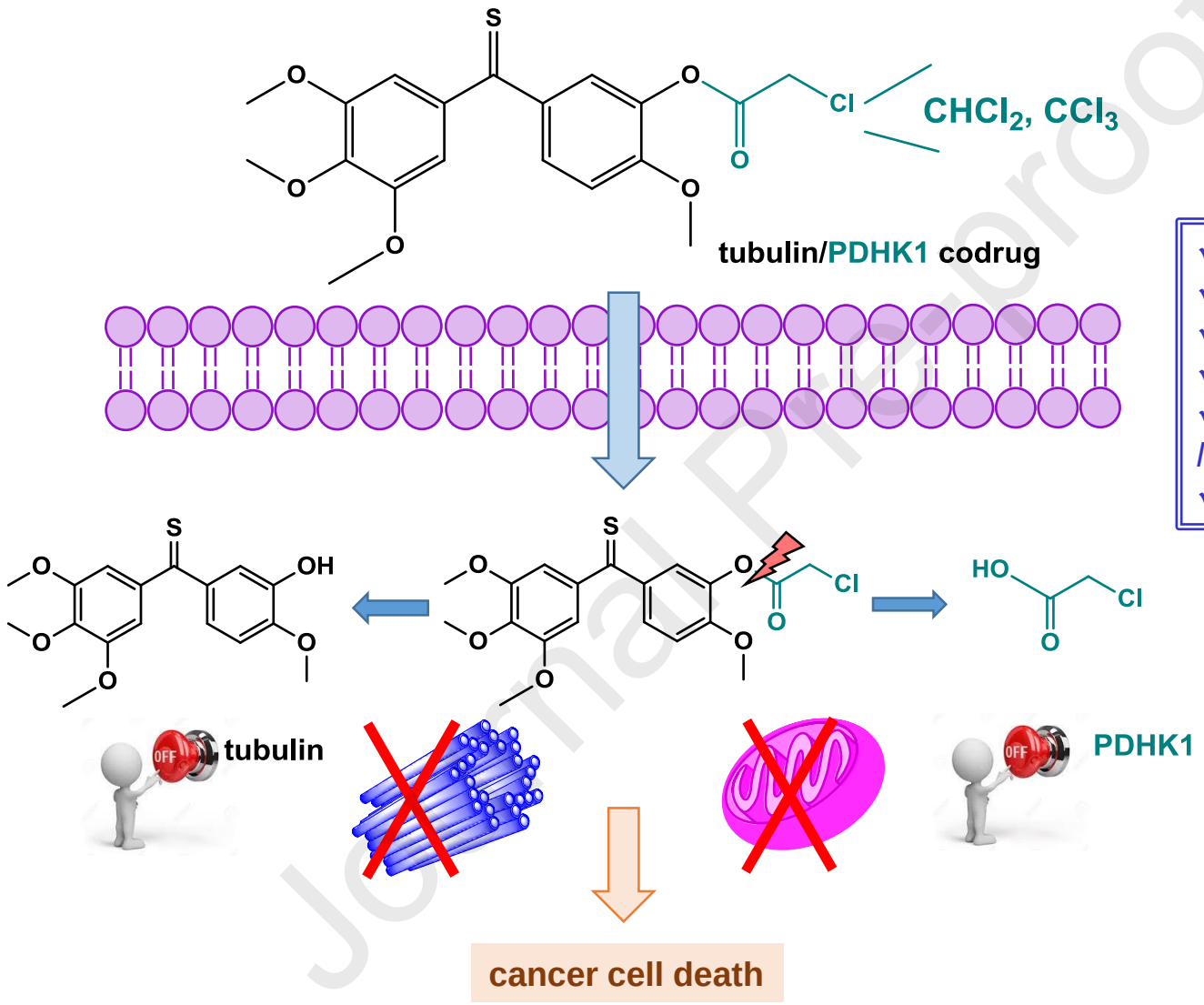

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Highlights

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: