Chapter

The Importance of the Chemiluminescent System of the Marine Chromophore, Coelenterazine

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Abstract

Chemiluminescence is the remarkable phenomenon that consists in the conversion of thermal energy into excitation energy due to a chemical reaction, which leads to emission of light. As no light-excitation source is required, only a low or negligible nonspecific signal is generated, which results in a high signal-to-noise ratio. So, several practical applications have been developed in the fields of biomedicine and bioanalysis. Coelenterazine is a chemiluminescent substrate widespread in the oceans, where reside about 80% of all luminescent organisms. Coelenterazine possess an imidazopyrazinone core that can be oxygenated into a high-energy peroxide intermediate, which quick decomposition generates a chemiexcited light-emitter. Interestingly, the imidazopyrazinone core can be found in eight phyla of bioluminescent organisms, demonstrating the importance of Coelenterazine and derivatives. More relevantly, the chemiluminescent system of Coelenterazine and related imidazopyrazinones have shown potential in biosensing, diagnostics and even as therapeutic agent. In this chapter, it will be provided insight into the most relevant steps of the chemiluminescent reaction of Coelenterazine and derivatives, as well as reporting developments regarding practical applications for this chromophoric system.

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1. The World of Luminescence

Luminescence consists in a term used to describe processes that result in the production of an optically active excited molecule, which will be capable of emitting luminous radiation of a certain wavelength. Thereby, it is possible to classify this occurrence in many distinct ways, such as fluorescence, phosphorescence, chemiluminescence and bioluminescence (among others), with these last two being of high interest in this chapter [1].

As for the importance of these phenomenon, they present intrinsic characteristics which allow the development of existing and novel techniques, leading to the enhancement of diagnostic and therapeutic methods. These consist in high sensitivity, specificity, fast reaction velocity and high quantum yield, with the possibility of modulating the color of the emitting light [1].

Chemiluminescence is a process associated with the production of light through a chemical reaction, responsible for the conversion of thermal energy in excitation energy which can occur in the presence of a catalyst or co-factor. During the reaction, the excitation of the molecule occurs and, upon the electron return to the fundamental state, will result in photon emission [1, 2].

The main mechanism associated with this type of reaction consists in the presence, on a reaction intermediate, of a peroxide bond (O-O), being that it is possible to classify the molecule containing this group in three distinct categories, depending on the number of additional carbonyl groups: dioxetane (zero carbonyl groups), dioxetanone (one carbonyl group) and dioxetanedione (two carbonyl group), as represented in Figure 1 [3]. This type of bond, upon the occurrence of a nonradiative energy transfer from the chemiluminescent donor, will be cleaved, through a spontaneous thermal decomposition, leading to the generation of the excited state products [2].

For the occurrence of the bond break, each type of molecule will release a different amount of energy, with dioxetane and dioxetanone, according to the calculations, releasing around 70 and 90 kcal mol^{-1}, being that the peroxide rings are capable of providing the necessary energy for the molecule excitation, during the decomposition process [4, 5].

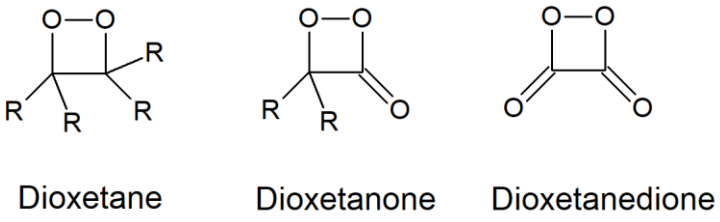

Figure 1. Schematic representation of types of chemiluminescent intermediates containing peroxide bonds.

As for the mechanism associated with the decomposition process of the peroxide ring, it consists in a stepwise biradical mechanism [6-8], which states that the reaction starts with a homolytic cleavage of the O-O bond in the peroxide ring, leading to biradical transition state, which will be followed by the breaking of the C-C bond, with this resulting in the light-emitting product [4, 5, 9].

The formation of excited singlet states by part of the peroxide was, initially, though to be derived from chemically induced electron-exchange luminescence (CIEEL) [5, 6], in which an electron transfer from oxidizable electron-rich moiety (if this group belongs to the molecule itself, this process is known as intramolecular CIEEL, being otherwise designated as intermolecular) to the peroxide will generate a radical ion pair, which is capable of undergoing back electron transfer from the carbonyl radical anion into the radical cation, leading to the formation of the singlet excited state, through charge annihilation [4, 7].

However, after the elaboration of new studies [10], it was noted that molecules which were associated with an efficient CIEEL manifested an inefficient luminescent emission, indicating that this wasn’t the mechanism behind efficient chemiexcitation [11]. This situation led to the proposal of novel mechanisms, such as charge transfer-initiated luminescence (CTIL), which suggests a gradual charge transfer and back charge transfer from the ionized electron rich moiety into the peroxide ring, being that these were also unable to properly explain the process of singlet excited state formation, since theoretical data indicates that charge transfer should also occur in the absence of the electron rich moiety [9]. Following this, work elaborated by Pinto da Silva et al. and followed by Magalhães et al. also confirmed that both CIEEL and CTIL would not explain the chemiexcitation process [12, 13]. In these studies, it was showcased that a neutral dioxetanone intermediate was responsible for the chemiexcitation [9]. This was explained

by theoretical calculations that this intermediate, through its thermal decomposition, gains access to a long potential energy surface (PES) region of ground (S_0) and excited (S_1) state degeneracy, being that this situation was thought to be potentiated by the attractive electrostatic interactions between the luminophore moieties and CO_2, which would promote the access to the PES region, with no correlation between energy and charge transfer within the dioxetanone and electron-rich moiety and chemiexcitation observed [4, 14, 15].

It is important to note that, for anionic dioxetanone, repulsive electrostatic interactions will be enhanced, causing a faster disassociation from the CO_2 molecule, which will prevent the access to the region of near degeneracy, reducing the efficiency of the chemiexcitation [4, 15].

Therefore, despite still not being fully understood, it is possible to observe that the chemiexcitation mechanism, rather than depending on electron and charge transfers, it is influenced by the interaction capacity between the dioxetanone keto groups and the CO_2 molecule [4, 15].

As for bioluminescence, it corresponds to a process similar to chemiluminescence, consisting in the emission of light, derived from a chemical reaction. However, the main difference corresponds to the fact the employed catalyst are enzymes, to promote the oxidation reaction, which as commonly known as luciferase, capable of targeting substrate molecules typically designated as luciferin, which highlights the higher complexity manifested by this process [3, 11, 16].

This type of reaction is usually present in living beings such as bacteria, insects and many others, with the most known example consisting in the light emitted by the fireflies, which have been studied in many previous experimental works, being associated with a higher light output when compared to chemiluminescence [3, 11, 17].

Additionally, it is important to note that alterations of these oxidizable motifs allow for the modification the color associated with the luminescent emission [3].

2. Examples of Chemiluminescent and Bioluminescent Systems

The chemiluminescent system that presents the most extensive analysis corresponds to the one that involves the substrate known as luminol, which is displayed in Figure 2. This compound can be oxidated in a basic environment, with the presence of hydrogen peroxide and a catalyst.

Afterwards, the oxidized luminol will interact with hydroxide anions, generating a dianionic intermediate capable of associating to oxygen, to produce an aminophthalate ion in the excited state, which emits intense blue light (with a wavelength around 425 nm) [3, 18].

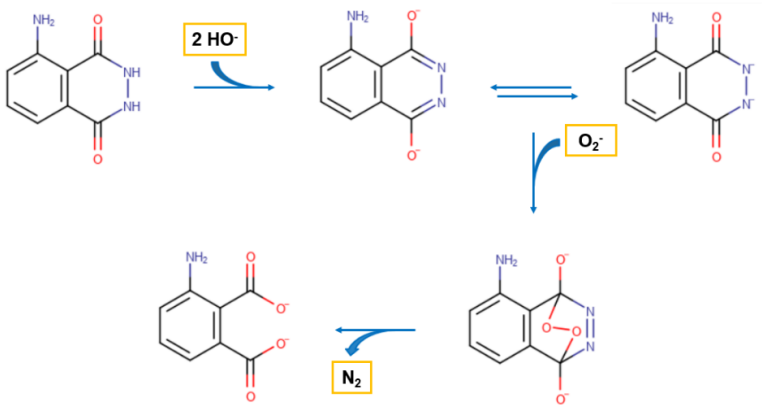

Figure 2. Schematic representation of the mechanism associated to the luminol chemiluminescent reaction.

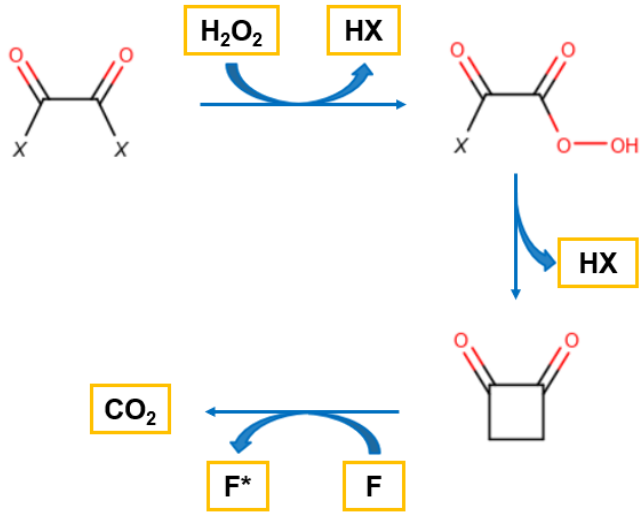

Figure 3. Schematic representation of the mechanism associated to the peroxyoxalate chemiluminescent reaction (X = OR or Cl group/F = Fluorescent molecule/F* = Fluorescent molecule in the excited state).

Another widely studied luminescent systems consists in the one based on peroxyoxalate compounds, represented in Figure 3, being that these can existing in an oxalyl ester or chloride form [6, 19]. In this process, the aforementioned molecule is capable of reacting with hydrogen peroxide (H_2O_2), through a nucleophilic substitution, yielding an oxalic peracid derivative, which, upon closure of the ring, will originate an intermediate dioxetanedione, that, when in contact with a fluorescent molecule, will lead to its excitation, leading to light emission [6, 19].

One of the main known bioluminescent reactions is the one associated to the firefly luciferin-luciferase system, highlighted in Figure 4. In this process, the luciferase will be responsible for catalyzing a two-step reaction [14]. It that starts with an AMPylation between the luciferin and an adenosine triphosphate molecule, generating an adenyl intermediate. Afterwards, this molecule will be oxidized by the presence of an oxygen molecule, which will result in the release of an adenosine monophosphate molecule, along with the formation of a dioxetanone. Finally, the thermolysis of the peroxide bond will occur, leading to the chemiexcitation of the light-emitting oxyluciferin, which manifested pH sensitive properties, with the maximum emission peak wavelength increasing with increase of the environment acidity [7, 20].

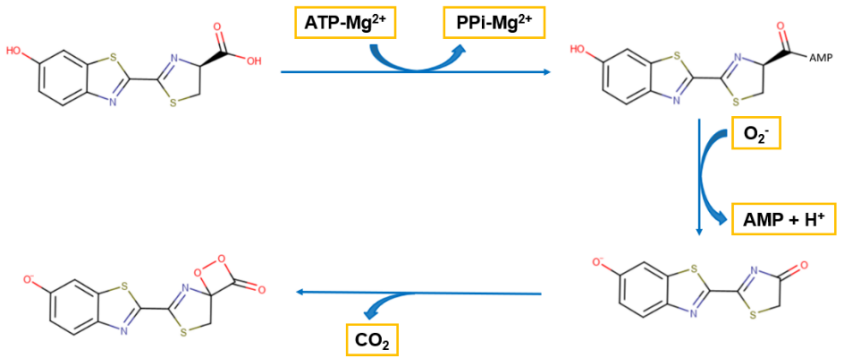

Figure 4. Schematic representation of the mechanism associated to the firefly luciferin bioluminescent reaction.

Another relevant bioluminescent reaction is the one that occurs in dinoflagellates, a species of marine plankton, being characterized by a single reaction step, as noted in Figure 5 In this situation, its luciferin will associate to the luciferase and, in the presence of oxygen, leads to the generation of a product with luminescent output, with a wavelength around 474 nm [19].

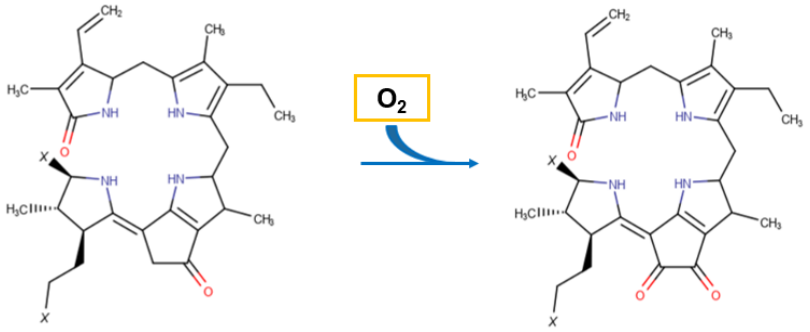

Figure 5. Schematic representation of the mechanism associated to the dinoflagellate luciferin bioluminescent reaction.

3. Coelenterazine and Its Luminous Emission

One of the most studied chemiluminescent and bioluminescent reactions, is the one that occurs with the substrate known as coelenterazine, along with its bioluminescent counterpart, which involves the presence of an enzyme designated luciferase. The reaction will originate a product capable of emitting a blue light of weak intensity. This compound [21] is observed to be present in several types of marine organisms with light-emitting properties, such as the jellyfish *Aequorea* (acquires the substrate from its diet) and the copepod *Metridia* (capable of synthesizing coelenterazine without requiring external sources) [4, 16, 21, 22].

Goto and coworkers were also able to determine, in a detailed manner, the structure of coelenterazine [23]. Through their analysis, the component of coelenterazine which was determined to present the main contribution for its luminous properties consisted in a structure known as the imidazopyrazinone core [17], as presented in Figure 6. This structure will be responsible for the interaction with the reactive oxygen species, to trigger the chemiluminescent reaction. This central core was also observed to be associated to the following groups: two phenols at the C2 and C6 positions, with a phenyl at C8, which correspond to the main targets of modification for the synthesis of coelenterazine analogues [24, 25].

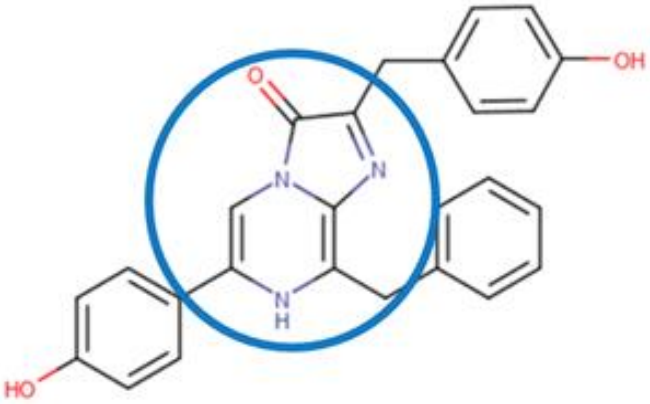

Figure 6. Representation of the coelenterazine molecule structure, with focus on the imidazopyrazinone core.

As for its chemiluminescent reaction, this substrate, in an initial step, is responsible for an electron or H-atom transfer into an oxidizing agent, which will generate a radical intermediate. Afterwards, that radical, will be oxygenated which will allow the production of a peroxide that, upon forming a four membered ring, results in an unstable molecule known as coelenterazine dioxetanone [26]. Finally, as this compound, due to its instability, will be thermically decomposed in a spontaneous manner, with rupture of the peroxide ring, leading to the release of a carbon dioxide molecule, along a coelenteramide molecule in the singlet excited state, capable of emitting a blue-green light (maximum intensity peak around 480 nm) upon relaxation, as represented in Figure 7 [11, 27].

Additionally, it is important to note that the chemiexcited coelenteramide will exist in an equilibrium between several chemical forms, which are based on a neutral, protonated and deprotonated amino and hydroxyl group and the phenolate-amide tautomerism [4].

As for the bioluminescent system associated to coelenterazine, it presents a significant difference in the fact that the chemical reaction will be triggered in the presence of the luciferase. In this process, will be induce the oxidation of the luciferase-bound coelenterazine, leading to the formation of excited state coelenteramide, which emits light of wavelength around 455 to 480 nm. It is important to note that the reaction will occur until the substrate is fully consumed. This bioluminescence reaction can be observed in marine organisms such as the shrimp *Oplophorus* and the coral *Renilla* [28].

Furthermore, the bioluminescent reaction involving this coelenterazine luciferin can also occur in association to a structure known as Ca^{2+}-regulated photoprotein, which can usually be observed the *Aequorea* jellyfish species. The luminescence output in this situation presents a maximum emission wavelength between 440 and 475 nm [27, 28].

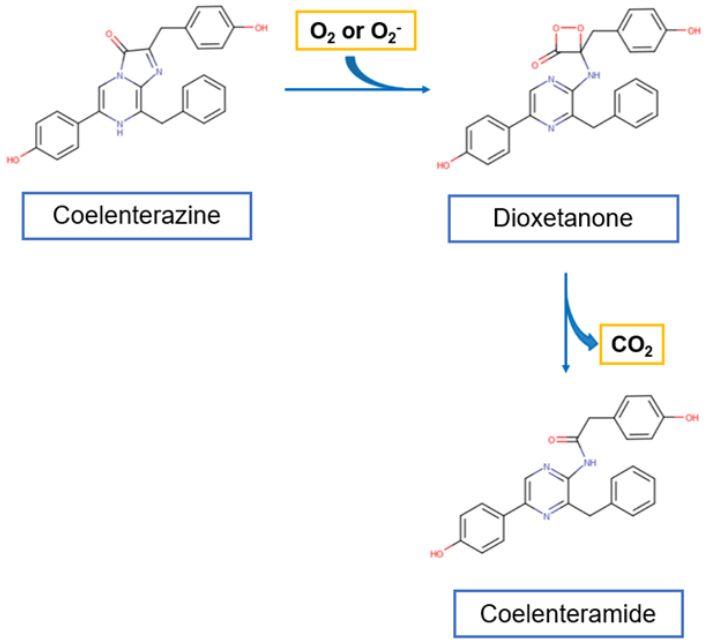

Figure 7. Schematic representation of the chemiluminescent reaction associated to coelenterazine.

In this marine organism, the photoprotein is known as aequorin, being that it corresponds to complex of apoaequorin (apoprotein) and a chromophore. This protein belongs to the EF-hand Ca^{2+}-binding protein superfamily, is composed of a single polypeptide chain that is composed by 189 amino acids, with a molecular weight of around 21 459 Da, which is organized in a helix-loop-helix motif, being classified as a monooxygenase [16].

The photoprotein is capable of tightly binding coelenterazine in its oxygenated form, known as 2-hydroperoxycoelenterazine, which will act as the chromophore [27, 28], meaning that the radiative emission is independent from the levels of molecular oxygen in the environment (evidencing its distinction from the enzyme luciferase). Another existing

difference is the fact that the bioluminescent reaction, in the photoprotein case, can only occur once, being that luciferase doesn't present this limitation, as mentioned previously [16, 28].

Additionally, the photoprotein can stabilize the peroxy intermediate with very high efficiency, which will allow the complex between enzyme and substrate in the form of an active photoprotein to be stored in the absence of Ca^{2+} in the surrounding environment, during a long period [16].

In the absence of the calcium ion, the photoprotein presents a basal light emission of very reduced levels, being known as "calcium-independent luminescence." However, upon the introduction of Ca^{2+}, the protein will undergo structural modifications at the active site, evidencing that the ion presents an important role as an allosteric modulator, which will induce small structural modification in the active site of the protein [29]. These alterations will lead to a promotion of the decarboxylation mechanism of 2-hydroperoxycoelenterazine, which will result in the formation of CO_2, along with the chemiexcited coelenteramide, being this compound bound to the protein. Upon relaxation, the emission of light will occur, presenting a wavelength between 465 and 495 nm, with an intensity that can be up to one million times higher than the basal levels [29]. Therefore, it can be said that the association of the ion can enhance the luminescence reaction, leading to an increased radiation intensity [16, 28].

In the following years, several novel coelenterazine analogues were sensitized, with each of them providing new insights on the relationship between structure and activity for this family of compounds [30-35]. With this in consideration, it is important to highlight the analogues 3 to 6 sensitized by Teranishi and Goto, during 1990, displayed in Figure 8. In these compounds, a dimethylene group was introduced, in order to bridge the phenyl group and pyrazine ring, leading to an increase of the conformational rigidity, being that this proved to be beneficial, since it allowed for an enhanced light emission, as noted in Table 1 [36].

Through modifications of the phenol group present in C8, it was verified that it was the structural motif responsible for the emission of light, being that its electron donating character is not necessary for a luminescence of enhanced intensity. Likewise, in terms of the presence of electron withdrawing (CF_3) or donating groups (OCH_3) in the C_6 phenol, displayed in Figure 9, they only presented a small influence over the chemiluminescent reaction, with the efficiency of the light emission being lower in the presence of electron withdrawing groups, as noted in Table 2 [37, 38].

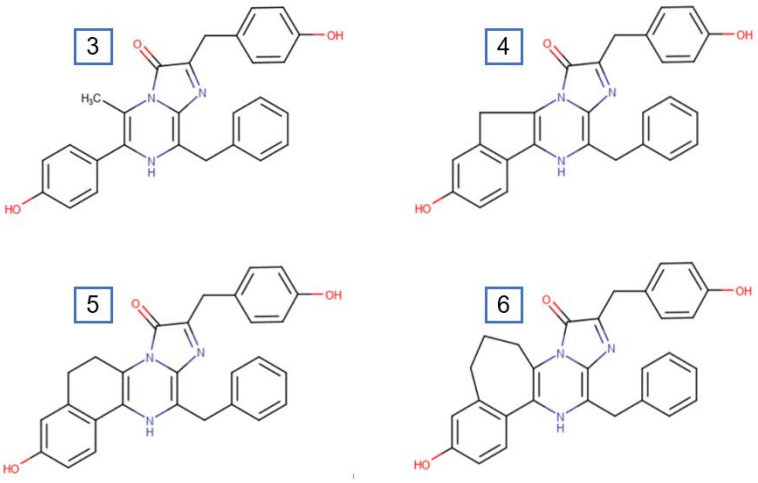

Figure 8. Schematic representation of the coelenterazine analogues with distinct levels of conformational rigidity.

Table 1. Characteristics (luminous output in relation to coelenterazine and peak wavelength) of the chemiluminescent reaction associated to the coelenterazine analogues with different levels of conformational rigidity, present on Figure 8 [36]

Coelenterazine Analogue	Chemiluminescence Quantum Yield	Emission $\lambda_{máx}$ (nm)
3	1.1	460
4	1.5	500
5	2.3	480
6	1.5	470

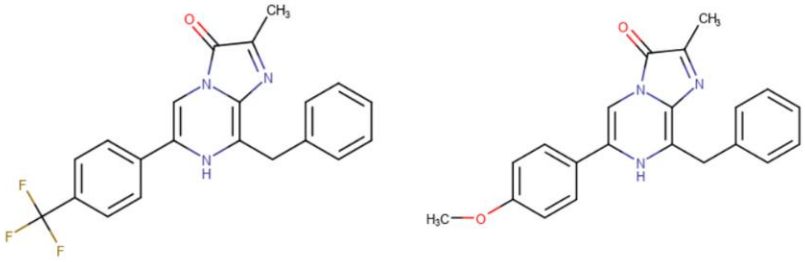

Figure 9. Schematic representation of two coelenterazine analogues, with electron withdrawing (left) and electron donating (right) groups.

Table 2. Characteristics (luminous output in relation to coelenterazine and peak wavelength) of the chemiluminescent reaction associated to the coelenterazine analogues containing CF3 and OCH3 groups, present on Figure 9 [38]

Coelenterazine Analogue	Chemiluminescence Quantum Yield	Emission $\lambda_{máx}$ (nm)
CF_3	0.05	454
OCH_3	0.21	473

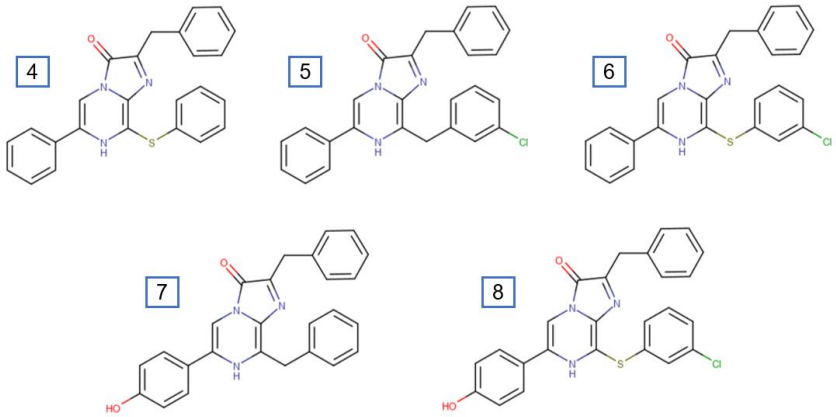

Figure 10. Schematic representation of the coelenterazine analogues containing a sulfur atom.

Table 3. Characteristics (luminous output in relation to coelenterazine and peak wavelength) of the chemiluminescent reaction involving the coelenterazine analogues, present on Figure 10 [39]

Coelenterazine Analogue	Chemiluminescence Quantum Yield	Emission $\lambda_{máx}$ (nm)
4	1.83	597
5	1.80	456
6	0.92	499
7	0.96	460
8	0.42	507

Additionally, in 2012, Guiliani et al. produced novel analogues, with the variations 4 to 8 showcased in Figure 10, that presented a sulfur heteroatom that replaced the methylene group in the C6 position, which was bonded to

the phenyl group in C8. In this situation, a significant bathochromic shift (towards red light wavelength) was observed, noted in Table 3, indicating the possibility of modulating the emission wavelength of the chemiluminescent product towards that of red light, which would enhance organic tissue penetration [39].

4. Practical Applications for the Coelenterazine Systems

Due to their chemiluminescent properties, chemiluminescence and bioluminescent systems involving coelenterazine have become an attractive target for the development of novel tools, capable of improving many scientific fields.

During this chapter, we will focus on two possible applications. One is related with the relatively recent discovery that modification of the coelenterazine system can lead to anticancer activity [26]. Another is the use of coelenterazine as a probe capable of sensing the presence of various type of species, with the superoxide anion being one of them (since is it essential for the chemiluminescent reaction to take place) [17, 40], since this species is usually overexpressed in certain diseases (such as cancer) [4, 26].

With this in consideration, before explaining in a detailed manner the coelenterazine roles in cancer treatment and diagnosis (associated with superoxide anion sensing), the main characteristics of this disease will be highlighted.

4.1. Cancer Plague over the World

Tumor corresponds to a term that usually refers to diseases which are caused by the uncontrollable development of cells, due to a defective cellular division process, originating lumps that can be classified as non-cancerous (benign, being that they only affect the tissues from where they originate) or cancerous (also called malign, since they capable of invading nearby and/or distant tissues, through a procedure known as metastasis, that normally occurs in the late stages of development) [41, 42].

The cancerous tumors can be divided according to the affected cell type, which lead to the following five classifications: carcinoma (organs and glands, such as breast and pancreas), sarcoma (connective tissues, such as

muscle and bone), melanoma (pigmented skin cells), lymphoma (white blood cells) and leukemia (blood cells) [41, 43].

This type of cancerous pathology consists in the main cause of death around the globe [44], associated to around 1 in 6 cases, responsible for around 10 million deaths as of 2020, being that the most common one consists in breast cancer (2.26 million), followed by lung, colorectal, prostate, skin and stomach cancer [41].

The development of cancer is associated to the conversion of normal cells in tumoral ones, through a multistage process. Initially, this procedure starts by the induction of damage in certain regulatory genes of the cell, such as tumor suppressor and DNA repair genes, or even in proto-oncogenes, which present an increased cell growth promoting activity when mutated. This mutation can be influenced by genetic factors, such as gene preposition to mutate, and environmental ones, which can be classified as physical (UV and ionizing radiation), chemical (such as asbestos) and biological carcinogens (certain virus, such as hepatitis, bacteria and parasites) [41, 45, 46].

In this situation, the mutated cells lack regulatory processes associated to their development cycle, allowing them to grow without the required growth signals. Additionally, they also acquire the possibility of bypassing programmed cellular death markers, such as chromosomes telomers shortening, along with the absence of cell contact inhibition (consisting in a growth inhibition signal upon sensing neighbor cells), while also being capable of evading the immune system, being that the combination of all these factors will contribute to their uncontrollable growth, causing the conversion into malignant cells, and consequent spreading through the neighbor tissues [47].

With this in consideration, the development of the tumor cells can be divided in three distinct phases, known as initiation, progression, and metastasis, that can be further classified in stages 0 to IV [42, 47].

During the first step, corresponding to the initiation, the mutation of the healthy cells will occur, due to the factors mentioned previously, leading to the generation of initiated cells, being that this type of cells will present an enhanced multiplication (hyperplasia), along with the distortion of its structure (dysplasia), leading to the advanced form known as carcinoma *in situ*, corresponding this situation to stage 0, since it isn't referred as cancer, due to the fact that it is still contained in the tissue of origin [42, 47].

Afterwards, during the progression stage, the mutated cell will experience an even more unregulated proliferation, while still being

contained in the area where the alterations first occurred, converting into preneoplastic cells, being this the stage II, which will eventually form cancer nodules, becoming neoplastic cells, giving rise to the stage III, where the cell will acquire the capacity of invading neighbor tissues [42, 47].

In the final stage, which consists in the stage IV, known as metastasis, the cancerous cell invasive capacity will increase, leading to the spreading into the blood stream, from where it will be capable of affecting distant tissues. The individuals experiencing this final cancer development stage, due to its high dissemination, present reduced chances of survival [42, 47, 48].

It is important to note that the unregulated tumor growth is associated to changes, not only in the signaling pathways, but also in the cell metabolism, leading to a distinct metabolic pathway (first discovered by Otto Warburgh), where the main source of ATP production will consist in aerobic glycosis (unlike normal cells, who obtain their energy from oxidative phosphorylation), which can be caused due to environmental and genetic changes [49]. This alteration will enhance the cell growth process, despite the lower energetic yield (which is compensated though an increased glucose consumption, known as "Warburgh Effect"), since it allows for a faster production of essential components, such as nucleic and amino acids [49]. Additionally, this modified metabolism will result in an increased production of reactive oxygen species, such as superoxide, which, in reduced amounts can promote cancer cell proliferation, while high amounts can provoke the degradation of the cell itself [48].

To prevent the cancer pathology, the most common approach consists of the avoiding contact with the existing carcinogens, mentioned previously, along with the employment of a healthy lifestyle (combination of balanced diet, which can provide antioxidants, and regular exercise) [41, 45].

As for disease diagnosis, it is very important to detect cancerous cells as early as possible, to increase the chances of a successful treatment, which can be possible through examination of cancer symptoms, through regular medical checkups, which consist in blood tests, X-ray, and biopsy. These can include situations of unusual weight loss, persistent sensation of tiredness and appearance of oddly shaped lumps in the skin [41].

4.2. Treatments and Their Limitations

In terms of treatments available to the general population, many different types exist, with each of them presenting distinct characteristic [45]. The most used therapy corresponds to chemotherapy [44], being this method based on the administration of drugs, through IV solution or an ingestible pill, capable of associating and disabling essential elements for cell development, leading to their degradation. Other method consists in radiotherapy [44], that requires the employment of a high level of radiation, which can induce cellular elimination, though damage to essential proteins. Immunotherapy also is a possible therapeutic pathway, being this based on immune response stimulation against tumoral cells, with T cell promotion, allowing specific targeting of the cancerous cells [42]. The process of hormonal therapy is a treatment that requires the application, or deprivation, of a specific hormone for the treatment of a certain cancer, through modulation of the human body biochemical pathways [42, 50].

Additionally, in cases where the cancerous tumor is still in the first stages of development, it is possible to perform its direct removal, through surgical methods, which would allow to avoid damage towards the healthy tissues [42, 47].

However, these therapies present significant side effects [42, 44], which are mainly derived from their reduced specificity, leading to the degradation of healthy cells, along with the cancerous ones, which is not desirable in a therapeutic method. These treatments also lead to a persistent sensation of fatigue and nausea, with hair loss and bloating also occurring with some methods. An additional obstacle consists in the fact that cancer cells present an heterogenous profile, being quite distinct in terms of characteristics across individuals, along with the fact that they are capable of manifesting resistance against drug-based treatments, through cell mechanisms such as drug removal [51-53].

Therefore, it was of very high importance to discover methods that could bypass these problems. One of the first answers consisted in the combination of different treatments for the treatment of the same type of cancer, which will be quite beneficial, since it should allow for the reduction of the individual does of each required drug, being this translated into a reduced individual toxicity for the compounds, along with the possibility of reducing the drug removal mechanism activation [51, 54].

However, despite having resulted in the improvement of the patient's survival chances, this technique also presented some significant

disadvantages. These consisted in a highly invasive nature and a reduced specificity towards the cancer cells, which led to considerable negative side effects over healthy ones [51].

Taking this into consideration, it was obvious that the search for an effective anticancer treatment, with reduced harmful effects over healthy cell, still had a long road ahead.

4.3. Photodynamic Therapy for a Better Future

In the search for ways of cancer treatment with higher degrees of efficacy, one other answer that came to be corresponded to the novel medicinal non-invasive technique known as photodynamic therapy [44], which was considered to be of high interest, being that, currently, it is being increasingly integrated for the treatment of various type of pathologies, with the most prominent consisting in cancer [45, 55].

The aforementioned procedure, as the name indicates, is based on the se of an external light source for the elimination of cancer cells. Considering this, the therapy requires the interaction between 3 distinct components [44]. First, a nontoxic photosensitizer is introduced into the site of lesion. Following this, the radiation of a certain wavelength will be applied (allowing the transition into an excited state of higher energy). Finally, the oxygen molecules (O_2), which are in the triplet state, present in the environment of the tissue to be treated will react with the photoexcited photosensitizer, yielding ROS capable of eliminating tumor cells [3, 55, 56].

This new therapeutic method presented many interesting advantageous properties. These corresponded to a minimally invasive nature, reduced occurrence of harmful effects over healthy cells and a high healing rate for these cells [44]. Additionally, the procedure also is associated to an enhanced spatiotemporal precision, since the photosensitizer can only be activated when exposed to the specific radiation, providing full control of the initiation of the treatment [3, 55].

The origin of the process of photodynamic therapy can be traced back to the ancient Egypt, being that proof exists of the application of extracted plant pigments, which presented photosensitizing properties, to the skin of individuals suffering from psoriasis, consisting in skin flaking, for their treatment [44, 54].

This procedure was first scientifically investigated in the 1970s at the hand of Thomas Dougherty, through the usage of hematoporphyrin

derivatives, which correspond to the first-generation photosensitizers [57]. This family of compounds was observed to be capable of preferential accumulation in pathogenic tissues, rather than healthy ones, which was discovered through the work of Policard [58]. The complex mechanisms associated to this procedure still not fully understood currently, being hypothesized that the increased vascular permeability, along with the reduced lymphatic drainage, which are commonly associated to cancerous tissues, can be responsible for this differential distribution phenomenon [54].

Additionally, it is important to note that these first-generation photosensitizers presented a slow clear rate from the healthy cells, meaning that the individual, during treatment, couldn't be under direct sunlight during several weeks, to prevent severe negative effects over these cells. Taking this into consideration, the development of new generations of hematoporphyrin derivatives, with faster clearing rates from the organism, was promoted [54].

As for the photodynamic therapy mechanism, it is necessary that the interaction between the 3 parts of the system, mentioned previously, is possible. Upon the irradiation of specific wavelength light over the photosensitizer, present in the target issue due to preferential distribution. Afterwards, the molecule will become transiently energized from its singlet ground state into a singlet excited state. Subsequently, the molecules present is this new state are capable, through a phenomenon known as intersystem conversion, of crossing to longer-lived triplet state. Finally, the molecule, in its triplet state, will be capable of interacting with triplet oxygen molecules present in the surrounding environment, leading to the generation of radical products, mainly singlet oxygen, capable of inducing cancer cell degradation through induction of a cascade of biochemical reactions, ending in cell death by apoptosis [54, 59].

This interaction between the excited triplet state photosensitizer and the surrounding dissolved oxygen can occur through two different reactions [44]. One of these consists in type I reactions, where the triplet state photosensitizer will transfer a hydrogen atom or an electron to molecular oxygen (or biological substrates), which will lead to the generation of reactive oxygen species (or radical species, which are capable of interaction with oxygen, to form other ROS), such as superoxide [3, 44, 54]. The other reactions are known as type II, being these the main ones associated to photodynamic therapy, where the excited photosensitizer induces an energy transfer into the near triplet oxygen molecules, leading to the generation of singlet oxygen species [3, 44, 54].

These radical molecules, through interaction with cell components such as proteins, lipids, and DNA, will induce photo-oxidative damage, which can cause the specific degradation of cancer cells, activating a biochemical signaling cascade, which ends in caspase-3 activation, that will result in either programmed (apoptosis) or non-programmed (necrosis) cellular decay, at low and high radiation intensity, respectively [59, 60].

The process of apoptosis can occur through distinct pathways. In the intrinsic pathway, which involves mitochondria-mediated signaling, the oxidative damage to DNA will induce p53 activation, responsible for the inhibition of anti-apoptotic proteins (BCL-2) and kinases required for cellular proliferation and survival (B-Raf), leading to caspase 9 activation, which, in turn, will activate caspase 3, causing the degradation of the cellular matrix and consequent programmed death. As for the extrinsic pathway, it presents a process similar to the intrinsic one, being that the main differences consist in the starting signal, which, in this case, corresponds to oligomeric death ligands, such as the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL or CD95L) and the caspase 3 activator, that consists in caspase 8 [59].

The nonprogrammed or necrotic pathway is independent from caspase 8/9, being that it requires, as a starting signal, the synergistic activation of receptor-interacting proteins 1 and 3 kinases, which will be activated through kinase cascade-induced phosphorylation, allowing for the formation of pores in the cellular membrane, inducing its degradation and respective damage response [59].

Additionally, this treatment was also capable of stimulating cytokines formations, which would lead to the enhancement of the immune response against the tumoral cells, promoting their degradation [59].

It is important to note that since healthy cells are not affected, the treated patient will present reduced scaring, promoting a positive cosmetic outcome when compared with the other types of therapies [60].

Another vantage associated to photodynamic therapy corresponds to the fact that it can be combined with other forms of cancer treatment, leading to the reduction of negative side effects associated to the individual application of each treatment [51, 52].

In the case of chemotherapy, it allows for a reduced dose of chemotherapeutic drug, to overcome the multi drug resistance associated to cancer cells, along with the reduction of the dosage necessary to obtain a therapeutic effect, while also being possible to be applied for patients with late-stage disease. With radiotherapy, the combination alleviates the

symptoms and the resulting pain associated to late-stage cancer, being also capable of increasing the sensibility of the damaged cells to radiation. As for immunotherapy, the cell degradation induced by photodynamic therapy can promote the activation of dendritic cells, leading to increased T cell activity, along with the possibility of associating monoclonal antibodies to the photosensitizer, in order to increase its specificity [51, 52].

4.4. The Appearance of New Obstacles

Unfortunately, photodynamic therapy presents new obstacles that need to be overcome. One problem consists in the fact that photosensitizers present maximum absorbance at a wavelength around 400 nm, equivalent to UV light, which is associated to a reduced skin penetration of the photosensitizing radiation. Therefore, they are associated to a limited activity spectrum, being only effective when applied to superficial pathologies (such as skin, esophageal non-small cell lung and other organ outer lining cancer). Other problem observed is that, due to its localized activity, this therapy can't affect metastatic tumors, which are characteristic of a more advanced stage of cancer, leading to a reduced effectivity towards late-stage patients [55, 56].

Another limitation of the photosensitizers is that, due to their slow removal from the organism, they present some degree of toxicity, which could cause problems during treatment. This procedure also requires the main components to be present in the same environment within the tissue to be treated, which can prove quite difficult to occur, since it is possible to occur unintended reactions during the transport.

Taking the existing problems into consideration, the scientific studies turned towards ways of being able to overcome these obstacles, in order to enhance the efficacy of photodynamic therapy towards the treatment of the various types of cancer.

For the limited penetration issue, several researchers proposed the usage of alternative type of radiation for the activation of the photosensitizer [2], such as near-infrared (also presents a reduced penetration, along with inefficient absorption by the photosensitizer, along with the possibility of inducing heat damage towards neighbor healthy tissues) [61], two-photon (the radiation needs to specifically target the damaged tissue, to avoid significant collateral damage) [62] and X-ray (it isn't capable of directly activating the photosensitizer, requiring the presence of an intermediate such

as nanoparticles, which manifest a reduced conversion efficacy to obtain the energy of the correct wavelength) [63].

4.5. Coelenterazine in Photodynamic Therapy for Treatment

For the improvement of photodynamic therapy efficacy, a research topic that is currently of high interest consists in performing this therapy without recurring to an external radiation source (allowing for a self-illuminating process). With this in mind, the molecule known as coelenterazine, due to its chemiluminescent/bioluminescent properties, came as an answer to this problem, since it could be integrated in the procedure, allowing for the creation of a self-illuminating systems [55].

Considering this, the bioluminescent system based on the interaction between *Renilla* luciferase and coelenterazine, characterized by an ATP independence (in contrast to the firefly system), was studied by Lai and co-workers for excitation source activity in photodynamic therapy, [3, 64].

In this study, which was performed in mice transfected with a human lung adenocarcinoma cell line, the enzyme was associated to carboxylate coated quantum dots, which can absorb the emitted photons through bioluminescence resonance energy transfer (BRET), making them the ones responsible for the photosensitizer activation, through a Forster resonance energy transfer [65, 66]. The main advantage of this effect is the fact that quantum dot emission can be more easily modified, allowing for the modulation of the radiation wavelength, in order to promote photodynamic therapy activity [3].

As for the obtained results, this combination presented significant cytotoxicity towards cancerous cell lines, which was superior when compared with the individual application of coelenterazine, while also being capable of inhibiting the formation of new blood vessels in the tumors, leading to their growth suppression [3, 65].

However, despite presenting a luminous emission capable of cytotoxicity of higher intensity when compared with the firefly luciferase-luciferin system, the *Renilla* luciferase-coelenterazine system still presented a photon output slightly lower than the one associated to standard photodynamic therapy [65], which could be derived from the occurrence of two distinct energy transfers, corresponding to BRET and FRET, leading to an efficiency reduction [3].

It should be noted that in the case of the bioluminescent system, since the luminescent reaction is continually activated, in the presence of oxygen, it will allow for the generation of light until the coelenterazine is fully consumed, being this also associated to a higher intensity when compared with chemiluminescence, evidencing the potential for photodynamic therapy [17]. Additionally, it is possible to conjugate the luciferase with quantum dots, allowing for an easier modification of the emission wavelength [65].

However, these bioluminescence-based systems present some problems, which are mainly associated to the reduced penetration and distribution through organic tissues. Therefore, it still would be necessary to improve this system, to obtain a more pronounced photodynamic effect [64].

It should be noted that the chemiluminescent reaction of coelenterazine, contrary to its bioluminescent reaction, can be triggered solely by superoxide anion, which is overexpressed in cancer cells [17, 67]. Additionally, cancer cells present antioxidant mechanism capable of removing this species, which not the case for singlet oxygen (the main cytotoxic ROS in photodynamic therapy) [2]. Given this, if coelenterazine could be modified in order for its chemiluminescent reaction, when triggered by superoxide anion, to generate singlet oxygen (instead of emitting light), a moderate oxidant could be replaced intracellularly by cytotoxic species capable of photodynamic effect. This could be possible if the triplet-to-singlet product ratio of the chemiluminescent reaction could be increased. One strategy for that could be enhancing the rate of intersystem crossing, during the chemiluminescent reaction, with approaches such as the heavy-atom effect [68].

With this in consideration, Pinto da Silva et al. demonstrated that addition of bromine heteroatoms to coelenterazine (with the aim of inducing the heavy-atom effect) resulted in the development of analogues with anticancer activity. One of the studies compared the activity of three novel distinct analogues (Clz-1, Clz-2 and Clz-3), represented in Figure 11, each presenting a bromine heteroatom to promote the heavy atom effect. The molecules were synthetized from the chemical reaction between 3-bromopyrazinamine derivatives and methylglyoxal, with hydrochloric acid (HCl) acting as a catalyst [52].

In terms of the obtained results, these compounds manifested blue light emission (with a wavelength between 420 and 440 nm) upon contact with the superoxide anion, which manifested through a flash profile. This situation confirmed that their chemiluminescent reaction is in fact derived from the presence of superoxide, with Clz-3 manifesting the higher

efficiency while Clz-2 was associated to the lowest, with Clz-1 being slightly lower than Clz-3 [52].

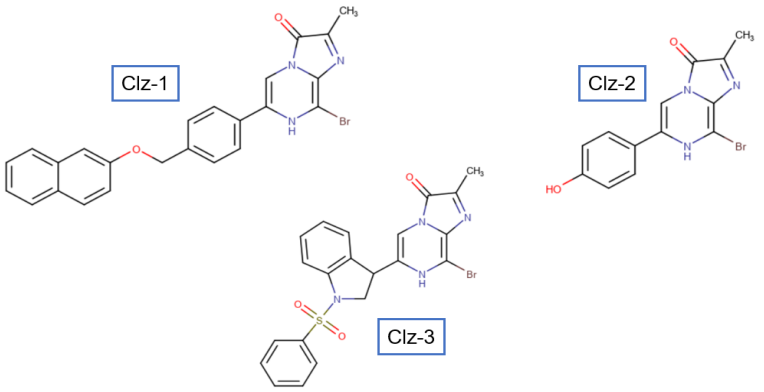

Figure 11. Three newly synthesized coelenterazine analogues which were studied for their anticancer activity.

Additionally, it was observed that the concentration of superoxide in the solution influenced the emission intensity of these molecules, being observed an emission decrease upon anion increase, in the cases of Clz-1 and Clz-3, indicating a susceptibility towards oxidation damage induced by the radical, while the opposite was verified for Clz-2, which showcased an emission increase with the superoxide amount [52].

In terms of the singlet oxygen production, through the usage of a fluorescence probe towards this species, it was verified that Clz-1 presented the highest generation levels, being followed by the other two analogues, which presented similar levels [52].

As for the anticancer activity, the analogues capacity was analyzed through the elaboration of cell viability assays in breast and prostate carcinoma cell lines, with the obtained results present in Table 4. In this case, all compounds presented a significant cell degradation activity seventy-two hours after their application in breast cells, with the required concentration of Clz-2 for the cytotoxic effect being three time lower than that of the reference drug, corresponding to tamoxifen. For the prostate cancer cells, they presented toxicity after only twenty-four hours, albeit in all cases an increase of cytotoxicity was associated to a longer incubation period, being that all three molecules presented a better activity than the reference drug consisting in metformin [52].

Table 4. Concentration levels required for cytotoxic activity by the reference drugs (Metformin and Tamoxifen) and the three newly synthesized coelenterazine analogues [52]

IC50	MCF-7		PC-3	
	24 h	72 h	24 h	72 h
Metformin	Not Determined		1.270 ± 0.416	0.813 ± 0.261
Tamoxifen	2.219 ± 0.194	11.07 ± 0.02	Not Determined	
Clz-1	>100	12.18 ± 0.06	0.048 ± 0.426	12.11 ± 0.15
Clz-2	47.31	3.00 ± 0.08	0.388 ± 0.459	1.647 ± 0.366
Clz-3	>100	49.59	0.530 ± 0.525	3.949 ± 0.362

Similar cell viability assays were performed to analyze the case of combination therapy with the reference drugs. In the case of breast carcinoma, which application of tamoxifen, an enhanced cytotoxicity was observed, being this especially at lower compounds concentration, while in the case of prostate carcinoma, with the usage of metformin, the toxicity levels were identical to the individual cases, being this activity selective towards the cancer cells [52].

Additionally, as showcased in the work by Magalhães et al. [26], the introduction of halogen atoms in novel analogues, based on the ones mentioned previously, could promote the transition into the excited triplet state, enhancing the anticancer activity and highlighting the importance of the heavy-atom effect for the successful elimination of the tumor cells by this family of compounds, along with the potential of the analogues for treatment.

Therefore, this situation indicates that the novel coelenterazine analogues were in fact capable of reacting with the superoxide anion dissolved in the cellular environment, showing their capacity of leading towards cancer remission, which will contribute towards the development of treatment procedures, such as external light-free photodynamic therapy.

4.6. Superoxide Anion: Ally and Enemy

ROS correspond to a group of molecules derived from an incomplete one electron reduction of a dioxygen molecule (O_2). This group of compounds presents a potent oxidative activity due to the presence of unpaired electrons.

Of this family, the one that presents a higher importance for the human organism is superoxide anion [27, 69].

First identified in 1968 by McCord and Fridovich, the superoxide anion, as mentioned previously, consists in a mildly reactive nucleophilic ion species, with a short half-life in aqueous solutions, being that in can be further converted into paramagnetic free radicals (hydroxyl radical) and diamagnetic molecules (hydrogen peroxide), with both types of compounds presenting a highly reactive nature [27].

In terms of production, as observed in eukaryotic cells, superoxide is mainly produced during the metabolic processes associated to mitochondrial respiration, being derived from the electron transport chain that occurs between complexes I and II. The radical can also be generated through reactions catalyzed by the action of certain enzymes, such as NADPH oxidase, xanthine oxidase and cytochrome P450 [69].

The characteristics manifested by the superoxide radicals confer to it the ability of acting as a signaling molecule in biochemical reactions essential for the normal functioning of the human organism. One of the main effects of this radical consists in the activation of the ras/rac-Raf1-MAPK pathway, which is responsible for the transductions of signals associated to the regulation of the cell cycle, tissue repair and cell migration to promote cell growth [69].

Other important role of this anion consists in the regulation of epigenetic process, through the modulation of DNA methylation and histone methylation/acetylation, being that superoxide anion can remove the proton of the C_5 position of cytosine, which will facilitate the introduction of methyl groups. Similarly, the anion also presents a role in its removal through oxidation of the group, in a procedure catalyzed by iron-dependent ten-eleven translocations dioxygenases family, which consists in a sequential oxidation of the methyl groups, leading to their conversion into hydroxy methyl and carboxyl groups [70, 71].

However, when the anionic radical is present in a very high amount in the cellular environment, it will present a significant danger towards the neighbor cells. In this situation, superoxide will interact and remove hydrogen atoms associated to essential macromolecules, such as DNA, RNA, fatty acids, and proteins, which will lead to their abnormal functioning and consequent degradation, leading to a signaling cascade for the activation of the programed cell death, known as apoptosis [70].

In this situation of overexpression, superoxide will also lead to a mutation that will cause the Ras protein to favor its active conformation,

even in the absence of growth factors, leading to an uncontrollable growth of the cell, which will eventually give rise to cancerous tumors [70].

To prevent these disturbances, the cells present an antioxidant system capable of removing excess superoxide present in the environment. This process is based on the sequential activity of two enzymes: first, the superoxide dismutase (SOD) will induce the reduction of the superoxide anion radical into hydrogen peroxide (H_2O_2), being this followed by the action of glutathione peroxidase (GPx), requiring glutathione as a substrate, in order to reduce H_2O_2 into water, which can be safely removed from the organism through the excretory system [70].

Additionally, superoxide anion is usually overexpressed in cases of certain pathologies, such as inflammation, diabetes mellitus and cancer [15], being this caused by the enhanced activity of NADPH oxidases [72, 73]. Therefore, due to its intrinsic association to these diseases, it is quite important to achieve its detection, which can occur through methods classified in two distinct fields [73].

4.7. Superoxide Anion Sensing Methods

Spectrophotometric methods are based on the measurement of the absorbance in the visible and ultraviolet range for the detection of superoxide, since this anion, upon reacting with probes such as Nitroblue tetrazolium, will form a colored complex that can be detected [74, 75]. The main advantage of this procedure is the fact that it allows for a monitorization of the species during a long period. However, it is also susceptible to interference from the solution environment, which could lead to the formation of overlapping spectrums, complicating the detection [73].

As for non-spectrophotometric methods, which don't require radiation absorption, these can be further divided in three different classes. The first one consists in the electrochemistry-based methods, capable of real-time detection, which encompasses the sensing of superoxide through the redox potential (with the employment of a molecule that will be reduced upon contact), direct detection (through reduction, while also allowing stability monitoring) and the application of a biosensor [73].

One other procedure corresponds to the analysis of light intensity derived from a chemical reaction involving the presence of the superoxide anion, which is known as luminescence. This term can be further divided in the procedures of electroluminescence, photoluminescence (based on the

application of a anion specific fluorescent probe, known as hydroethidine,) and chemiluminescent (which is associated to the highest sensitivity when compared with the previous methods, leading to reduced interference, being that it can be further improved with the integration of nanomaterials) [73].

For biologic systems, such as the human organism, sensing through chemiluminescence presents significant advantages since, along with its superior sensitivity, it is capable of specifically interacting with the intercellular sites where the generation of superoxide occurs, leading to an enhanced detection [73].

With this in consideration, the analysis of chemiluminescent molecules started to be promoted, to improve superoxide anion detection. In 1993, the experimental work pertaining to the first molecule to successfully be applied as a chemiluminescent probe towards this anion was published, corresponding this compound to CLA, shortly followed by the molecule known as MCLA [17]. The study was based on the application of this probe in stimulated Kupffer cells of a rat liver, which promotes its production of superoxide, being that MCLA was capable of sensing the radical with a higher sensitivity when compared with CLA [17]. Afterwards, in 1998, derivates of MCLA were produced, presenting an even higher sensitivity [25]. However, they presented a luminous emission which was not suitable for the application in biological tissues. Therefore, it still is required the investigation of new types of probes, capable of bypassing the existing problems [76, 77].

4.8. Coelenterazine as a Superoxide Probe for Diagnosis

The other main application to be described here for coelenterazine and analogues is that as sensing probe for superoxide anion [78].

As mentioned previously, it was noted that coelenterazine presents sensitivity for superoxide, making it capable of specifically targeting cells affected by certain pathologies, such as inflammation, diabetes melitus and cancer, since these present an increased production of reactive oxygen species, due to their modified metabolism [15, 79]. This property would allow for a more selective chemiluminescent reaction, with reduced potential side effects, leading to the emission of light that could be picked up by a detector for disease diagnosis [80].

In previous works, coelenterazine and its derivatives, such as coelenterazine-v and coelenterazine-h, were applied towards the sensing of

superoxide, through their chemiluminescent reaction. This procedure generally resulted in a significant capacity for its detection [27, 81].

However, coelenterazine-based compounds present a significant problem, consisting in the fact that the emission in an aqueous environment is attenuated [17, 82]. Therefore, it would be necessary perform additional studies to achieve a way of enhancing the luminous emission of this family [83].

With this in consideration, our investigation group synthetized a novel coelenterazine analogue known as MeOBr-Cla, through modifications on the C_6 positions, showcased in Figure 12. The molecule presented an intense light emission, which could solve the problem of reduced emission. Therefore, this compound was analyzed to verify if was capable of acting as a probe towards superoxide [82].

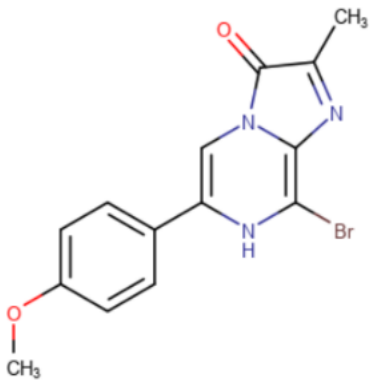

Figure 12. Schematic representation of the novel coelenterazine analogue known as MeOBr-Cla.

This compound was produced through a Suzuki-Myaura cross coupling induced between a commercial sample of 5-bromopyrazin-2-amine and a specific phenylboronic acid, which led to the generation of the first intermediate, corresponding to a coelenteramine known as MeO-Clm. Afterwards, a bromide heteroatom was introduced into the molecule near the amine group, through the addition of a N-Bromosuccinimide. Finally, a cyclization reaction, with HCl acting as a catalyst, was performed with methyglyoxal to yeld the pretended MeOBr-Cla, being that its correct synthesis was confirmed through $^{1}H/_{1}C$ NMR and FT-MS spectroscopy [82].

The MeOBr-Cla analogue, according to the results present in scientific article by José Pedro Silva et al. in protic conditions (water), manifested a

very high increase of luminescence upon contact with the superoxide anion, with an intense decay after the initial contact, indicating an instant chemiluminescent reaction [82].

In terms of the environment surrounding the reaction, studies involving three solutions for each pH range (acidic, neutral, and basic, respectively) were performed. It was observed that an increase of the solution's pH would result in the decrease of light emission, as showcased in Table 5. As for the initial reaction velocity, it was verified to superior in the acidic medium, with decreasing values at higher pH [82].

Table 5. Luminescence output (intensity and area), values of MeOBr-Cla, in aqueous solutions of different pH, with increasing superoxide anion amounts [82]

Superoxide Anion (mg)	Emission Intensity	Emission Area
Acetate Buffer pH 5.2		
5	$2.38 \times 10^6 \pm 1.30 \times 10^5$	$8.15 \times 10^6 \pm 2.54 \times 10^6$
10	$2.05 \times 10^6 \pm 3.92 \times 10^4$	$4.00 \times 10^6 \pm 2.38 \times 10^5$
15	$1.72 \times 10^6 \pm 6.99 \times 10^4$	$2.20 \times 10^6 \pm 1.75 \times 10^5$
Phosphate Buffer pH 7.4		
5	$9.87 \times 10^5 \pm 9.00 \times 10^4$	$1.89 \times 10^6 \pm 1.64 \times 10^5$
10	$5.64 \times 10^5 \pm 2.97 \times 10^4$	$1.02 \times 10^6 \pm 1.25 \times 10^5$
15	$7.69 \times 10^5 \pm 4.16 \times 10^4$	$1.25 \times 10^6 \pm 5.39 \times 10^4$
NaOH 0.1 M		
5	$5.39 \times 10^3 \pm 3.05 \times 10^2$	$9.69 \times 10^5 \pm 8.55 \times 10^4$
10	$3.76 \times 10^3 \pm 1.17 \times 10^2$	$5.77 \times 10^5 \pm 4.32 \times 10^4$
15	$3.59 \times 10^3 \pm 1.00 \times 10^2$	$4.45 \times 10^5 \pm 7.88 \times 10^3$

This luminescence and initial velocity reduction with the increase of the environment basicity degree could be derived from 2 possible factors. In a way, it is known that at a higher pH the analogue molecule will acquire a negative charge, since the imidazopyrazinone core will become ionized due to the deprotonation of the NH group, which would cause a repulsion effect towards the superoxide anion (due to also presenting a negative charge), preventing the interaction and formation of the intermediate dioxetanone. Another possible explanation is the fact that the basic pH of the solution will induce the formation of an anionic dioxetanone, which is associated to a less effective pathway for the generation of the light-emitting molecule [82].

In terms of the emission intensity, it was noted that increasing amounts of superoxide anion resulted in luminescence variations, being that higher

amounts of the anion would result in a reduction of the light emission derived from the novel analogue, being this effect observed for all pH ranges. This decrease indicates that the molecule presents a susceptibility towards oxidation damage induced by the superoxide, being this behavior in contrast to what can be observed with the original coelenterazine molecule [82].

Additionally, when compared with a commercial sample of native coelenterazine (Clz), which consists in a commonly utilized probe towards superoxide, MeOBr-Cla presented an identical chemiluminescent profile to Clz, with similar emission intensity variations, albeit with a significantly higher photon output in all pH media, which can be seen on Table 6. Interestingly, it was noted that the compounds presented opposite behaviors with increasing amounts of the anionic radical, being that, with its increase, the commercial sample manifested an increased light production, indicating that it isn't as susceptible to oxidation damage. Despite that, its emission intensity still lower than that of the novel analogue, highlighting the potential of this molecule as a probe towards superoxide [82].

Table 6. Luminescence output (intensity and area), values of MeOBr-Cla, relative to Clz, in aqueous solutions of different pH, with increasing superoxide anion amounts [82]

Superoxide Anion (mg)	Emission Intensity	Emission Area
Acetate Buffer pH 5.2		
5	$1.11 \times 10^4 \pm 2.31 \times 10^3$	$3.16 \times 10^3 \pm 1.42 \times 10^3$
10	$6.80 \times 10^3 \pm 5.10 \times 10^2$	$1.04 \times 10^3 \pm 1.53 \times 10^2$
15	$5.18 \times 10^3 \pm 5.57 \times 10^2$	$3.96 \times 10^2 \pm 7.03 \times 10^1$
Phosphate Buffer pH 7.4		
5	$6.62 \times 10^2 \pm 1.12 \times 10^2$	$1.49 \times 10^2 \pm 3.04 \times 10^1$
10	$2.45 \times 10^2 \pm 2.54 \times 10^1$	$1.11 \times 10^2 \pm 2.72 \times 10^1$
15	$1.29 \times 10^2 \pm 2.72 \times 10^1$	$5.30 \times 10^1 \pm 5.25 \times 10^0$
NaOH 0.1 M		
5	$2.53 \times 10^1 \pm 5.91 \times 10^0$	$9.43 \times 10^1 \pm 2.77 \times 10^1$
10	$2.13 \times 10^1 \pm 3.39 \times 10^0$	$9.81 \times 10^1 \pm 1.73 \times 10^1$
15	$2.46 \times 10^1 \pm 3.26 \times 10^0$	$8.05 \times 10^1 \pm 1.24 \times 10^1$

To confirm that these chemiluminescent characteristics were in fact derived from the intrinsic properties of MeOBr-Cla, similar assays to the previous ones were performed with the usage of an aprotic solvent, with the same pH ranges, in the absence of the superoxide anion. The aprotic solvent

was chosen since it is known that this type of environment can trigger a spontaneous chemiluminescent reaction, with a stable detectable emission [82, 83].

In these conditions, it was verified that MeOBr-Cla still presented an intense light emission associated to a quick burst, in all pH values, identical to what was observed with the protic solvent, which is present in Table 7. However, the main difference consisted in the decay rates after the initial emission, which were significantly slower in this environment, being this translated into a longer half-life time. The slowest rate observed at higher pH, with acidic and neutral pH presenting similar decay values [82].

Table 7. Luminescence output (intensity and area), velocity and duration for MeOBr-Cla in DMF solutions of different pH [82]

Solvent	Emission Intensity (RLU)	Emission Area	Initial Velocity (RLU/s)	Half-Life (ms)
DMF + Acetate Buffer pH 5.2	$4.77 \times 10^4 \pm 2.68 \times 10^3$	$9.98 \times 10^6 \pm 5.53 \times 10^5$	$9.92 \times 10^3 \pm 1.77 \times 10^3$	$251^a \pm 5$
DMF + Phosphate Buffer pH 7.4	$3.23 \times 10^4 \pm 2.15 \times 10^3$	$7.14 \times 10^6 \pm 4.61 \times 10^5$	$2.70 \times 10^4 \pm 7.33 \times 10^3$	$546^a \pm 90$
DMF + NaOH 0.1 M	$5.67 \times 10^4 \pm 7.24 \times 10^3$	$6.85 \times 10^6 \pm 5.75 \times 10^5$	$4.69 \times 10^4 \pm 9.28 \times 10^3$	107.05 ± 14.70

Interestingly, faster kinetics of the chemiluminescent reaction were observed with the increase of the pH, which means that the production of the chemiexcited compound and emission of light would be more rapidly achieved. Despite that, this information indicates that the luminescent emission occurs during a longer period, rather than an emission of higher intensity, being this supported by the slower decay. The situation is most likely derived from the chemical equilibrium between the neutral and anionic dioxetanone intermediate [82].

It was also observed, through the chemiluminescent emission spectra, that, in the acidic environment, the two overlapping peaks at around 410 and 460 nm were present, which indicated the existence of two distinct species, corresponding to the neutral and anionic amine groups, respectively, of the chemiexcited product. As for the basic medium, only a single peak was observed on the spectrum, with the emission wavelength corresponding to 460 nm, meaning that the only existing species consisted in the anionic coelenteramide derivative, since these conditions are associated to a complete deprotonation [82].

Taking this into consideration, along with the previous studies, the MeOBr-Cla analogue does in fact present a chemiluminescent reaction, influenced only by its intrinsic properties, that acts as a response towards the presence of the superoxide anion, along with a quite intense light emission in aqueous environment, when compared with coelenterazine and other analogues previously applied in the field of detection.

Therefore, this novel coelenterazine analogue would be capable of acting as a sensing probe, capable of detecting the presence of superoxide anion, through interaction with this species that results in intense light emission, capable of being detected by measuring systems. This activity would allow for an enhanced detection of superoxide in cases where it is overexpressed, such as the ones mentioned previously, corresponding to inflammation, diabetes melitus and cancer. With all of this said, MeOBr-Cla consists in a molecule that allowed for a significant advance in the field of superoxide anion-related diagnosis (as in cancer) [82].

Conclusion

The chemiluminescent and bioluminescent systems involving the molecule known as coelenterazine present unique properties, which are associated to its reactional mechanism, that acts as a response towards the presence of oxygen-based species, resulting in a product capable of light emission.

With this in consideration, this compound presents a potential for the application in the field of cancer treatment, since the light emitting product can experience an intersystem conversion into the excited triplet state, capable of ROS production with tumor cell elimination activity. This transition was also observed to be promoted by the introduction of halogen atoms in novel coelenterazine analogues, due to a phenomenon known as the heavy-atom effect, leading to an enhanced degradation of cancer cells.

The light emission by itself is also quite valuable, since, in the chemiluminescent system, it results from the interaction with the superoxide anion, which is overexpressed in certain diseases, such as inflammation, diabetes melitus and cancer. Therefore, coelenterazine was also successfully applied as a probe capable of sensing the presence of superoxide anion, with marked improvement already underway, mainly in the single molecule application and detection in aqueous environment, through the introduction of specific groups in the creation of new analogues.

Therefore, it can be said that in the field of luminescent based chemical reactions, the coelenterazine family of chromophores presents interesting properties which are capable of resonating with markers characteristic of cancer, leading to a marked improvement in the existing diagnostic and therapy methods, along with the possibility of paving the way for the future development of novel processes of higher efficacy.

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