SYNTHESIS AND CYTOTOXIC ACTIVITY OF 5-BENZYLIDENE-2-[(PYRIDINE-2-YLMETHYLENE)HYDRAZONO]-THIAZOLIDIN-4-ONE DERIVATIVES

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Circumvention of multidrug resistance is a new field of investigation in cancer chemotherapy, and safe and potent multidrug resistance inhibitors are needed for clinical use. 4-Thiazolidinone ring system is a core structure in various biological active compounds with a wide range of biological and pharmacological properties. As a part of our continuing research directed toward the structural development of 4-thiazolidinone as a multitemplate for lead discovery, we report here the synthesis and cytotoxic activity of a new isomeric series of 5-benzylidene-2-[(pyridine-2-ylmethylene)hydrazono]-thiazolidin-4-ones 4a-h.

The compounds 4a-h were obtained by Knoevenagel condensation of the 4-thiazolidone intermediates 3a-b (R= H; R= C₆H₅) with commercially available aromatic aldehydes (benzaldehyde, 4-nitrobenzaldehyde, 4-dimethylaminobenzaldehyde and 4-chlorobenzaldehyde). The reaction was carried out in ethanol at reflux with piperidine as catalyst. The chemical structures were established using IR, ¹H NMR and HRMS spectroscopy. All the compounds have been tested in vitro by MTT assay for their cytotoxicity against HEp-2 (mucoepidermoid carcinoma of larynx) cell lines.

The 5-benzaldheyde-4-thiazolidione analogues 4a-h were prepared with satisfactory yield, and characterized based on their physical, analytical and spectral data. IR spectra showed absorption band corresponding to the stretching mode ν(C=O) of the lactam group at about 1697-1715 cm⁻¹. In their ¹H NMR spectra, the introduction of 5-arylidene moiety was confirmed through the absence of the signal of 5-CH₂ protons of 3a-b and the presence of resonances assigned to the methine hydrogen appearing
as singlet (8.51-8.64 ppm). The in vitro cytotoxic activity showed that the compounds 4b and 4c were effective against the HEp-2 cell lines with IC₅₀ 0.7 μg/mL and 0.5 μg/mL, respectively, and thus were much more active than etoposide (IC₅₀= 6.10 μg/mL). The remaining compounds showed no significant antiproliferative activity even at 25 μg/mL.

Eight 5-benzylidene-2-[(pyridine-2-ylmethylene)hydrazono]-thiazolidin-4-ones 4a-h were synthetized and tested for their in vitro cytotoxic activity. Compounds 4b and 4c could be good starting points for developing better anticancer agents, because they were more effective than etoposide in HEp-2 cells. Modifications to improve the potency for these derivatives by structural diversification are currently in our laboratory.

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_Synthesis, Cytotoxic and Antimicrobial activities of 5-benzylidene-2-[(pyridine-2-ylmethylene)hydrazono]-thiazolidin-4-one Derivatives_

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**SUMMARY.** A novel series of 5-benzylidene-2-[(pyridine-2-ylmethylene)hydrazono]-thiazolidin-4-ones 3a-i has been synthesized. 2-[(Pyridine-2-ylmethylene)hydrazono]-thiazolidin-4-ones 2a-c were also obtained and used as intermediates to give the target compounds. The in vitro cytotoxic activity was evaluated for both series. The
findings obtained showed that the compounds 2a, 2b, 3b and 3c were effective against the HEp-2 cell lines with IC\textsubscript{50} in the 1.6 - 0.5 μg/mL range, whereas the compounds 2a (IC\textsubscript{50} = 3.6 μg/mL), 2b (IC\textsubscript{50} = 2.4 μg/mL) and 3f (IC\textsubscript{50} = 3.5 μg/mL) showed good inhibitory effects against HEp-2 and HT-29 cell lines. As complementary biological test, all 4-thiazolidinones were evaluated for antimicrobial activity against various bacteria and fungal species.

KEY WORDS: antimicrobial activity, antiproliferative activity, cytotoxicity, 4-thiazolidinone

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INTRODUCTION

Cancer involves a pathological breakdown in the processes which control cell proliferation, differentiation and death of particular cells. Worldwide, approximately 10 million people are diagnosed with cancer annually and more than 6 million die of the disease every year; currently, over 22 million people in the world are cancer patients.

Many cancers can be cured by surgery, radiotherapy or chemotherapy, especially if they are detected early. Multidrug resistance (MDR) is a major factor in the failure of many forms of chemotherapy. Actually, Multidrug resistance in cancer is a phenomenon in which administration of a single chemotherapeutic agent causes cross-resistance of cancer cells to a variety of therapies even with different mechanisms of action. Development of MDR against standard therapies is a major challenge in the treatment of cancer. Thus, circumvention of multidrug resistance is a new field of investigation in cancer chemotherapy, and safe and potent multidrug resistance inhibitors are needed for clinical use.
4-Thiazolidinones arouse great interest in medicinal chemistry, as numerous compounds that have this heterocycle in their structure show important biological activity, such as antimicrobial and antitumoral properties. Indeed, Zhou et al. reported several 4-thiazolidinones (Fig. 1a) that selectively killed both non-small cell lung cancer cell line H460 and its paclitaxel-resistant variant H460taxR at an IC50 between 0.21 and 2.93 μM while showing much less toxicity to normal human fibroblasts at concentrations up to 195 μM. The authors also revealed that the nitrogen substitution in the 4-thiazolidinone ring blocked cytoselective anticancer activity and although the R' site was lenient to substitution, the R'' site required an -NMe2 group at the 4-position for optimal activity.

Based on these facts, our research group has recently investigated the cytotoxic properties of the 4-thiazolidinone scaffold, utilizing a series of 5-benzylidene-2-[(pyridine-4-ylmethylene)hydrazono]-thiazolidin-4-ones (Fig. 1b). In particular, compound I (R= Ph, R1= NMe2) showed excellent anticancer activity against human lung carcinoma (NCI-H292) cell line with IC50= 1.38 μg/mL. This compound showed equipotent to methotrexate (IC50= 1.31 μg/mL) and 2-fold more potent than etoposide (IC50= 2.75 μg/mL) at inhibiting cancer cell growth.

As a part of our continuing research directed toward the structural development of 4-thiazolidinone as a multitemplate for lead discovery, we report here the synthesis and cytotoxic activity of a new isomeric series of 5-benzylidene-2-[(pyridine-2-ylmethylene)hydrazono]-thiazolidin-4-ones 3a-i and their precursors 2-[(pyridine-2-ylmethylene)hydrazono]-thiazolidin-4-ones 2a-c (Fig. 2). As complementary work, all 4-thiazolidinones were evaluated for antimicrobial activity against various bacteria and fungal species.

**MATERIALS AND METHODS**

**Synthesis**

The synthesis of the thiosemicarbazone derivatives (1a-c) are reported in recent work by our research group. Compounds 2a-c and 3a-i were synthesized according to the synthetic pathway described in Scheme 1. The synthesis of 2-[(pyridine-2-ylmethylene)hydrazono]-thiazolidin-4-ones 2a-c was performed by condensation of 2-formylpyridine thiosemicarbazones 1a-c with 2-chloroacetic acid in the presence of sodium acetate anhydrous in ethanol, and isolation in good yield ranging from 67 to 82%. The final compounds 5-arylidene-4-
thiazolidinones 3a-i were obtained by Knoevenagel condensation of the previous products with commercially available aromatic aldehydes (benzaldehyde, 4-dimethylaminobenzaldehyde and 4-nitrobenzaldehyde) in yields varying from 44 to 65%. The reaction was carried out in ethanol at reflux with piperidine as catalyst. All compounds were identified by IR, HRMS and NMR spectroscopy.

**Materials and Instruments**

The melting points were determined on BÜCHI-535 apparatus and are uncorrected. IR spectra were measured on BRUKER IFS-66 IR spectrophotometer. HRMS were recorded on Varian MAT 711 spectrometer 70 eV electron impact. NMR were recorded on UNITY PLUS-300 MHz-VARIAN spectrometer by using tetramethylsilane as internal standard. The chemical shifts are reported in δ units, and coupling constants (J) are reported in hertz. TLC development was conducted on 0.25 mm silica gel plates (60F254, Merck).

**General synthetic procedure process for compounds 2a-c**

A solution of 4.54 mmol of thiosemicarbazones 1a-c, 4.54 mmol of 2-chloroacetic acid, and 6.9 mmol of sodium acetate anhydrous in 25 mL of ethanol was stirred until reflux till the completion of the reaction (8-24 h). After, the solution was cooled to 0 °C, and the precipitate was collected with filter under vacuum and washed with hot methanol and water.

**General synthetic procedure process for compounds 3a-i**

A solution of 1.36 mmol of 4-thiazolidinones 2a-c, 1.36 mmol of aldehyde aromatic, and two drops of piperidine in 10 mL of ethanol was stirred until reflux till the completion of the reaction (4-10 h). After, the solution was cooled to 0 °C, and the precipitate was collected with filter under vacuum and washed with hot ethanol and water.

**Cytotoxic activity**

The human larynx carcinoma (HEp-2) and the Human colon adenocarcinoma (HT-29) cell lines were purchased from the Adolfo Lutz Institute, São Paulo, Brazil. A DMEM (Dulbecco’s Modified Eagle’s Medium), enriched with 10% of fetal bovine serum, 1% of L-glutamine and 1% of antibiotics (penicillin and streptomycin),
was used for cell cultivation and to perform the tests. The cytotoxic activity was investigated using the MTT assay (3-(4,5-dimethylthiazole-2-yl)-2,5- diphenyltetrazolium bromide). Cell suspensions were diluted to 105 cells/mL, suitably prepared and distributed in plates of culture with 96 wells (225 μL in each well), then incubated at 37 ºC in a humid atmosphere with 5% of CO₂. After 24 h, 25 μL of either the synthesized compounds or the reference drugs (doxorubicin and etoposide) was added to each well. The plates were incubated again at 37 ºC for 72 h. Then, 25 μL of MTT solution (5 mg/mL) was added to each well, and the mixture was incubated at 37 ºC for 2 h. At the end of this period, the culture medium with the MTT excess was aspirated and after that, 100 μL of DMSO was added to each well to dissolve the formazan crystals. The optical density (OD) of the wells was measured at 540 nm and compared to the control (cells with medium only). The data represent the mean of three experiments in triplicate and were expressed as means ± SD. IC₅₀ and their 95% confidence intervals were determined from nonlinear regression using the program SigmaPlot version 11. The IC₅₀ value was defined as the concentration at which 50% survival of cells was observed.

**Antimicrobial Activity**

Bacteria and fungal species used in the antimicrobial evaluation were obtained from Departamento de Antibióticos and Instituto de Micologia cultures collections, Universidade Federal de Pernambuco, Brazil. Namely, *Staphylococcus aureus* (UFPEDA 02), *Bacillus subtilis* (UFPEDA 86), *Escherichia coli* (UFPEDA 224), *Pseudomonas aeruginosa* (UFPEDA 416) *Klebsiella pneumoniae* (UFPEDA 396), *Mycobacterium smegmatis* (UFPEDA 71), *Candida albicans* (UFPEDA 1007), *Candida Krusei* (UFPEDA 1002), *Malassezia Furfur* (UFPEDA 1320), *Aspergillus Niger* (UFPEDA 2003), species. The antibacterial and antifungal activities are reported preliminary utilizing disc diffusion method. In this method, disks containing known amounts of an antimicrobial agent were placed on the surface of an agar plate that has been inoculated with a standardized suspension of microorganisms tested. Paper discs with only DMSO were used as negative controls. The MZI (Mean Zone Inhibition) for rifampicin (antibacterial), and nystatin (antifungal) was referred to as a reference value (mm). All experiments were carried out three times and repeated if the results differed. For MIC assays, a stock solution (1 mg/mL) of test compounds was prepared in DMSO solvent. Further, the serial dilution of test compounds was carried out and the concentrations used ranged from 10 for 1000 μg/mL. Test compounds at various concentrations were added to culture medium in a test tube and different strains were inoculated at 10⁸ bacteria/mL concentration. Tryptic Soy Agar was utilized for culture medium. The tubes were incubated at 37 ºC
for 24 h and then examined for the presence or absence of growth organisms tested. The MIC values were obtained from the lowest concentration of the test compounds where the tubes remained clear, indicating that the bacterial or fungal growth was completely inhibited at this concentration. The MIC values were expressed in μg/mL.

RESULTS AND DISCUSSION

The 4-thiazolidiones 2a-c and 3a-i were prepared with satisfactory yield, and characterized based on their physical, analytical and spectral data. The data for 2b and 3b are described below:

Data for 3-methyl-2-(2-(pyridin-2-ylmethylene)hydrazono)thiazolidin-4-one 2b: Reaction time: 24 h. Gray solid, 77%; mp 157-158 °C. Rf: 0.55 (ethyl acetate:hexane, 3:7). IR (KBr) cm⁻¹: n: 1717 (NC=O), 1620 and 1552 (C=N), 1298 (NCS), 1118 (N-N=C), 1055 (CS); ¹H NMR (400 MHz, DMSO-d₆, 400 MHz/ppm) δ: 8.43 (s, 1H, CH=N), 3.71 (s, 2H, S-CH₂), 3.23 (s, 3H, CH₃); pyridine ring: 8.58 (d, 1H, J = 8.0 Hz) 8.03 (d, 1H, J = 8.0 Hz), 7.70 (t, 1H, J = 8.0 Hz), 7.26 (t, 1H, J = 8.0 Hz); ¹³C NMR (DMSO-d₆, 100 MH /ppm): δ 172.06 (C=O), 166.14 (C=N), 32.42 (CH₂-S), 29.91 (CH₃); pyridine ring: 152.81 (Cq), 148.88, 137.09, 124.84, 121.73; HRMS (ES+): 257.0475 [M+Na]⁺ C₁₀H₁₀N₄OS requires 257.0458.

Data for 5-(4-(dimethylamino)benzylidene)-2-(2-(pyridine-2-ylmethylene)hydrazono)thiazolidin-4-one 3b: Reaction time: 14 h. Red solid; yield 56%; mp 255-257 °C. Rf: 0.6 (ethyl acetate:hexane, 1:4). IR (KBr) cm⁻¹: ν: 1694 (NC=O), 1625 and 1525 (C=N), 1583 (C=C), 1294 (NCS), 1214 (N-N=C), 1004 (CS); ¹H NMR (300 MHz DMSO-d₆) δ: 12.18 (s, 1H, NH), 8.40 (s, 1H, CH=N), 7.78 (s, 1H, HC=C), 7.45 (d, J = 8.0 Hz, 2H, Ar-H), 6.90 (d, J = 8.0 Hz, 2H, Ar-H), 3.05 (s, 6H, N(CH₃)₂); pyridine ring: 8.67 (d, J = 6.4 Hz, 1H), 8.07 (d, J = 6.4 Hz, 1H), 7.93 (t, J = 6.4 Hz, 1H), 7.64 (t, J = 6.4 Hz); HRMS (ES+): 374.1054 [M+Na]⁺ C₁₈H₁₅N₅OS requires 374.0801.

IR spectra of the compounds 2a-c revealed the presence of lactam C=O stretching bands and NCS bending vibration of the ring at 1709-1721 cm⁻¹ and 1292-1383 cm⁻¹, respectively. This is considered to be a strong confirmation of the thiazolidinone nucleus formation. The ¹H NMR spectra revealed the presence of a single signal at 3.71-3.98 ppm corresponding to methylene moiety of thiazolidinone nucleus. On the other hand, peaks resonated in the range 32.43-38.34, 165.77-167.20 and 171.69-174.04 ppm in the ¹³C NMR spectra were assigned for S–CH₂, C=N and C=O moieties.
IR spectra of 3a-i showed absorption band corresponding to the stretching mode $\nu$(C=O) of the lactam group at about 1694-1715 cm$^{-1}$. The exocyclic C=C bond was also assigned by means of the IR spectra, which showed a stretching band in the region of 1533-1599 cm$^{-1}$. In their $^1$H NMR spectra, the introduction of 5-arylidene moiety was confirmed through the absence of the signal of 5-CH$_2$ protons of 2a-c and the presence of resonances assigned to the methine hydrogen appearing as singlet (7.63-7.81 ppm). Unfortunately, the very low solubility of compounds 3a-i in usual solvents was a limitation for a valuable record of their $^{13}$C NMR spectra. HRMS data fully agree with the proposed structure.

Initially, the compounds 2a-c and 3a-i were evaluated for antiproliferative activity against the HEp-2 (mucoepidermoid carcinoma of larynx) and HT-29 (Human colon adenocarcinoma) cell lines. Among the twelve compounds tested, five drugs, named 2a, 2b, 3b, 3c and 3f, showed excellent to moderate cytotoxic effects (Table 1). The remaining seven compounds showed no significant antiproliferative activity even at 25 μg/mL. The known antitumor agents, doxorubicin and etoposide were included as reference compounds. The IC$_{50}$ values of 2a, 2b 3b and 3c against HEp-2 cell line were only in the 1.6 - 0.5 μg/mL range, and thus were much more active than etoposide (IC$_{50}$= 6.10 μg/mL). As for the relationship between cytotoxicity and the different substitution on the ring phenyl of the compounds 3b (IC$_{50}$= 0.7 μg/mL) and 3c (IC$_{50}$= 0.5 μg/mL), we did not observe a significant difference between electron-donating ((CH$_3$)$_2$N) and electron-withdrawing (NO$_2$) groups for the contribution to anticancer activity. In contrast, compound 3f (IC$_{50}$= 13.2 μg/mL) was 2-fold less active than reference drug. On the other hand, none of the compounds (IC$_{50}$= 20.4 – 2.4 μg/mL) were more effective than reference doxorubicin (IC$_{50}$= 0.4 μg/mL) in HT-29 cells, but compounds 2a, 2b and 3f showed good inhibitory effects with IC$_{50}$= 3.6 μg/mL, IC$_{50}$= 2.4 μg/mL and IC$_{50}$= 3.5 μg/mL, respectively.

After, the compounds 2a-c and 3a-i were also tested to antimicrobial activity by the disc diffusion method against various bacteria and fungal species. The mean zone inhibition (MZI) for rifampicin (antibacterial) and nystatin (antifungal) was referred to as a reference value (in mm). Except for compounds 2a and 2b, none of the tested compounds displayed good inhibition of the growth (MZI above or equal to 16 mm) of Gram positive and Gram negative bacteria, and fungi in relation to the reference drugs. The thiazolidinone 2a showed significant MZI for Mycobacterium smegmatis (20 mm) and for Candida albicans (17 mm), but this compound demonstrated higher values of MIC (>500 μg/mL and 62 μg/mL, respectively) when compared with rifampicin (MIC= 125 μg/mL) and nystatin (MIC= 50 μg/mL). Besides, the activity against C. albicans was found for compound 2b (16
mm), but this N-methyl-4-thiazolidinone derivative showed higher value of MIC (500 μg/mL) regarding the nystatin. It has been known that the introduction of arylidene moieties at 5 position of the 4-thiazolidinone ring enhanced the antimicrobial activity 20. As regards the relationships between the structure and the detected antimicrobial activity, the 5-benzylidene derivatives 3a–i did not show neither antibacterial nor antifungal efficacy.

**CONCLUSION**

Nine new 5-benzylidene-2-[(pyridine-2-ylmethylene)hydrazono]thiazolidin-4-ones 3a-i were synthetized and tested together with starting 2-[(Pyridine-2-ylmethylene)hydrazono]thiazolidin-4-ones 2a-c for their in vitro cytotoxic and antimicrobial activities. Compounds 2a, 2b, 3b and 3c could be good starting points for developing better anticancer agents, because they were more effective than etoposide in HEp-2 cells. Antimicrobial assessment indicated that compounds 2a against *M. smegmatis* and *C. albicans*, and 2b against *C. albicans* showed considerable antibiotic activity.

Relative to antimicrobial activity, except for compounds 2a and 2b, none of the 4-thiazolidinones prepared showed considerable antibiotic activity.

According to results of antimicrobial activity, only compounds 2a and 2b showed significant inhibition against *M. smegmatis* and *C. albicans*, and *C. albicans*, respectively, but demonstrated higher values of MIC when compared with standard drugs.

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**REFERENCES**


Figure 1. 4-Thiazolidinones with anticancer properties

I  \( R = \text{C}_6\text{H}_5, R_1 = \text{N}(\text{CH}_3)_2 \)
Figure 2. Reagents and conditions: (i) ClCH$_2$CO$_2$H, anhydrous AcONa, EtOH, reflux, 8-24 h (67-82%); (ii) ArCHO, EtOH, piperidine (cat.), reflux, 4-10 h (44-65%)
Table 1
Cytotoxic activity in tumor cells of some compounds 2 and 3 \(^a\)

<table>
<thead>
<tr>
<th>compound</th>
<th>Cell Lines IC(_{50}) (μg/mL)(^b)</th>
<th>HEP-2</th>
<th>HT-29</th>
</tr>
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<tbody>
<tr>
<td>2a</td>
<td>1.6</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>0.5</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>0.7</td>
<td>14.7</td>
<td></td>
</tr>
<tr>
<td>3c</td>
<td>0.5</td>
<td>20.4</td>
<td></td>
</tr>
<tr>
<td>3f</td>
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<td>3.5</td>
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</tr>
<tr>
<td>Doxorubicin</td>
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</tr>
<tr>
<td>Etoposide</td>
<td>6.10</td>
<td>nt</td>
<td></td>
</tr>
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</table>

\(^a\)The data represented the mean of three experiments in triplicate. \(^b\) IC\(_{50}\) and their 95% confidence intervals were determined from nonlinear regression using the program SigmaPlot version 11. The IC\(_{50}\) value was defined as the concentration at which 50% survival of cells was observed. nt = not tested.