Synthesis and Characterization of New Phthalhydrazothiazole Derivatives: A Preliminary Investigation on Their Activity against Hepatocellular Carcinoma

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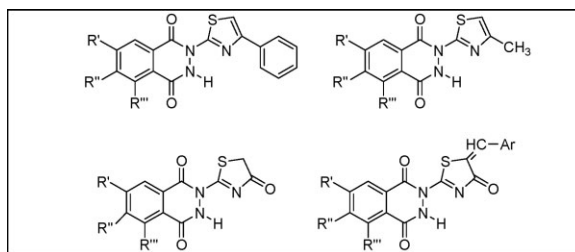[a]Dipartimento Farmaco Chimico Tecnologico, Via Ospedale, Cagliari 09124, Italy
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The synthesis of new 2-(4-substituted thiazol-2-yl)-2,3-dihydrophthalazine-1,4-diones, 2-(4-oxo-4,5-dihydro-thiazol-2-yl)-2,3-dihydrophthalazine-1,4-diones, and 2-(5-arylidene-4-oxo-4,5-dihydrothiazol-2-yl)-2,3-dihy-drophthalazine-1,4-diones is reported. The introduction of different substituents on the phthalazine, the thiazole and the thiazolinone has been studied. The new compounds have been characterized and evaluated for their antiproliferative activity against hepatocellular carcinoma, one of the most lethal tumors. The activity shown by some of these compounds towards liver tumor cells is encouraging.

INTRODUCTION

Thiazoles play a prominent role in nature and have broad applications in agricultural and medicinal chemistry. As a matter of fact, thiazole derivatives show antitumor [1,2], anti-hypertensive [3], anti-inflammatory [4,5], anti-hyperlipidemic [6] and other biological properties [7,8].

Phthalazine derivatives, similarly to other members of the isomeric diazine series, have found wide application as therapeutic agents [9–21]. The synthesis and biological evaluation of semicarbazide derivatives that exhibit anticancer activity has been reported [22–24].

Moreover, a number of molecules bearing a tridentate ligand system, structurally related to thiazolophthalazines and thiazolinonephthalazines [25–32], show an inhibitory activity towards tumors; it has been shown that complexation of these compounds results in derivatives that are potent cytotoxic agents [33].

Following our interest in the synthesis and biological activity of heterocyclic compounds [34–39], we have synthesized several new 2-(4-substituted thiazol-2-yl)-2,3-dihydrophthalazine-1,4-diones, 2-(4-oxo-4,5-dihydro-thiazol-2-yl)-2,3-dihydrophthalazine-1,4-diones, and 2-(5-arylidene-4-oxo-4,5-dihydrothiazol-2-yl)-2,3-dihydro-phthalazine-1,4-diones, in order to assess their capability of inhibiting tumor cell growth, and in particular their activity against hepatocellular carcinoma.

Hepatocellular carcinoma is one of the five most common cancers worldwide; it is one of the most common cancers in males in the world; it has an annual incidence worldwide of more than 500,000 cases and is very poorly treated with survival rates of only 23% at one year and less than 5% at five years [40].

According to literature data, the highest diffusion is observed in China and eastern Asia, middle Africa and some countries of western Africa, while lower incidence is evident in Japan, Europe, and America. Nevertheless a rising trend is observed, due to the increasing of HCV infection [41]. The prognosis is generally poor, especially in the African and Chinese population, where survival time may be as short as eleven weeks from the onset of symptoms.

In this article, we investigate a straightforward and efficient synthesis of compounds containing thiazole and phthalazine moieties in the same molecule, as we consider this aspect particularly interesting.

RESULTS AND DISCUSSION

Several 2-(4-substituted thiazol-2-yl)-2,3-dihydroph-tha-lazine-1,4-diones, 2-(4-oxo-4,5-dihydrothiazol-2-yl)-2,3-dihydrophthalazine-1,4-diones, and 2-(5-arylidene-4-oxo-4,5-dihydrothiazol-2-yl)-2,3-dihydrophthalazine-1,4-

Scheme 1
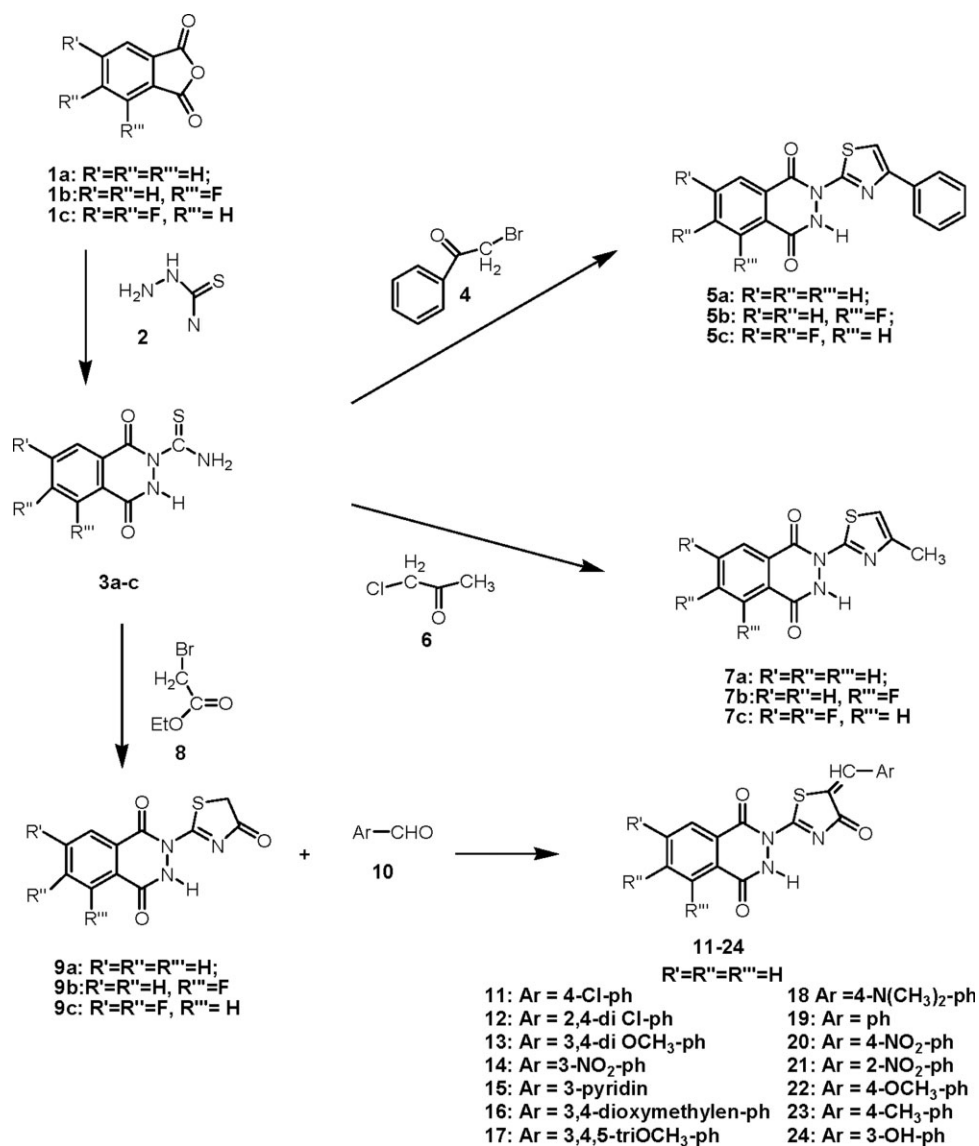
diones have been synthesized and their capability of inhibiting tumor cell growth investigated.

The first step in the synthetic pathway (Scheme 1) consists of the reaction of equimolecular amounts of phthalic anhydrides **1a–c** with thiosemicarbazide **2** in isopropanol, in the presence of catalytic amounts of acetic acid. By this method [39], 2-carbothioamidophthalazines **3a–c** can be prepared easily.

Compounds **3a–c** are then reacted either with α-halogen ketones or with α-halogen esters to form the substituted thiazole ring derivatives (**5a–c**, **7a–c**), and the thiazolinonic derivatives **9a–c,** respectively.

In the case of compound **3b** and its derivatives two possible regioisomers can be obtained. Apparently, according to chromatographic and spectral data, only one of the two possible isomers is formed, but the exact structure has not been investigated at this stage of the study.

Compounds **11–24** were obtained by reacting compound **9a** with different aryl aldehydes in acetic acid and acetic anhydride. All the obtained compounds were purified by crystallization from an appropriate solvent, and were fully characterized with the aid of ^{1}H NMR spectroscopy, mass spectrometry, and elemental analysis.

Compounds **5b–c**, **7a–c**, **9a–c**, **11–18** have been evaluated for their anti-proliferative activity towards the *FaO Reuber hepatoma* cell line, which maintains the characteristics of hepatocytes, both *in vivo* and *in vitro*, and retains the ability to undergo apoptosis [42].

To demonstrate the most promising structures and substitutions for biological activity, we investigated

Table 1

Percentage of cell vitality with respect to the control (NRU assay).

Cp	Concentration (mM)					
	1 (mM)	0.5 (mM)	0.25 (mM)	0.1 (mM)	0.05 (mM)	0.01 (mM)
7a	94.83	94.83	96.43	98.57	100	100
9a	60.02	64.86	91.35	93.87	97.69	98.01
5b	42.68	94.73	100	100	100	100
7b	98.71	100	100	100	100	100
9b	100	100	100	100	100	100
5c	100	100	100	100	100	100
7c	65.20	69.12	82.03	91.70	99.77	100
9c	100	100	100	100	100	100
11	62.17[a]	42.83[a]	36.53	44.41	94.84	95.12
12	40.68	38.20	41.68	58.08	74.94	100
13	100[b]	88.83	90.29	94.33	100	100
14	100	100	100	100	100	100
15	100	100	100	100	100	100
16	67.18	100	100	100	100	100
17	45.86	71.93	84.30	98.76	95.98	100
18	95.36	94.12	89.87	91.11	89.95	88.55

[a] Poorly soluble at 1 mM e 0.5 mM.
[b] Precipitates at 1 mM.

three different scaffolds, namely 2-(4-phenylthiazol-2-yl)-2,3-dihydrophthalazine-1,4-diones, 2-(4-thiazolinone-2-yl)-2,3-dihydrophthalazine-1,4-diones, and 2-(4-arylidenthiazolinone-2-yl)-2,3-dihydrophthalazine-1,4-diones.

Some of the tested compounds (Table 1) show a fairly good cytotoxic activity against FaO cells in the NRU assay.

Compound **11**, 2-[5-(4-chlorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione shows the best activity with a 44% cell survival at a concentration of 0.1mM.

A similar behavior was observed in compound **12**, 2- [5-(2,4-dichlorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione, indicating that the presence of one or two chlorine atoms, in the 5-arylidene moiety, increases activity against hepatoma cells.

It should be observed on the other hand that introduction of different substituents in the same scaffold generally leads to a decrease in biological activity, as observed for compounds **14, 15,** and **18**, which have a 3-nitrobenzylidene-, a 3-pyridylene-, and a 4-dimethylaminobenzylidene in position 5 of the thiazolidinone moiety, respectively.

In the case of compounds **16** and **17**, good activity is observed only at relatively high concentrations.

Compound **9a**, which contains an unsubstituted thiazolinonic cycle, showed a moderate biological activity.

The substitution of the thiazolinonic ring with a 4-phenylthiazole or 4-methylthiazole leads to a moderate increase in biological activity. This is particularly evident for compounds **5b** and **7c**, where one or two fluo-

rine atoms are introduced in the phthalazine moiety, respectively.

Moreover, the presence of an opportunely functionalized aromatic moiety both in the thiazole and in the thiazolinone rings seems important for biological properties.

Thus activity is strongly influenced by the nature of the substituents, and the presence of halogen atoms both on the phthalazine and on 5-arylidenthiazolinone leads to an increase in activity.

These results give an indication towards the design of new and potentially more active compounds, and suggest that 2-(4-phenylthiazol-2-yl)-2,3-dihydrophthalazine-1,4-diones, 2-(4-thiazolinone-2-yl)-2,3-dihydrophthalazine-1,4-diones, and 2-(5-arylidenthiazolinone-2-yl)-2,3-dihydrophthalazine-1,4-diones could be considered promising scaffolds for cytotoxic compounds.

EXPERIMENTAL

Melting points were uncorrected and were determined on a Reichert Kofler thermopan apparatus. ^{1}H NMR spectra were recorded on a Bruker AMX (300 MHz) using tetramethylsilane (TMS) as internal standard (chemical shifts in δ values). Electron ionization (EI) mass spectra were obtained by a Fisons QMD 1000 mass spectrometer (70 eV, 200 μA, ion source temperature 200°C). The samples were introduced directly into the ion source. Elemental analyses were obtained on a Perkin-Elmer 240 B microanalyzer.

All synthesized compounds were purified by crystallization from an appropriate solvent (ethanol, ethanol/water or ethanol/acetic acid).

Compounds **3a–c** were prepared as reported in the literature [39].

General procedure for the synthesis of compounds 5a–c. The compounds **3a–c** (3 mmol) and 2-bromoacetophenone (3.5 mmol) in 100 mL of isopropanol suspension is refluxed, under vigorous stirring, until complete dissolution of reagents and for further 4 h. After cooling to room temperature, a solid is obtained, which is filtered off, washed with isopropyl ether several times, and dried.

The following listed compounds were synthesized using the same procedure.

2-(4-Phenylthiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione (5a). Whitish solid, m.p. 300°C. MS m/z = 321, yield = 70%. ^{1}H NMR (DMSO-*d*6): δ 7.36 (t, 1H, phth., *J* = 7.7); 7.47 (t, 2H, phenyl, *J* = 7.7); 7.90 (s, 1H, C5H-thiaz.); 7.96–8.05 (m, 4H, phth. + phenyl); 8.10 (d, 1H, phth., *J* = 7.3); 8.38 (d, 1H, phth., *J* = 7.3); 12.42 (s, 1H, NH, D2O-exch.). *Anal.* Calcd for C17H11N3O2S: C, 63.54; H, 3.45; N, 13.08. Found: C, 63.71; H, 3.43; N, 13.13.

5- (or 8-) Fluoro-2-(4-phenylthiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione (5b). Pale yellow solid, m.p. 210–211°C. MS m/z = 339, yield = 79%. ^{1}H NMR (DMSO-*d*6): δ 7.36 (t, 1H, phth., *J* = 7.7); 7.47 (t, 2H, phenyl, *J* = 7.7); 7.90 (s, 1H, C5H-thiaz.); 7.96–8.05 (m, 3H, phenyl); 8.10 (d, 1H, phth. *J* = 7.3); 8.38 (d, 1H, phth. *J* = 7.3); 12.42 (s, 1H, NH, D2O-exch.). *Anal.* Calcd for C17H10FN3O2S: C, 60.17; H, 2.97; N, 12.38. Found: C, 59.99; H, 2.96; N, 12.35.

6,7-Difluoro-2-(4-phenylthiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione (5c). Beige solid, m.p. 229–231°C. MS m/z = 357, yield = 72%. ^{1}H NMR (DMSO-*d*6): δ 7.42–7.44 (m, 1H, phth.); 7.51–7.55 (m, 2H, phenyl); 7.99 (s, 1H, C5H-thiaz.); 8.04–8.07 (m, 2H, phenyl + phth.); 8.14 (t, 1H, phenyl, *J* = 8.1); 8.42 (t, 1H, phenyl, *J* = 8.1); 12.82 (s, 1H, NH, D2O-exch.). *Anal.* Calcd for C17H9F2N3O2S: C, 57.14; H, 2.54; N, 11.76. Found: C, 56.99; H, 2.55; N, 11.75.

General procedure for the synthesis of compounds 7a–c. A suspension of compounds **3a–c** (2.71 mmol) and chloroacetone (3.25 mmol) in 140 mL of isopropanol is refluxed, under vigorous stirring, until complete dissolution of reagents and then for further 5 h. The mixture is allowed to cool down to room temperature thus obtaining a solid, which is filtered off, washed with isopropyl ether several times, and dried. The following listed compounds were synthesized using the same procedure.

2-(4-Methylthiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione (7a). Beige solid, m.p. 249–251°C. MS m/z = 259, yield = 52%. ^{1}H NMR (DMSO-*d*6): δ 2.38 (s, 3H, CH3); 7.08 (s, 1H, C5H-thiaz.); 7.95–8.08 (m, 3H, phth.); 8.37 (d, 1H, phth. *J* = 7.3); 12.61 (s, 1H, NH, D2O-exch.). *Anal.* Calcd for C12H9N3O2S: C, 55.59; H, 3.50; N, 16.21. Found: C, 55.80; H, 3.48; N, 16.15.

5- (or 8-) Fluoro-2-(4-methylthiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione (7b). Beige solid, m.p. 243–245°C. MS m/z = 277, yield = 89%. ^{1}H NMR (DMSO-*d*6): δ 2.13 (s, 3H, CH3); 6.61 (s, 1H, C5H-thiaz.); 7.76–7.96 (m, 2H, phth.); 7.98–8.03 (m, 1H, phth.); NH not detected. *Anal.* Calcd for C12H8FN3O2S: C, 51.98; H, 2.91; N, 15.15. Found: C, 52.19; H, 2.90; N, 15.11.

6,7-Difluoro-2-(4-methylthiazol-2-yl)-2,3-dihydrophtha-lazine-1,4-dione (7c). Beige solid, m.p. 254–258°C. MS m/z = 295, yield = 77%. ^{1}H NMR (DMSO-*d*6): δ 2.27 (s, 3H, CH3); 6.50 (s, 1H, C5H-thiaz.); 7.65 (dd, 1H, phth., *J* = 7.8, 2.4);

8.00 (dd, 1H, phth., *J* = 7.8, 2.7); 10.72 (s, 1H, NH, D2O-exch.). *Anal.* Calcd for C12H7F2N3O2S: C, 48.81; H, 2.39; N, 14.23. Found: C, 48.55; H, 2.41; N, 14.17.

General procedure for the synthesis of compounds 9a-c. A mixture of compounds **3a–c** (0.013 mol) and ethyl bromoacetate (0.016 mol) were suspended in isopropanol (120 mL) and refluxed for 2 h. A white product was isolated by filtration and crystallized from acetic acid. The following listed compounds have been synthesized using the same procedure.

2-(4-Oxo-4,5-dihydrothiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione (9a). White solid, m.p. 311–312°C. MS m/z = 261, yield = 93%. ^{1}H NMR (DMSO-*d*6): δ 4.10 (s, 2H, CH2-thiaz.); 7.83–7.91 (m, 4H, phth.); 12.52 (s, 1H, NH, D2O-exch.). *Anal.* Calcd for C11H7N3O3S: C, 50.57; H, 2.70; N, 16.08. Found: C, 50.78; H, 2.68; N, 16.11.

5- (or 8-) Fluoro-2-(4-oxo-4,5-dihydrothiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione (9b). White solid, m.p. 309°C d. MS m/z = 279, yield = 82%. ^{1}H NMR (DMSO-*d*6): δ 3.85 (s, 2H, CH2-thiaz.); 7.71–7.76 (m, 2H, phth.); 7.89–7.92 (m, 1H, phth.); 11.77 (s, 1H, NH, D2O-exch.). *Anal.* Calcd for C11H6FN3O3S: C, 47.31; H, 2.17; N, 15.05. Found: C, 47.52; H, 2.18; N, 14.99.

6,7-Difluoro-2-(4-oxo-4,5-dihydrothiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione (9c). Whitish solid, m.p. 298°C d. MS m/z = 297, yield = 84%. ^{1}H NMR (DMSO-*d*6): δ 3.97 (s, 2H, CH2-thiaz.); 8.15–8.26 (m, 2H, phth.); 11.88 (s, 1H, NH, D2O-exch.). *Anal.* Calcd for C11H5F2N3O3S: C, 44.45; H, 1.70; N, 14.14. Found: C, 44.21; H, 1.69; N, 14.18.

General procedure for the synthesis of 2-(5-arylidene-4-oxo-4,5-dihydrothiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione derivatives (11–24). These derivatives were prepared starting from 2-(4-oxo-4,5-dihydrothiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione (4 mmol) and the appropriate aryl aldehyde (4 mmol) in acetic acid (20 mL) and acetic anhydride (2 mL). The reaction mixture was stirred and heated to reflux for 30 min. A colored precipitate was obtained, which is filtered off and crystallized from the appropriate solvent. Compounds **11–24** were synthesized using this procedure.

2-[5-(4-Chlorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (11). Light yellow solid, m.p. 346–347°C d. MS m/z = 383–385, yield = 64%. ^{1}H NMR (DMSO-*d*6): δ 7.52 (d, 2H, aryl, *J* = 8.5); 7.58 (d, 2H, phth., *J* = 7.3); 7.76 (s, 1H, aryl—CH=); 7.90–7.97 (m, 4H, aryl + phth.); 11.80 (1H, s, NH, D2O-exch.). *Anal.* Calcd for C18H10ClN3O3S: C, 56.33; H, 2.62; N, 10.95. Found: C, 56.14; H, 2.64; N, 10.90.

2-[5-(2,4-Dichlorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (12). Orange solid, m.p. 345–347°C d. MS m/z = 417–420, yield = 54%. ^{1}H NMR (DMSO-*d*6): δ 7.52 (d, 2H, aryl, *J* = 8.5); 7.59 (d, 2H, phth., *J* = 7.3); 7.76 (s, 1H, aryl—CH=); 7.88–7.97 (m, 3H, aryl + phth.); 11.80 (1H, s, NH, D2O-exch.). *Anal.* Calcd for C18H9Cl2N3O3S: C, 51.69; H, 2.17; N, 10.05. Found: C, 51.92; H, 2.15; N, 10.10.

2-[5-(3,4-Dimethoxybenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (13). Yellow solid, m.p. 308–311°C d. MS m/z = 409, yield = 68%. ^{1}H NMR (DMSO-*d*6): δ 3.76 (s, 3H, OCH3); 3.78 (s, 3H, OCH3); 6.84 (d, 1H, aryl, *J* = 8.1); 7.07 (d, 1H, aryl, *J* = 8.1); 7.24 (s, 1H, aryl); 7.73 (s, 1H, aryl—CH=); 7.76–7.94 (m, 4H, phth.); 11.78 (s, 1H, NH, D2O-exch.). *Anal.* Calcd for C20H15N3O5S:

C, 58.67; H, 3.69; N, 10.26. Found: C, 58.55; H, 3.70; N, 10.30. *Anal*. Calcd for $C_{10}H_{10}OS$: C, 67.38; H, 5.65; S, 17.99. Found: C, 67.06; H, 5.75; S, 17.69.

2-[5-(3-Nitrobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (14). Mustard colored solid, m.p. 320–323°C d. MS m/z = 394, yield = 49%. ^{1}H NMR (DMSO-d_6): δ· 7.72 (t, 1H, aryl, J = 8.1); 7.88–7.96 (m, 6H, phth. +aryl━CH=); 8.24 (d, 1H, phth., J = 7.3); 8.45 (s, 1H, aryl); 11.78 (s, 1H, NH, D_2O-exch.). *Anal*. Calcd for $C_{18}H_{10}N_4O_5S$: C, 54.82; H, 2.56; N, 14.21. Found: C, 55.01; H, 2.54; N, 14.26.

2-[5-(3-Pyridinylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (15). Light yellow solid, m.p. 297–299°C d. MS m/z = 350, yield = 67%. ^{1}H NMR (DMSO-d_6): δ· 7.48 (t, 1H, pyr. J = 7.8); 7.80 (s, 1H, ━CH=aryl); 7.83–7.94 (m, 5H, phth. + pyr.); 8.58 (dd, 1H, pyr, J = 4.6, 1.9); 8.82 (d, 1H, pyr., J = 1.9); 11.78 (s, 1H, NH, D_2O-exch.). *Anal*. Calcd for $C_{17}H_{10}N_4O_3S$: C, 58.28; H, 2.88; N, 15.99. Found: C, 57.98; H, 2.90; N, 16.04.

2-[5-(3,4-Dioxymethylenbenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (16). Orange solid, m.p. 333°C d. MS m/z = 393, yield = 75%. ^{1}H NMR (DMSO-d_6): δ 6.09 (s, 2H, CH_2); 7.04 (d, 1H, aryl, J = 8.1); 7.13 (d, 1H, aryl, J = 8.1); 7.20 (s, 1H, aryl); 7.68 (s, 1H, ━CH=aryl); 7.85–7.93 (m, 4H, phth.); 12.25 (s, 1H, NH, D_2O-exch.). *Anal*. Calcd for $C_{19}H_{11}N_3O_5S$: C, 58.01; H, 2.82; N, 10.68. Found: C, 57.62; H, 2.80; N, 10.72.

2-[5-(3,4,5-Trimethoxybenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (17). Yellow solid, m.p. 320–322°C d. MS m/z = 439, yield = 56%. ^{1}H NMR (DMSO-d_6): δ· 3.70 (s, 3H, OCH_3); 3.76 (s, 3H, OCH_3); 3.78 (s, 3H, OCH_3); 6.85 (s, 1H, aryl); 6.97 (s, 1H, aryl); 7.73 (s, 1H, aryl━CH=); 7.85–7.92 (m, 4H, phth.); 12.22 (s, 1H, NH, D_2O-exch.). *Anal*. Calcd for $C_{21}H_{17}N_3O_6S$: C, 57.40; H, 3.90; N, 9.56. Found: C, 57.19; H, 3.88; N, 9.53.

2-[5-(4-Dimethylaminobenzylidene)-4-oxo-4,5-di-hydro-thiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (18). Yellow solid, m.p. 293–294°C d. MS m/z = 392, yield = 70%. ^{1}H NMR (DMSO-d_6): δ 3.37 (s, 6H, NCH_3); 6.74 (d, 2H, aryl, J = 8.1); 7.36 (d, 2H, aryl, J = 8.1); 7.62 (s, 1H, aryl━CH=); 7.89–7.92 (m, 4H, phth.); 11.78 (s, 1H, NH, D_2O-exch.). *Anal*. Calcd for $C_{20}H_{16}N_4O_3S$: C, 61.21; H, 4.11; N, 14.28. Found: C, 60.97; H, 4.09; N, 14.34.

2-(5-Benzylidene-4-oxo-4,5-dihydrothiazol-2-yl)-2,3-dihydrophthalazine-1,4-dione (19). Orange solid, m.p. 331–333°C d.MS m/z = 349, yield = 50%. ^{1}H NMR (DMSO-d_6): δ 7.50 (d, 2H, phenyl, J = 7.7); 7.60 (d, 2H, phth., J = 7.3); 7.80 (s, 1H, phenyl━CH=); 7.93–7.98 (m, 5H, phenyl + phth.); 11.79 (1H, s, NH, D_2O-exch.). *Anal*. Calcd for $C_{18}H_{11}N_3O_3S$: C, 61.88; H, 3.17; N, 12.03. Found: C, 62.12; H, 3.15; N, 11.98.

2-[5-(4-Nitrobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (20). Dark yellow solid, m.p. 345–348°C d. MS m/z = 394, yield = 60%. ^{1}H NMR (DMSO-d_6): δ 7.82 (d, 2H, aryl, J = 8.8); 7.87 (s, 1H, aryl━CH=); 7.88–7.98 (m, 4H, phth.); 8.26 (d, 2H, aryl, J = 8.8); 11.78 (s, 1H, NH, D_2O-exch.). *Anal*. Calcd for $C_{18}H_{10}N_4O_5S$: C, 54.82; H, 2.56; N, 14.21. Found: C, 54.57; H, 2.55; N, 14.17.

2-[5-(2-Nitrobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (21). Orange solid, m.p. 327°C

d. MS m/z = 394, yield = 37%. ^{1}H NMR (DMSO-d_6): δ· 7.84–8.09 (m, 9H, phth. +aryl━CH=); 12.27 (s, 1H, NH, D_2O-exch.). *Anal*. Calcd for $C_{18}H_{10}N_4O_5S$: C, 54.82; H, 2.56; N, 14.21. Found: C, 54.88; H, 2.55; N, 14.18.

2-[5-(4-methoxybenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (22). Beige solid, m.p. 301–304°C d. MS m/z = 379, yield = 50%. ^{1}H NMR (DMSO-d_6): δ· 3.86 (s, 3H, OCH_3); 7.02 (d, 2H, aryl, J = 8.4); 7.51 (d, 2H, aryl, J = 8.8); 7.70 (s, 1H, aryl━CH=); 7.86–7.96 (m, 4H, phth.); 11.78 (s, 1H, NH, D_2O-exch.). *Anal*. Calcd for $C_{19}H_{13}N_3O_4S$: C, 60.15; H, 3.45; N, 11.08. Found: C, 59.88; H, 3.47; N, 11.06.

2-[5-(4-Methylbenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione (23). Yellow solid, m.p. 331–333°C d. ^{1}H NMR (DMSO-d_6): δ· 2.32 (s, 3H, CH_3); 7.28 (d, 2H, aryl, J = 8.1); 7.45 (d, 2H, aryl, J = 8.1); 7.72 (s, 1H, aryl━CH=); 7.76–7.97 (m, 4H, phth.); 11.79 (s, 1H, NH, D_2O-exch.). *Anal*. Calcd for $C_{19}H_{13}N_3O_3S$: C, 62.80; H, 3.60; N, 11.56. Found: C, 63.11; H, 3.59; N, 11.60.

2-[5-(3-Hydroxybenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl]-2,3-dihydrophthalazine-1,4-dione. (24). Yellow solid, m.p. 354°C d. MS m/z = 365, yield = 42%. ^{1}H NMR (DMSO-d_6): δ· 7.22 (d, 1H, aryl, J = 7.7); 7.35 (s, 1H, aryl); 7.44–7.49 (d, 2H, aryl+aryl-OH, D_2O-exch.); 7.52 (t, 1H, aryl, J = 7.7); 7.75 (s, 1H, ━CH=aryl); 7.88–7.94 (m, 4H, phth.); 11.78 (s, 1H, NH, D_2O-exch.). *Anal*. Calcd for $C_{18}H_{11}N_3O_4S$: C, 59.17; H, 3.03; N, 11.50. Found: C, 58.97; H, 3.05; N, 11.45.

Biological assay. The FaO rat hepatoma cell line H-35 Reuber (ICLC ATL99001) was purchased from Interlab Line Collection (Servizio Biotecnologie, IST, Genoa, Italy). FaO cells were cultured in flasks (capacity 75 cm^2) containing 15 mL Dulbecco's modified Eagle's medium (DMEM plus Glutamax I, Invitrogen S.r.l., Milan, Italy) and then supplemented with 100 UI/mL penicillin, 0.1 mg/mL streptomycin and 10% foetal bovine serum (Mascia Brunelli, Milan, Italy). Cells were incubated at 37°C in 5% CO_2 and 95% O_2 atmosphere. All experiments were carried out 24 h after seeding. Before the assay, the cells were treated with a phosphate buffer (PBS) and the culture medium was substituted with DMEM without serum.

Cell vitality. The vitality of adhesive cells was determinated with the *Neutral Red Uptake* assay (NRU) as reported by Borenfreund and Puerner [43]. As far as the NRU assay is concerned, the Neutral Red solution (0.4% NR in H_2O) was diluted 1:80 with phosphate buffer (PBS) and incubated at 37°C overnight. Then the solution was centrifuged (2600 rpm) to remove micro crystals that are insoluble in coloring. Once treated, the culture medium was vacuumed and substituted with a NR solution. The cells were incubated in the NR solution at 37°C for 30 min to allow coloring incorporation of the lisosomes in the vital cells of the flask. The RN solution was removed, the cells were rapidly washed with 1% formaldehyde and a calcium chloride solution was added. To remove incorporated NR from vital cells, a mixture (solution) of 1% acetic acid in ethanol 50% was added. A sample collected from every well was read using a Perkin-Elmer, 540 nm spectrophotometer. The values obtained for the treated cells were expressed as percentages of the value obtained for the control cells. All experiments were repeated at lease three times and were made in triplicate.

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