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Comparative investigation into the anticancer activity of analogs of marine coelenterazine and coelenteramine

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ABSTRACT

Cancer is still one of the most challenging diseases to treat, making the pursuit for novel molecules with potential anticancer activity an important research topic. Herein, we have performed a comparative investigation into the anticancer activity of analogs of marine coelenterazine and coelenteramine. The former is a well-known bioluminescent substrate, while the latter is a metabolic product of the resulting bioluminescent reaction. While both types of analogs showed anticancer activity toward lung and gastric cancer cell lines, we have obtained data that highlight relevant differences between the activity of these two types of compounds. More specifically, we observed relevant differences in structure–activity relationships between these types of compounds. Also, coelenteramine analogs showed time-dependent activity, while coelenterazine-based compounds usually present time-independent activity. Coelenterazine analogs also appear to be relatively safer toward noncancer cells than coelenteramine analogs. There was also seen a correlation between the activity of the coelenterazine-based compounds and their light-emission properties. Thus, these results further indicate the potential of the marine coelenterazine chemi-/bioluminescent system as a source of new molecules with anticancer activity, while providing more insight into their modes of action.

1. Introduction

Cancer treatment is still considered one of the most challenging problems in modern medicine [1]. So, the development and use of new methods for the effective treatment of different types of cancer is a hot research topic [2,3]. Despite this, there is still the risk that patients cannot escape therapy failure and be subjected to serious side-effects [4–6]. Given this, researchers have been increasingly focused on the identification of new compounds with anticancer activity, to improve the survival rates and quality of life of cancer patients [7]. However, the development of new anticancer drugs is complex and with high failure rates, due to problems with toxicity or efficacy [8]. One of the reasons for this is that different compounds can lead to off-targets effects [8], and so, more detailed investigations at preclinical stages of the mode of action of new molecules with anticancer activity is essential [8,9].

In recent years, the members of this team have been active in the design, synthesis, and study of new molecules with anticancer activity based on the chemi-/bioluminescent system of marine coelenterazine [10–16]. Chemiluminescence consists in the emission of radiation that results from chemiexcitation due to a chemical reaction [17,18], while in bioluminescence the emission results from a biochemical reaction in living organisms [19,20]. Coelenterazine (Fig. 1) is one of the most studied chemi-/bioluminescent substrates and is present in many marine organisms [21–23]. Interestingly, coelenterazine is capable of both chemiluminescence (either in aprotic solvent or when triggered by reactive oxygen species) [24–26] and bioluminescence (as in the presence of luciferase enzymes) [23,27]. Despite this, both proceed *via* identical reaction mechanisms: first, the oxygenation of coelenterazine

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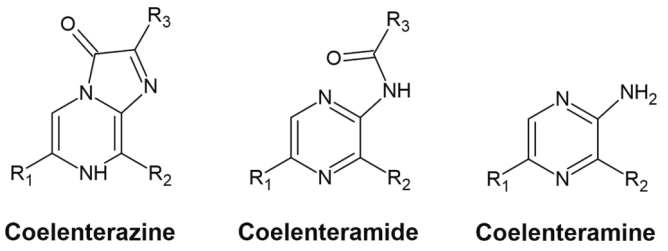

Fig. 1. Chemical structures of marine coelenterazine, coelenteramide and coelenteramine. R_1: phenol; R_2: benzyl; R_3: *p*-cresol.

to yield a high-energy peroxide intermediate; second, the rapid decomposition of the latter intermediate into the chemiexcited light-emitter, coelenteramide (Fig. 1) [28]. Another component of this system is coelenteramine (Fig. 1), which is both a metabolic product of the bioluminescent reaction and an intermediate of the chemical synthesis of coelenterazine [29–31].

More specifically, our group has been focused on the development of novel brominated coelenterazine analogs to be used in self-activating photodynamic therapy of cancer (PDT) [10–13,15]. PDT itself is a minimally invasive cancer treatment, in which a photosensitizer generates cytotoxic reactive oxygen species due to light irradiation [32,33]. While PDT is selective, the low penetration depth of light into tissues limits its use to more superficial tumors [34,35]. Given this, our objective has been to eliminate this restriction by removing the dependence of PDT regarding light irradiation, by modifying the coelenterazine system. Namely, the chemiluminescence of coelenterazine can be solely triggered by superoxide anion [25], which is overexpressed in cancer cells [36,37]. The addition of bromine heteroatoms to the structure of the analogs was performed to increase chemiexcitation to triplet states, instead of singlet excited states, due to the heavy-atom effect [38].

Direct chemiexcitation to triplet states is expected to allow the resulting chemiluminophores to interact with molecular oxygen and generate highly cytotoxic singlet oxygen [10–13,15]. Quite interestingly, several coelenterazine analogs showed relevant anticancer activity toward different cancer cell lines, such as prostate, breast, lung, gastric, and neuroblastoma [10–13,15]. Moreover, an interesting safety profile was also found, as no cytotoxicity was observed for corresponding noncancer breast cell lines [10]. Combining these analogs with chemotherapeutic drugs also leads to an increase in anticancer efficiency [11,15]. Thus, this type of coelenterazine analogs have shown significant potential to be used as a starting point for the development of light-free and improved PDT.

Nevertheless, we have also recently synthesized and evaluated a brominated analog of coelenteramine [13]. As this type of compound has an aminopyrazine core instead of an imidazopyrazinone one (Fig. 1), it is incapable of chemiluminescence, and so, it was not expected to possess anticancer activity. However, it was unexpectedly found that this coelenteramine analog (Fig. 2, **Clm-2**) showed higher anticancer activity, toward gastric and lung cancer, regarding both its corresponding coelenterazine version (Fig. 2, **Clz-2**) and other coelenterazine analogs [13]. Given the structural similarities between types of compounds, this finding could have repercussions in the development of coelenterazine analogs and the understanding of their mechanism of action. That is, if brominated coelenterazine and coelenteramine analogs share the same mode of action, the anticancer activity of the former could not result from a photodynamic effect (with implications in their use in light-free PDT). If, on the contrary, these two types of analogs possess different modes of action, it would mean that the bromination of different components of the coelenterazine system can produce compounds with different anticancer potential and targets. So, further understanding of the properties of these analogs is required.

More recent investigations indicated that brominated coelenterazine and coelenteramine analogs do possess different modes of action. For one, while **Clm-2** (Fig. 2) showed higher potency than **Clz-2** (Fig. 2) for lung and gastric cancer cell lines [13], the inverse was found for breast and prostate cancer [10,12,14]. In fact, **Clm-2** did not show any observed activity toward breast cancer [14]. Furthermore, while the activity of **Clm-2** is dependent on the 4-bromophenyl moiety (Fig. 2) and additional structural modifications impair its anticancer activity [13,14], the activity of coelenterazine analogs was found to be retained even with relevant structural modifications [10,11,13]. The mechanism of action of **Clm-2** was also recently related to targeting membrane lipids by synchrotron-radiation FTIR [16]. Nevertheless, this issue is still not fully characterized.

Herein, this work aims to further investigate the potential similarities and differences related to the anticancer modes of action of brominated coelenterazine and coelenteramine analogs (Fig. 2). To this end, four different coelenterazine – coelenteramine pairs of analogs (Fig. 2) were comparatively evaluated in terms of anticancer activity toward both gastric AGS and lung A549 cancer cell lines. Each compound of each pair is just differentiated in terms of possessing either an imidazopyrazinone or an aminopyrazine core while presenting the same structural modifications (Fig. 2). So, the analysis of these four pairs will help us to investigate the specific activities relative to each type of compound (coelenterazine or coelenteramine). Furthermore, the activity of each type of compound was linked to the presence of bromine heteroatoms [12,13], despite its presence by itself not being enough to induce cytotoxicity [15]. So, to further understand if the anticancer activity of these compounds can be further tuned by additional modifications with bromine heteroatoms, each pair of analogs was distinguished from each other by the position and/or the number of bromine heteroatoms (Fig. 2). This type of comparative investigation into the anticancer activity regarding both coelenterazine and coelenteramine was performed here for the first time.

2. Materials and methods

2.1. Chemical synthesis

The synthesis of both **Clm** and **Clz** compounds was performed by following synthetic routes already validated by our team [10–15,32]. More specifically, the syntheses of **Clm-2/3/4** and **Clz-2/4** are already described in [10–14,31]. An identical procedure was followed for both **Clz-1** and **Clz-3**, which is provided in detail in the Supplementary

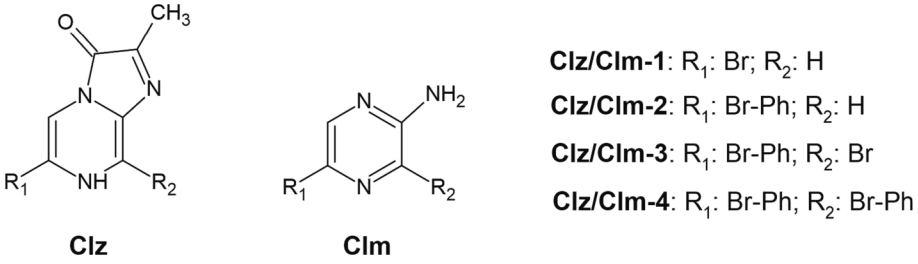

Fig. 2. Chemical structures of the studied coelenterazine (**Clz**) – coelenteramine (**Clm**) pairs of analogs (**Clz**/**Clm-1** to **Clz**/**Clm-4**). Br-Ph: 4-bromophenyl moiety.

Materials. In short, these **Clz** compounds were obtained through the formation of the imidazopyrazinone core by reacting the synthesis intermediates (the corresponding **Clm-1** and **Clm-3** structures) with methylglyoxal in acidic media, to afford **Clz-1** and **Clz-3**. The structural characterization of the final **Clz** compounds was performed by both ^{1}H NMR spectroscopy and FT-MS spectrometry, which spectra are provided in the Supplementary Materials. **Clm-1** is commercially available.

2.2. Cell culture

A549, AGS and MRC-5 cell lines were purchased from ATCC (Manassas, VA, USA) and maintained in DMEM + GlutaMAX™ (A549, AGS) or MEM + GlutaMAX™ (MRC-5) media with 1 % penicillin/streptomycin and 10 % FBS, at 37 °C, in a humidified atmosphere of 5 % CO_2.

2.3. Assessment of cellular viability – MTT assay

Cells were seeded in 96-well plates; human non-small cell lung cancer (A549) at a density of 10,000 cells/well at 24 h, 5,000 cells/well at 48 h, and 2,500 cells/well at 72 h; human gastric cancer cells (AGS) were seeded at a density of 15,000 cells/well at 24 h, 10,000 cells/well at 48 h, and 5,000 cells/well at 72 h; MRC-5 cells were seeded at 20,000 cells/well at 72 h and allowed to attach for 24 h under the conditions described above. After the respective incubation period, 0.5 mg/mL MTT solution was added and incubated for 2 h. The formazan in each well was dissolved in a solution of 3:1 DMSO:isopropanol. Lastly, the absorbance at 560 nm was read in a Thermo Scientific ™ Multiskan ™ GO microplate reader. Results were expressed as percentage of the respective control and correspond to the mean ± standard error of the mean (SEM) of at least three independent experiments performed in triplicate.

2.4. Luminometry

Chemiluminescent kinetic measurements were made with a homemade luminometer equipped with a Hamamatsu HC135-01 photomultiplier tube, in a setup that also includes a sample holder, an automatic burette, and a PC for data acquisition. The reactions were performed at room temperature at least in sextuplicate. Assays were performed in DMF containing 1 % of sodium acetate buffer solution, pH 5.2. Light was integrated and recorded at 0.1 s intervals, while final volumes of 500 mL were considered. Compound concentration of 2.5 μM was used for all experiments. Light was measured for at least 250 s.

2.5. Fluorescence spectroscopy

Fluorescence measurements were performed by using a Horiba Jovin Fluoromax 4 spectrofluorimeter (integration time of 0.1 s and slits of 2 nm) with quartz cuvettes. **Clz** compound concentrations of 5 μM were used in all experiments. The measurements were focused on shorter-term stability, which was assessed by measuring the initial spectra in different acidic buffer solutions at Day 1, followed by daily measurements in Days 2 and 3. Between measurements, the solutions were left at room temperature.

3. Results and discussion

3.1. Cytotoxicity toward lung and gastric cancer cells

The four pairs of **Clz** – **Clm** analogs were screened for their cytotoxicity towards both A549 (non-small cell lung) and AGS (gastric) cancer cell lines. Experiments were performed at increasing incubation periods: 24, 48 and 72 h. These experiments were performed with the same concentration of 100 μM for all compounds, as consistent with a previous study [13]. It should be noted that here we are not focused on the overall absolute anticancer efficiency of these specific molecules, but on their relative performance. That is, we are currently more focused on relative differences in activity between **Clm** and **Clz** types of analogs, as well as the general effect exerted by different number and/or position of bromine heteroatoms than on the quantitative anticancer activity of these eight molecules. This is explained by the fact that we are currently focused on trying to understand the general mechanism of these two classes of molecules, and how to potentially improve their activity, and not claim that these specific compounds already possess optimized anticancer properties.

In Fig. 3 are presented the results for A549 lung cancer cell line. Starting by analyzing the **Clm** molecules, we can see that they present a time-dependent cytotoxicity effect (increase of potency with increasing incubation time), which is consistent with previous results for **Clm-2** [13]. The less potent, while also being the simplest structure, **Clm** compound is **Clm-1**. At these conditions, is apparently devoid of toxicity at 24 and 48 h, while reducing the cellular viability to ~ 60 % at 72 h. Substituting the bromine heteroatom of **Clm-1** by a 4-bromophenyl moiety (yielding **Clm-2**, Fig. 2) increases the potency of these compounds, as the cellular viability decreased to ~ 50 % and ~ 30 %, at 24 h/48 h and 72 h, respectively. Increasing the number of bromine heteroatoms, by direct inclusion of one at the R_2 position (**Clm-3**, Fig. 2) has a negative effect on the potency of these compounds, as cellular viability is higher than ~ 60 % at 24 h and reaches ~ 35 % at 72 h, as consistent with previous results [13]. Substituting that bromine heteroatom at R_2 position by another 4-bromophenyl moiety (**Clm-4**, Fig. 2) has an even worse effect, which was observed also for breast and prostate cancer [14]. **Clm-4** appears to be devoid of toxicity at 24 h, while only decreasing the cellular viability to ~ 70 % at both 48 h and 72 h. In short, **Clm-2** is the most potent **Clm** analog here studied, which is consistent with previous studies [13,14]. This further highlights the importance of the 4-bromophenyl moiety in the R_1 position for the anticancer activity [13,14], while indicating that increasing the number of bromine heteroatoms does not help in increasing their activity.

As for the **Clz** analogs (Fig. 3), one interesting result that distinguishes them from **Clm** compounds, it is that their cytotoxicity is not always time-dependent. More specifically, **Clz-1** appears to essentially be inactive during the three incubation periods, with minor activity (ca. 20 %) at 48 h. The reduction in cellular viability caused by **Clz-2** is ~ 50 % at 48 and 72 h. **Clz-4** reduced the cellular viability to ~ 70/80 % in the considered incubation periods. The exception appears to be **Clz-3**. At **24 h**, the cellular viability was reduced to ~ 80 %, followed by further reduction to ~ 60 % at 48 h, and finally to ~ 35 % at 72 h.

As for the impact of the different substituents on the cytotoxicity of **Clz** analogs, we can see some similarities and differences regarding **Clm** compounds (Fig. 3). Regarding the similarities, **Clz-1** (as **Clm-1**) appears to be generally devoid of toxicity. Replacing the bromine heteroatom by the 4-bromophenyl moiety (yielding **Clz-2**, Fig. 2) also increases the potency of **Clz** compounds. However, the addition of a second bromine heteroatom (included in the R_2 position, yielding **Clz-3**, Fig. 2) further increases the potency of these compounds at 72 h (contrary to what was observed for **Clm** analogs). Nevertheless, replacing that bromine heteroatom by another 4-bromophenyl moiety (in the R_2 position, yielding **Clz-4**, Fig. 2) negatively affects the potency of **Clz** analogs.

So, we can already see some relevant differences between **Clz** and **Clm** compounds. For one, the cytotoxicity of compounds appears to be generally increased with incubation, while that of the latter appears to be more time-independent. The relationship between structure and activity also shows some differences between compounds. In fact, the most potent **Clz** and **Clm** analogs are not part of the same **Clz** – **Clm** pair. That is, the most potent **Clm** is single-brominated **Clm-2**, which presents a 4-bromophenyl moiety and a hydrogen atom at R_1 and R_2 positions, respectively. As for **Clz** analogs, the most potent one is double-brominated **Clz-3**, which shows a 4-bromophenyl moiety and a bromine heteroatom at R_1 and R_2 positions, respectively. Finally, it should also be highlighted some differences in the relative potency between **Clz** and **Clm** analogs in the different pairs. That is, at 72 h there

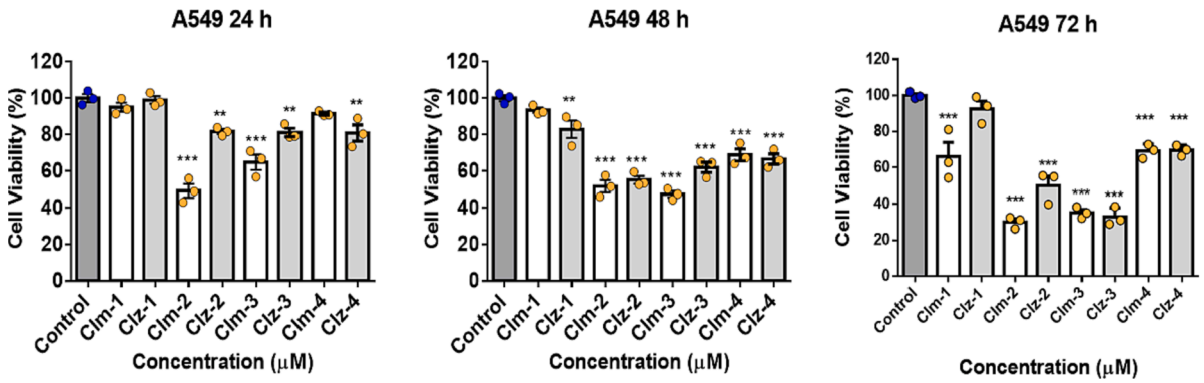

Fig. 3. Cytotoxicity evaluation of the eight **Clz** and **Clm** analogs, in A549 cells. A549 cells were treated with different compounds at the same concentration of 100 μM for 24, 48 and 72 h. Cell viability was assessed by MTT assay. Data are given as the mean ± SEM (n = 3). Statistical analysis was conducted between each treatment and the control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

are relevant differences between the cytotoxicity induced by **Clm** and **Clz** analogs in the **Clz-1**/**Clm-1** and **Clz-2**/**Clm-2** pairs, with **Clm** analogs presenting higher potency. However, no effective differences were seen for the **Clz-3**/**Clm-3** and **Clz-4**/**Clm-4** pairs.

The results regarding the cytotoxicity of **Clz** and **Clm** analogs toward AGS cancer cells are presented in Fig. 4. We can also see a time-dependent effect for **Clm** analogs, more specifically with both **Clm-2** and **Clm-3**. **Clm-2** is also the most potent **Clm** compound, followed by **Clm-3** (consistent with previous results [13]). By their turn, neither **Clm-1** nor **Clm-4** present relevant cytotoxicity. Also, the cytotoxicity presented by **Clm-2** and **Clm-3** appears to be generally in line with what was observed for A549 cells. We would also like to point out that there can be seen a slight increase in cellular viability (~50 % to 65 %) for AGS cells when treated with Clm-2 at 24 h and 48 h incubation, respectively. While this variation is not particularly pronounced, when can only hypothesize as to the reasons for such result. One hypothesis is that, putatively, cells may have initially engaged in a process of cell survival or rescue, as it is known to happen in some cases with autophagy after an insult, having such process subsequently failed and resulting in cells resuming the toxic effects of the molecule in higher time-points.

As for **Clz** analogs (Fig. 4), the most potent analog is still **Clz-3**, which contrasts with **Clm-2** as the most active **Clm** analog. This further demonstrates that the anticancer activity of these types of compounds do behave differently with structural modifications. In fact, the **Clz** pair of **Clm-2** (**Clz-2**) presents only very low activity toward AGS cancer cells. Moreover, only the cytotoxicity of **Clz-3** appears to be time-dependent, to the contrary of **Clz-1**, **Clz-2** and **Clz-4**. Nevertheless, it should be pointed out that both **Clz-1** and **Clz-4** appear to be devoid of cytotoxicity toward AGS cancer cells. In fact, the **Clz** analogs appear to be in general less active toward AGS cells than for A549 ones. We once again observe significant differences in the relative activity between members of **Clz** – **Clm** pairs. That is, while **Clz-2** appears to be relevantly less activity than **Clm-2** toward AGS cells, the efficiency of both **Clz-3** and **Clm-3** appears quite comparable. Finally, we also highlight that cells treated with Clz-1 at 24 h present average viability slightly higher than 100 %, never reaching statistical significance when compared to the control. This is a common finding, associated with the intrinsic variation that is known to take place when conducting several independent experiments using biological materials such as cells.

3.2. *Cytotoxicity toward noncancer fibroblast cells*

After analyzing the potential cytotoxicity of the studied **Clz** and **Clm** analogs toward cancer cells, it is also important to evaluate their effect on noncancer cells. To that end, we have then evaluated their cytotoxicity toward the noncancer MCR-5 fibroblast cell line, which results are presented in Fig. 5. Here we have focused on the incubation period of 72 h, at which the highest cellular viability reductions were obtained regarding cancer cells. We have also focused only on the **Clz-2**/**Clm-2** and **Clz-3**/**Clm-3** pairs, as they were the ones to consistently present cytotoxicity toward the studied cancer cell lines.

Results shown in Fig. 5 indicate that the four considered analogs possess cytotoxicity toward the studied noncancer cells, with variable degrees. Moreover, the type of analogs with the highest potency was once again that of **Clm** compounds. However, there was observed a change in relative potency, as now **Clm-3** is the most potent one, instead of **Clm-2**. Interestingly, **Clz-3** is still more potent than **Clz-2**, when considering **Clz** analogs. So, there appears to be some differences for **Clm** analogs in structure–activity relationship between noncancer and

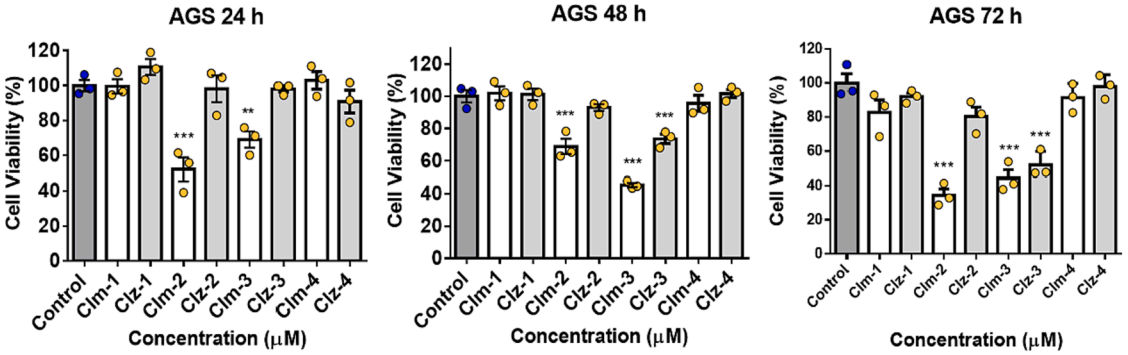

Fig. 4. Cytotoxicity evaluation of the eight **Clz** and **Clm** analogs, in AGS cells. AGS cells were treated with different compounds at the same concentration of 100 μM for 24, 48 and 72 h. Cell viability was assessed by MTT assay. Data are given as the mean ± SEM (n = 3). Statistical analysis was conducted between each treatment and the control. ** $p < 0.01$, *** $p < 0.001$.

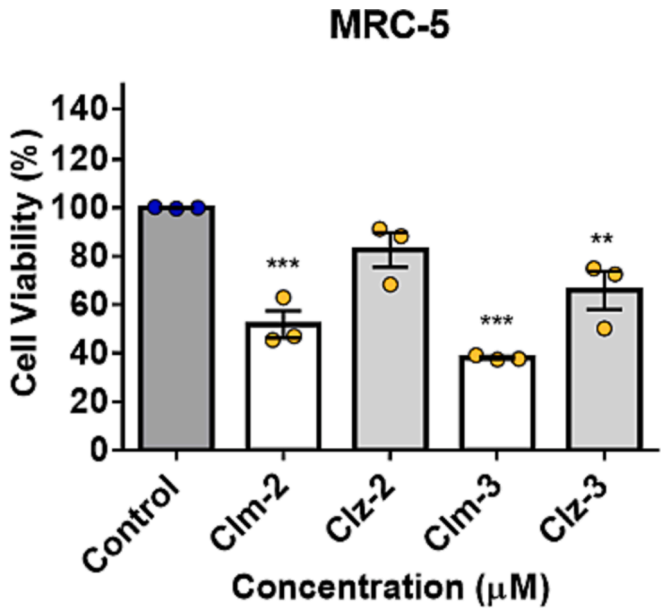

Fig. 5. Cytotoxicity evaluation of four **Clz** and **Clm** analogs, in MCR-5 cells. MCR-5 cells were treated with different compounds at the same concentration of 100 μM for 72 h. Cell viability was assessed by MTT assay. Data are given as the mean ± SEM (n = 3).

cancer cells, which are not seen for **Clz** analogs.

Contrary to what was observed for cancer cells, for MCR-5 cell lines both pairs presented relevant differences in efficiency between **Clz** and **Clm** analogs. That is, now **Clm-3** is capable of greater reductions in cellular viability than **Clz-3**, while both compounds showed similar potency toward AGS and A549 cell lines. **Clm-2** remains more active than **Clz-2**. Furthermore, **Clz** analogs appear to be safer for noncancer cells than **Clm** compounds. More specifically, the activity of **Clm-3** toward MCR-5 cells appears to be similar to what was observed toward A549 and AGS cells. **Clm-2** appears less active toward noncancer cells than for cancer ones but was still able to reduce the cellular viability of the former to ~ 50 %. However, **Clz-2** and **Clz-3** only reduced the cellular viability of MCR-5 cells to ~ 80 %/~70 % (Fig. 5), while reducing the viability of cancer cells down to ~ 50 %/~35 % (Figs. 3 and 4).

It should be noted that in a previous study, **Clm-2** only showed residual toxicity (~10 %) toward noncancer cells at 24 h [13], albeit a different noncancer cell line was used. The increased cytotoxicity here observed at 72 h (Fig. 5) can be a result of intrinsic differences across the noncancer cell line used or an indication that the activity of this compound towards noncancer cells is also time-dependent, as for cancer cells [13].

3.3. *Evaluation of the chemiluminescence of **Clz** analogs*

After analyzing the cytotoxicity of the eight studied analogs, toward both noncancer and cancer cells, the results highlight relevant differences between the anticancer activity of **Clz** and **Clm**. That is, we have observed relevant differences in activity between **Clz** and **Clm** compounds. For one, there are differences in the structure–activity relationships between the different types of compounds, as the addition of a second bromine heteroatom at the R_2 position (Fig. 2) decreases the anticancer activity of **Clm** analogs (Figs. 3 and 4, and consistent with [13,14]), while increasing that of **Clz** compounds (Figs. 3 and 4). This is in line with previous studies that indicate that the anticancer activity of **Clm** analogs is related to a 4-bromophenyl moiety at R_1 position, while showing a relevant structural flexibility for **Clz** analogs [10,11,13,14]. Furthermore, the cytotoxicity induced by **Clm** analogs appears to be generally time-dependent (Figs. 3 and 4) [13], while that of **Clz** compounds does not (Figs. 3 and 4). **Clz** analogs also appear to be relatively safer for noncancer cells than **Clm** compounds (Fig. 5). Finally, the relative efficiency between **Clm** analogs appears to be somewhat different for cancer and noncancer cells, while remaining the same for **Clz** analogs.

Thus, to consider different modes of action for **Clz** and **Clm** analogs, we return to our previous interpretation that the anticancer activity of the former is related with its chemiluminescent reaction [10–13]. As for **Clm** analogs, we refer to a recent study by us in which the anticancer activity of **Clm-2** was related to cellular lipids, by synchrotron-radiation FTIR, by affecting their organization and composition due to oxidative stress [16]. So, our focus here is now trying to understand if we can relate the anticancer activity shown by the present **Clz** analogs with their chemiluminescent properties, to further assess their potential mode of action.

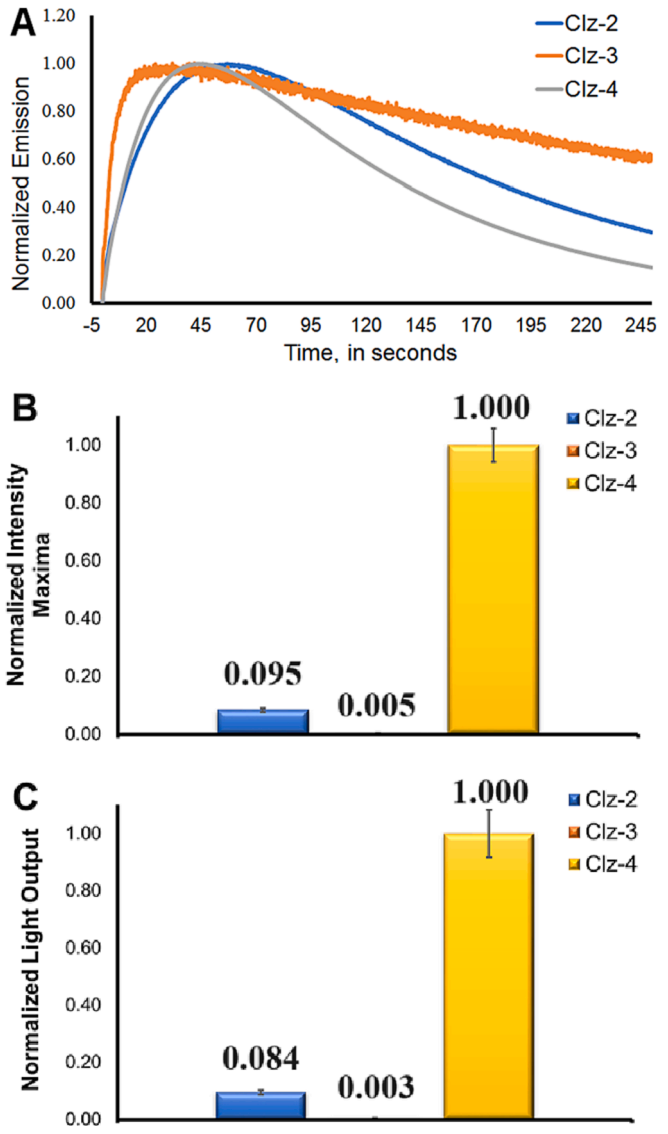

Fig. 6. Representative, and normalized, chemiluminescent kinetic profile for **Clz-2**, **Clz-3** and **Clz-4**, in DMF-sodium acetate buffer (a); Normalized, with **Clz-4** as reference, light-emission intensity maxima for **Clz-2**, **Clz-3,** and **Clz-4**, in DMF-sodium acetate buffer (b); Normalized, with **Clz-4** as reference, total light output (area) of the chemiluminescent reaction of **Clz-2**, **Clz-3** and **Clz-4**, in DMF-sodium acetate buffer (c). Total light output (area) was measured for a period of 250 s. In Fig.s B and C are presented the relative intensities/outputs for each compound, with **Clz-4** as reference.

Given this, we have measured the chemiluminescence of **Clz-2**, **Clz-3** and **Clz-4** (Fig. 6) in DMF – sodium acetate buffer pH 5.2 mixture. These assays were performed in aprotic polar solvent, in which coelenterazine and other imidazopyrazinones can undergo a chemiluminescent reaction by interacting with dissolved molecular oxygen [10,26,39]. Addition of acidic buffer is also common in the measurement of the chemiluminescence of this type of compounds [15,40,41]. We have focused here on the measurement of the chemiluminescence of **Clz-2**/**Clz-3**, as the compounds that showed anticancer activity (Figs. 3 and 4), and **Clz-4**, as a negative control (due to residual/absent activity). It should be further remembered that **Clz-2** has already presented anticancer activity toward other tumor types, such as breast and prostate [10,12].

Fig. 6A presents the representative and normalized, chemiluminescent kinetic profiles for the three analogs. It is observed for the three **Clz** the quick burst of light to light-intensity maximum, followed by decay, which is typical for coelenterazine and other imidazopyrazinones [15,42]. However, there appear to be relevant differences in kinetics between **Clz** compounds. While **Clz-2** and **Clz-4** appear to share similar kinetic profiles, with a quicker decay, **Clz-3** shows a slower decay to basal levels. Among the former, **Clz-4** shows the faster decay. Finally, the light-emission intensity maximum is reached first by **Clz-3**, followed by **Clz-4**, and then by **Clz-2**.

It should be noted, once again, that **Clz** compounds are proposed to possess anticancer activity due to chemiluminescence-based generation of triplet states, which is based on the enhancement of intersystem crossing during chemiexcitation due to the heavy-atom effect [10–13]. Furthermore, triplet excited states are generally more easily quenched in solution than singlet excited states [43–45]. Given this, it would be expected that **Clz** compounds with more efficient anticancer activity are associated with lower chemiluminescence, due to a higher triplet-to-singlet product ratio [10,12]. To that end, we have measured both the chemiluminescence light-intensity maxima and total light output (area) for **Clz-2**, **Clz-3**, and **Clz-4** [10,31,42]. The results are provided in Fig. 6.

Quite interestingly, we did observe a relationship between the anticancer activity and the relative light-emission intensity maxima for the three **Clz** compounds (Fig. 6B). Namely, **Clz-4** (with residual/absent anticancer activity) is the compound that presents the highest light-emission intensity maximum of the three compounds (relative/normalized maximum of 1), which is significantly higher than the values presented by the two compounds that showed anticancer activity: **Clz-2** and **Clz-3**. Furthermore, we can even see that **Clz-2** (with lower anticancer activity) presents a relevantly higher intensity maximum than the most potent **Clz** compound, **Clz-3**: relative maxima of 0.095 and 0.005, respectively (Fig. 6B).

The total light output was measured and shown in Fig. 6C. The results are quite similar to those obtained for light-emission intensity maxima and continue to show **Clz-4** as the compound with significantly more efficient chemiluminescence (relative/normalized output of 1). As for **Clz-2** (relative output of 0.084) and **Clz-3** (relative output of 0.003), their chemiluminescence is relatively residual, especially for the latter. Thus, the chemiluminescence data is in line with the observed anticancer activity of these **Clz** compounds and their proposed mode of action, as higher anticancer potency is associated with lower light-emission intensity [10–12]. It should also be noted that both **Clz-2** and **Clz-4** were previously shown to generate light-emission when triggered by superoxide anion [10,31], and so, the different anticancer activities of the **Clz** compounds here measured should not be related with a potential ability/inability of being triggered by superoxide anion. Nevertheless, we should point out that there is the possibility that these compounds exhibit anticancer via more than one single mechanism. That is, their cytotoxicity effect may result in part due to other unknown (so far) mechanisms of action, instead of just via the proposed chemiluminescence-based one. This possibility should be further explored and clarified in the future, in works focused on **Clz**-based compounds.

3.4. Stability measurements

For the development of new potential drugs, and to further understand their mode of action, it is also relevant to understand their stability. To that end, we have measured the shorter-term stability of **Clz** compounds. This was performed by measuring their fluorescence in sodium acetate buffer solution (pH 5.2) during 3 sequential days, following a previously used procedure [13]. Assays were performed in this buffer solution to try to mimic the tumor environment, which is associated with lower pH than normal cells [46,47].

We have first focused on **Clz-2** and **Clz-3**, which were the ones that showed higher potency. Initial measurements were performed on Day 1, followed by measurements on Days 2 and 3 [13]. In the meantime, samples were left at room temperature. The resulting variations in fluorescence intensity are presented in Fig. 7. These results show that both compounds show instability in acidic buffer solution, with variable degrees. More specifically, the fluorescence of both compounds is enhanced after Day 1. For **Clz-2**, the fluorescence intensity increases by ~ 30 % between Day 1 and Day 2, while remaining stabilized between Day 2 and Day 3. As for **Clz-3**, the results indicate significantly higher instability, as the fluorescence intensity more than doubled between Day 1 and Day 2, followed by another increase (albeit quite smaller) on Day 3.

Structurally, the only difference between **Clz-2** and **Clz-3** is the inclusion of a bromine heteroatom in the R_2 position (Fig. 2), and so, the difference in stability between compounds should be related with this change. It was recently observed for a group of different **Clm** compounds that some of them showed relevant instability in acidic buffer, by enhancement of their fluorescence [13]. Interestingly, that instability was typically observed for **Clm** compounds with bromine heteroatoms directly bound to the aminopyrazine core at R_2 position [13], similar to what is observed for **Clz-3**. So, the reason behind the increased instability of **Clz-3** should be related with similar reasons as for those **Clm** compounds. In fact, **Clm-3**, the pair of **Clz-3**, was one of the compounds that showed instability in acidic buffer [13].

It should be noted, however, that this instability does not appear to be related neither with the anticancer potency nor with the time-dependency of anticancer activity, at least for **Clm** compounds. That is, it was observed, both in this study and in previous one [13], that **Clm-2** is more potent than **Clm-3** and that the two compounds show time-dependent activity. However, only **Clm-3** showed instability in acidic buffer [13]. Thus, if the higher instability of **Clz-3** is related with either/both its higher potency or/and its time-dependent effect, it would show relevant differences regarding the mode of action of **Clm** compounds.

To try to assess if we can correlate the anticancer activity of **Clz** compounds with their stability/instability in acidic buffer, we have also measured the fluorescence intensity variation for **Clz-1** and **Clz-4** between Day 1 and 3. The results are presented in Fig. 7. Both compounds showed fluorescence enhancement over time, in a magnitude quite similar to **Clz-2.** These results further indicate that the higher instability of **Clz-3** is indeed related with the presence of a bromine heteroatom in the R_2 position. More relevantly, the differences in intensity are quite similar for **Clz-1**, **Clz-2,** and **Clz-4**, while the observed anticancer activity was different. Thus, the observed variations in fluorescence intensity should not be related with the anticancer activity of these compounds. Nevertheless, the fact that instability was observed could help to explain why **Clz** compounds tend to present time-independent activity (due to potential degradation over time), and that their efficiency is limited due to lower stability. So, this topic should be explored in more detail in the future development of novel **Clz** compounds.

4. Conclusions

Here we have performed a comparative investigation into the anticancer activity of analogs of marine coelenterazine and coelenteramine. The second is a metabolic product of the bioluminescent reaction of

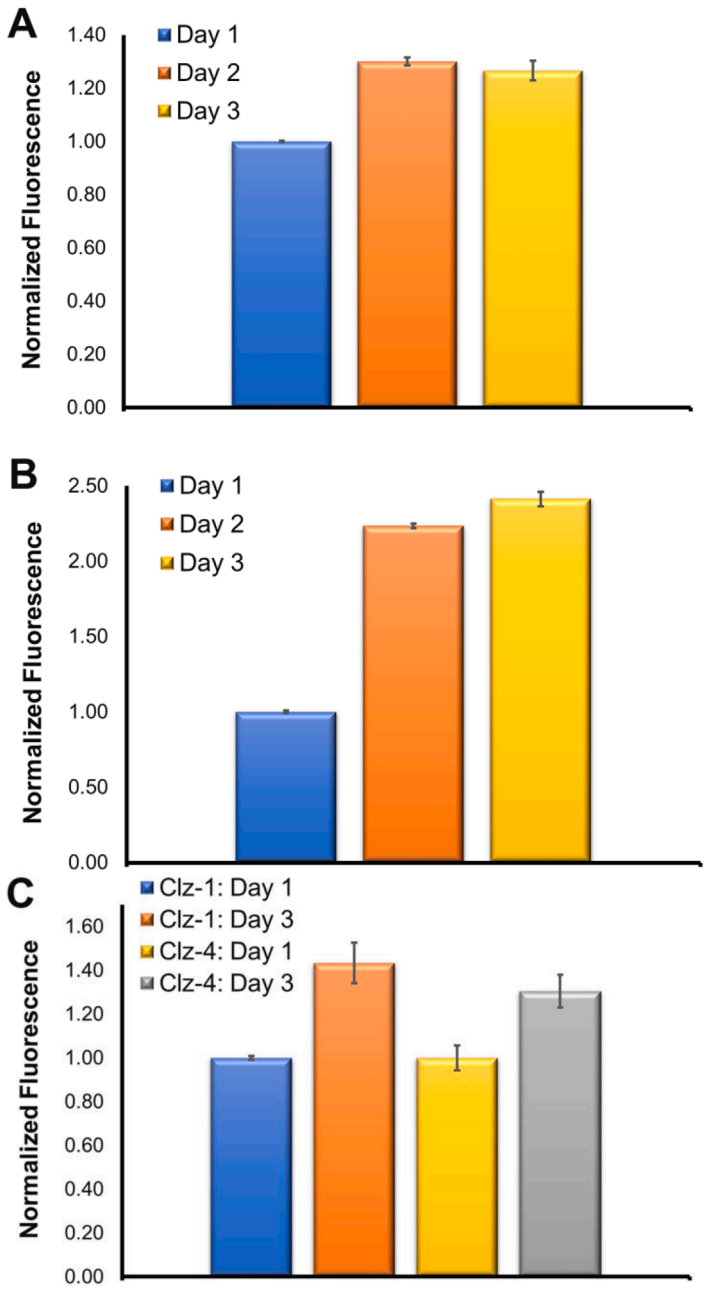

Fig. 7. Normalized, with Day 1 as reference, fluorescence intensity maxima of **Clz-2**, measured every **24 h** (a); Normalized, with Day 1 as reference, fluorescence intensity maxima of **Clz-3**, measured every **24 h** (b); Normalized, with Day 1 as reference, fluorescence intensity maxima of **Clz-1** and **Clz-4**, measured every 24 h (c). All assays were performed in sodium acetate buffer solution, pH 5.2, with a compound concentration of 5 μM.

coelenterazine. To that end, we have evaluated the cytotoxicity toward lung and gastric cancer cell lines of four coelenterazine – coelenteramine pairs of analogs. The molecules of each pair are only differentiated in terms of possessing either an imidazopyrazinone or aminopyrazine core. As for the different pairs, they are distinguished from each other by the number and/or position of bromine heteroatoms. The main objectives here were then to further clarify if coelenterazine and coelenteramine analogs possess the same mode of action, and to obtain information regarding the structure–activity relationship of these compounds.

The obtained results indicate that despite their structural similarity, the two types of analogs show relevant differences in their anticancer activities. More specifically, we observed relevant differences in structure–activity relationships between these compounds. That is, the addition of a second bromine heteroatom at the R_2 position decreases the activity of coelenteramine analogs while increasing the potency of the coelenterazine-based molecules. This is in line with previous results that indicate that the activity of the former is related to the presence of a 4-bromophenyl moiety at the R_1 position, while the activity of the latter is less dependent on the structure of the analogs. Furthermore, the activity of the coelenteramine molecules was found to be time-dependent, which did not appear to be the case for coelenterazine analogs. Also, the latter analogs appear to be safer toward noncancer cell lines than the former. Finally, the anticancer activity of the coelenterazine analogs could be correlated with their chemiluminescent properties, as consistent with their proposed mode of action, while coelenteramine analogs are non-chemiluminescent.

Thus, we obtained insight into the activity of these two types of compounds that should be useful for the future optimizations of their anticancer potential, while providing insight that indicates that they possess different modes of action. Finally, this study further

demonstrated the potential of the marine coelenterazine system as an attractive source of novel analogs with potential anticancer activity.

CRediT authorship contribution statement

Carla M. Magalhães: Writing – original draft, Visualization, Investigation. **Renato B. Pereira:** Writing – original draft, Investigation. **El Hadi Erbiai:** Investigation. **Patricia González-Berdullas:** Investigation. **Joaquim C.G. Esteves da Silva:** . **David M. Pereira:** Writing – review & editing, Supervision, Investigation. **Luís Pinto da Silva:** .

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2023.107083.

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