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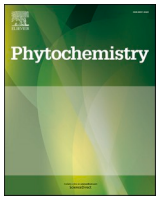

A review of nemorosone: Chemistry and biological properties

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ABSTRACT

Nemorosone is a bicyclic polyprenylated acylphloroglucinol derivative originally isolated from *Clusia* spp. and it can be obtained through chemical synthesis employing different synthetic strategies. Since its discovery, it has attracted great attention both from a biological and chemical viewpoint. In the present article, we attempted to review various chemical and biological topics around nemorosone, with an emphasis on its antiproliferative activities. For this purpose, relevant data was collected from different scientific databases including Google Scholar, PubMed, Scopus and ISI Web of Knowledge. This natural compound has shown activity against several types of malignancies such as leukemia, human colorectal, pancreatic, and breast cancer because it modulates multiple molecular pathways. Nemorosone has both cytostatic and cytotoxic activity and it also seems to induce apoptosis and ferroptosis. Additionally, it has antimicrobial capabilities against Gram-positive bacteria and parasites belonging to genus *Leishmania*. Its promising antiproliferative pre-clinical effects deserve further attention for anticancer and anti-parasitic drug development and translation to the clinic.

1. Introduction

Nemorosone, a bicyclic polyprenylated acylphloroglucinol (BPAP), was first reported from four species of the genus *Clusia* (Oliveira et al., 1996). It is found in some species of Higher Plants belonging to the Clusiaceae family and it is a usual component of *Clusia* floral resins. Nemorosone is also present in tropical propolis from several countries including Cuba, Brazil and Venezuela, because these floral resins are used by female bees for the construction of the nest (Cuesta-Rubio et al., 2002a,b; Trusheva et al., 2004; Ishida et al., 2011).

Since its discovery, nemorosone has attracted attention both from a biological and chemical viewpoint. In the last two decades, nemorosone has been proven to show cytostatic and cytotoxic activity against several cell lines (Andreu et al., 2015; Frión-herrera et al., 2020; Holtrup et al., 2011; Popolo et al., 2011). On the other hand, its characteristic bicyclic system bearing acyl and prenyl substituents has made nemorosone a challenging starting point for synthetic chemistry efforts (Simpkins et al., 2010; Tsukano et al., 2007; Uwamori et al., 2012; Wen et al., 2019).

Some reviews have been published that cover both chemical and biological properties of polycyclic polyprenylated acylphloroglucinols (PPAPs) including nemorosone (Ciochina and Grossman, 2006; Phang et al., 2020; Yang et al., 2018). However, to the best of our knowledge, no comprehensive review specifically focusing on nemorosone is available in the recent literature. This review is an attempt to address this gap as it provides an extensive overview of the current knowledge regarding this compound. The natural occurrence, chemical structure, biosynthesis, chemical synthesis and biological activity of nemorosone during the last decades are covered in this work.

2. Methodology

An extensive survey of the “Clusiaceae”, “acylphloroglucinols”, “nemorosone”, was conducted in multiple bibliographic databases

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(including Embase, Medline, Scopus, and Web of Science Core Collection), chemistry databases (SciFinder-n, Reaxys) and gray literature sources. The search terms "Clusiaceae", "acylphloroglucinols", "nemorosone", "anti-cancer" were used for data collection. In total 110 publications were included from year 1996 to Marchr 2023. Eligible work of any study type included primary research data on nemorosone relevant to chemistry, biological and anti-cancer activity.

3. Natural occurrence

Nemorosone (1) is found in plants belonging to the Clusiaceae family (Fig. 1), and it is one of the major constituents of floral resins of *Clusia* spp. (Cuesta-Rubio et al., 2001; Oliveira et al., 1996, 1999). Initially, it was isolated as a methyl derivative from *C. rosea* Jacq, *C. grandiflora* Splitg., *C. insignis* Mart and *C. nemorosa* G. Mey, (Oliveira et al., 1996). Additionally, honey bees land on flowers of *Clusia* spp. in tropical countries to collect resins and produce propolis (Roubik, 1992). For this reason, female bees contribute to increasing both the natural sources of nemorosone and its intake in communities that use propolis for medicinal purposes. To the best of our knowledge, the ingestion of *Clusia* floral resins is not a practice in traditional medicine. Therefore, honey bees could contribute in a decisive way in the consumption of nemorosone by human beings.

It is very interesting to note that although several PPAPs containing a bicyclo[3.3.1]nonane-2,4,9-trione core are present in different genera of Clusiaceae such as *Hypericum*, *Garcinia*, *Rheedia* and *Monorobea* (Yang et al., 2018), nemorosone has been only reported from genus *Clusia* thus far.

4. Chemical structure

Nemorosone, a mixture of two tautomers 1-benzoyl-3,5,7-tri-(3-methyl-2-butenyl)-4-hydroxy-8,8-dimethylbicyclo[3.3.1]non-3-en-2,9-dione and 1-benzoyl-3,5,7-tri-(3-methyl-2-butenyl)-2-hydroxy-8,8-dimethylbicyclo[3.3.1]non-2-en-4,9-dione, belongs to a group of natural products that contain acylphloroglucinol cores decorated with isoprenyl or geranyl side chains, named PPAPs. From a structural perspective, the PPAPs group contains very dissimilar compounds related by a common biosynthetic pathway. Nemorosone is a water-insoluble compound, but soluble in different organic solvents such as methanol, ethanol, chloroform and n-hexane. In nature, the compound is a mixture of two tautomers, as a white powder or crystals (Cuesta-Rubio et al., 2001).

Nemorosone features a bicyclic core with a benzoyl group at C-1 and three prenyl side chains attached at C-3, C-5 and C-7 (Fig. 1). The prenyl side chains at C-3 and C-5 are prone to be oxidized and further cyclized with *O*-2 or *O*-4 to form additional heterocycles, mainly furan and pyran derivatives. The compound is stable in alcohols, both in pure solutions or ethanol extracts, but it has been proven to be unstable in some apolar solvents. Interestingly, some compounds isolated from different plants, such as garcinielliptone l, hyperinone B and propolone A, are obtained when nemorosone is dissolved both in *n*-hexane and chloroform (Piccinelli et al., 2009). Therefore, some of these compounds might well be artefacts originated as a result of the methods employed to extract or purify them.

Nemorosone has three stereogenic centers (C-1, C-5 and C-7), and so there are a theoretical total of 8 optical stereoisomers, but the existence of bridged cycles reduces the possibilities to 4 stereoisomers only. In general, the optical stereoisomers isolated from natural sources are named as nemorosone, *ent*-nemorosone and 7-*epi*-nemorosona, respectively. Clusianone and 7-*epi*-clusianone are the unique constitutional isomers of nemorosone reported so far. Nemorosone II has been obtained by chemical synthesis, but it has not been isolated from natural sources (Taylor, 2011).

Pagano et al. (2008) carried out a structural and conformational investigation of nemorosone using a combination of X-ray and quantum mechanical methods (Pagano et al., 2008). The basic bicyclic ring system in nemorosone required that the substituents at C-1 and C-5 are equatorial. One of the rings adopts a half-chair conformation while the other exhibits a chair-boat conformation equilibrium. Unlike 7-*epi*-clusianone, in which both conformers are nearly isoenergetic, the nemorosone chair conformer shows to be more stable that the boat one (~16 kcal/mol) (Piccinelli et al., 2005). This behavior seems to be determined by the configuration at C-7: in nemorosone chair conformer the prenyl group is equatorial (exo) while in 7-*epi*-clusianone is axial (endo). As expected, 1,3-diaxial repulsions present in 7-*epi*-clusianone chair conformer decrease its stability, and a similar behavior to 7-*epi*-nemorosone would be expected. Therefore, the configuration of the stereogenic center at C-7 seems to play an important role in the conformational equilibrium of these bicyclo[3.3.1]nonane derivatives.

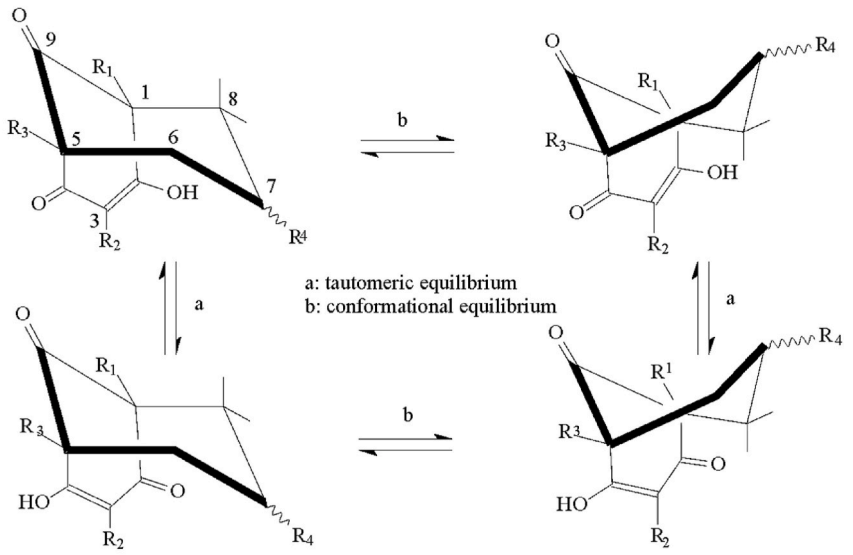
In general, the relative stereochemistry of chiral carbon at C-7 can be easily determined by different NMR considerations: (a) $^{3}J_{H6ax\text{-}H7}$ is 6–8 Hz when the C-7 substituent is in the axial position (endo) and 10–13 Hz when this one is in the equatorial position (exo) (Cuesta-Rubio et al., 2005), respectively; (b) the difference in chemical shift of the two H-6 atoms (0.3–1.2 ppm, exo; 0,0-0,2 ppm; endo) and the chemical shift of C-7 (41–44 ppm, exo; 45–49 ppm, endo), the later known as Grossman and Jacobs' rule (Ciochina and Grossman, 2006; Grossman and Jacobs, 2000).

5. Classification and biosynthetic pathway

In an initial classification published in 2001, some bicyclo[3.3.1]nonane derivatives were divided into types A, B and C depending on the relative position of the benzoyl group in benzophenone derivatives, and the absence of type C was suggested (Cuesta-Rubio et al., 2001). Subsequently, the classification system was improved considering that the acyl group varies in PPAPs (Ciochina and Grossman, 2006). Eventually, Yang et al. (2018) provided a general classification for PPAPs and suggested that all of them may be divided into three groups (I-III): group I contains BPAPs and related *seco*-BPAPs; group II includes derivatives with adamantane and homoadamantane skeletons, whereas group III contains other related metabolites derived from MPAPs (Yang et al., 2018). The initial 2001 classification of types A and B is still, but as subclassification. Nowadays, it is accepted that nemorosone belongs to group I (Type A), which comprises the highest percentage of PPAPs known so far (Yang et al., 2018). It is very important to note that the last compounds initially classified as type C PPAPs were later revised to corresponding type A structures using NMR spectroscopic and quantum computational chemistry methods (Yang et al., 2017).

The classification system mentioned above uses a biosynthetic criterion. Biosynthetically, it has been suggested that all PPAPs have a common pathway that involves condensation of three malonyl-CoA units and one acyl-CoA unit (Adam et al., 2002; Phang et al., 2020; Yang et al., 2015, 2018). Thus, the initial polyketide cyclizes into acylphloroglucinol via Dieckmann condensation, originating a common precursor which may later undergo C-alkylation with prenyl or higher diphosphates and cyclization to originate diverse skeletons. The biosynthetic pathway that has been proposed by several authors demands the existence of type A or B BPAPs only, regardless of the substituent position (at C-3 or C-5, phloroglucinol moiety) involved in the two possibilities of cyclization originating the bicyclic skeleton (Fig. 1). Besides, the position of the prenyl group at C-7 in BPAPs can be *endo* or *exo*. The phloroglucinol moiety shows a symmetric substitution patten, in which three phenol groups activate the ring carbons at the *ortho* and *para* positions, so that both C-3 and C-5 become equally susceptible to alkylation. In the case of nemorosone and its optical stereoisomers, C-alkylation with prenyl and isogeranyl groups is suggested, alongside only one cyclization (Fig. 1).

A



Compound	R_1	R_2	R_3	R_4
Nemorosone	Benzoyl	Prenyl	Prenyl	Prenyl (endo)
7-*epi*-nemorosone	Benzoyl	Prenyl	Prenyl	Prenyl (exo)
Clusianone	Prenyl	Benzoyl	Prenyl	Prenyl (endo)
7-*epi*-clusianone	Prenyl	Benzoyl	Prenyl	Prenyl (exo)

B

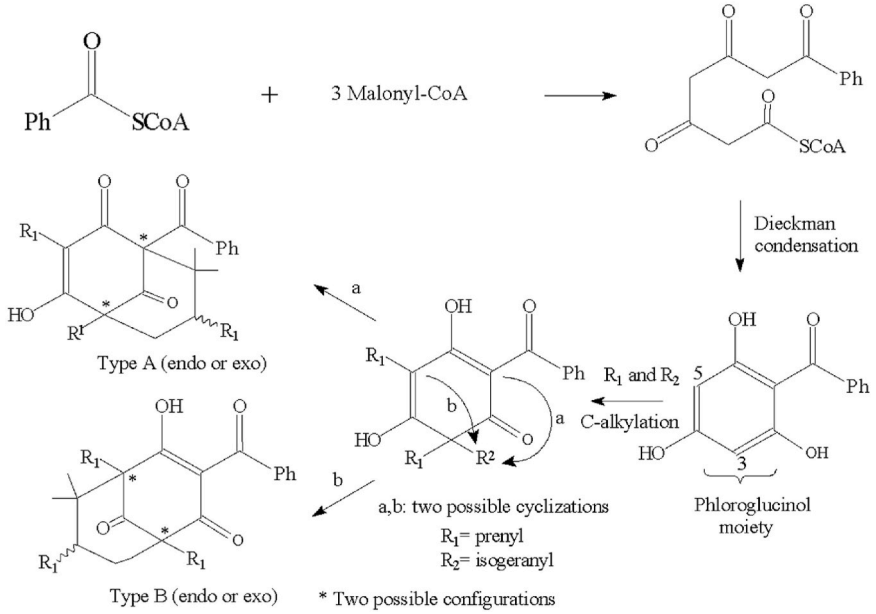


Fig. 1. A: Nemorosone and its main isomers in conformational and tautomeric equilibriums. B: Biosynthetic pathway originating type A and B bicyclic polyprenylated acylphloroglucinol (BPAPs) with a bicyclo[3.3.1]nonane core.

6. Chemical synthesis

Nemorosone, exhibits an oxygenated and highly functionalized bicyclo[3.3.1]nonane-2,4,9-trione core and a broad range of biological activities, in the same manner as other PPAPs. Both considerations have drawn significant attention in organic synthesis. Initially, much of the synthetic investigation was focused toward the construction of the bicyclo[3.3.1]nonane core due to the complexity of forming this bicyclic skeleton with the correct positioning of the substituents (Ciochina and Grossman, 2003; Kraus et al., 2003; Nicolaou et al., 2005; Spessard and Stoltz, 2002). Some research teams have developed a variety of synthetic methodologies for the construction of the bicyclo[3.3.1]nonane core or the total syntheses of BPAPs. The first total synthesis of nemorosone starting from 3,5-dimethoxyphenol was developed by Danishefsky's group, 11 years after its isolation from *Clusia* spp. (Tsukano et al., 2007). Several comprehensive review papers that cover the chemical syntheses of PPAPs have been published (Ciochina and Grossman, 2003; Njardarson, 2011; Phang et al., 2022; Richard et al., 2012; Simpkins, 2013; Tsukano et al., 2010; Yang et al., 2018). Because of that, the present review provides only a short-summarized comparison of the total synthesis strategies employed to construct nemorosone and its optical stereoisomers (Table 1).

To date, both nemorosones (*ent*-nemorosone and (±)-7-*epi*-nemosone) have been obtained by total synthesis employing different synthetic strategies; Simpkins et al. (2010); Sparling et al. (2015); Uwamori et al. (2012); Wen et al. (2019); Zhang and Porco (2012). Undoubtedly, several ingenious methods to functionalize the phloroglucinol moiety at multiple positions have been carried out, and it is worth highlighting the obtained results. As expected and due to the complexity of the total synthesis, all strategies are characterized by numerous steps (7–29), the use of a wide variety of chemicals, wide temperature ranges between −78 and 120 °C and low overall yield (<10%). On the other hand, nemorosone can be obtained with high yield (30–45%) by direct crystallization from *C. rosea* floral resins (unpublished results), which makes this natural source very attractive in relation to the synthetic one. *C. rosea* has a wide distribution in Cuba because this tree is employed as an ornamental plant. Some public entities guarantee its reproduction, commercialization and distribution. Each tree produces hundreds of flowers every year and from each flower about 2 g of floral resins can be collected. Its propagation by branch cuttings constitutes another favorable aspect for the reproduction and conservation of this species. Therefore, it seems that there are already some solid conditions which will assure an adequate collection of floral resins from *C. rosea* in the future.

Table 1
Total syntheses of nemorosone and its optical stereoisomers carried out by different investigation groups.

Synthesis strategies[a]	Key steps and main characteristics	Reference
Danishefsky group	Starting with 3,5-dimethoxyphenol. The key skeleton-building stages were allylative de-aromatization and iodinative cyclization. 17 steps. (±)-nemorosone and (±)-clusianone are obtained from a common intermediate.	Tsukano et al. (2007)
Simpkins group	Synthesis via a bridgehead substitution process, involving initial iodination and subsequent lithium-iodine exchange followed by acylation. 17 steps. A versatile intermediate is transformed into (±)-nemorosone and (±)-clusianone.	Simpkins et al. (2010)
Nakada group	Starting with methyl 2,6-dimethoxy benzoate. Three main steps: intramolecular cyclopropanation, stereoselective alkylations and regioselective ring-opening of cyclopropane. 29 steps. Produce (±)-nemorosone	Uwamori et al. (2012)
Porco group	Starting with 5-methoxyresorcinol. Key steps include retro-aldol-vinyl cerium addition to a hydroxy adamantane core scaffold and palladium-mediated deoxygenation. 12 steps. Produce (±)-7-*epi*-nemorosone.	Zhang and Porco (2012)
Shair group	Starting with isoprenyl alcohol. A scalable Lewis acid catalyzed epoxide-opening cascade cyclization is employed. 15 steps. Nemorosone and hyperforin are obtained from a common intermediate. Produce (−)-nemorosone (*ent*-nemorosone).	Sparling et al. (2015)
Porco group	Starting with 5-methoxyresorcinol. Regiodivergent photocyclization of dearomatized acylphloroglucinol substrates using an excited-stated intramolecular proton transfer process. 7 steps. Shortest synthesis to date. Produce (−)-nemorosone.	Wen et al. (2019)

[a] Formal syntheses were not included.

7. Pharmacological activity of nemorosone

The role of natural compounds as drugs or as a basis for the development of new drugs has been recently reviewed by Newman and Cragg (2020). According to this analysis, 929 products have a natural origin and 952 are classified as synthetic drugs with natural product pharmacophores or as drugs that mimic a natural product (Newman and Cragg, 2020). In correspondence with the increased popularity of specialised metabolites from plants for drug design (Veeresham, 2012), the pharmacological activity of nemorosone has been investigated. Currently, this compound has been shown several therapeutic activities, mainly of antiproliferative nature (Fig. 2). Other activities have also been reported in the scientific literature, which will be also commented.

7.1. Therapeutic potential of nemorosone in cancer

Since the first reports on the biological activities of nemorosone, the antimicrobial property of this compound was recognized as a natural mechanism of self-protection of bees (resin-collecting bees that use nemorosone to build their nest) against pathogens (Lokvam et al., 2000; Oliveira et al., 1996). Shortly after, its cytotoxic activity against epithelioid carcinoma (HeLa), epidermoid carcinoma (Hep-2), prostate cancer (PC-3) and central nervous system (U251) cancer cells was demonstrated (Cuesta-Rubio et al., 2002a,b). Following these initial works, a significant number of studies have been carried out that also support the anti-neoplastic and apoptosis-inducing effects of nemorosone in various systemic malignancies such as leukemia, neuroblastoma, colorectal, breast, pancreatic, and hepatic cancer (Table 2) (Díaz-Carballo et al., 2008a; Díaz-Carballo et al., 2008b; Holtrup et al., 2011; Frión-herrera et al., 2020; Frión-Herrera et al., 2019a; Frión--Herrera et al., 2019b; Pardo-Andreu et al., 2011; Popolo et al., 2011).

On the other hand, despite the fact that nemorosone has been mentioned in several reviews (mainly those focused on the properties of propolis) (Fernández-Acosta et al., 2019; Patel, 2016; Salatino et al., 2011; Shrestha et al., 2021; Silva-Carvalho et al., 2015; Watanabe et al., 2011), no systematic review of the literature has focused exclusively on the anticancer potential of nemorosone and its molecular mechanisms of action. Therefore, the aim of this section is to integrate and analyze the existing information on the efficacy and safety of nemorosone in the treatment of cancer, as well as to establish its molecular targets. Table 2 shows a chronological summary of the different anticancer effects reported for nemorosone, organized according to the types of cells studied; while Fig. 3 shows the main molecular pathways modulated by nemorosone in cancer cells. Both Table 3 and Fig. 3 aim to summarize and clarify the state of the art on the anticancer effects of nemorosone.

7.2. Cell cycle regulation

Cancer is a group of diseases in which the components of the cell cycle machinery are frequently altered due to the accumulation of driver mutations (Molinari, 2000; Otto and Sicinski, 2017). This can result in a

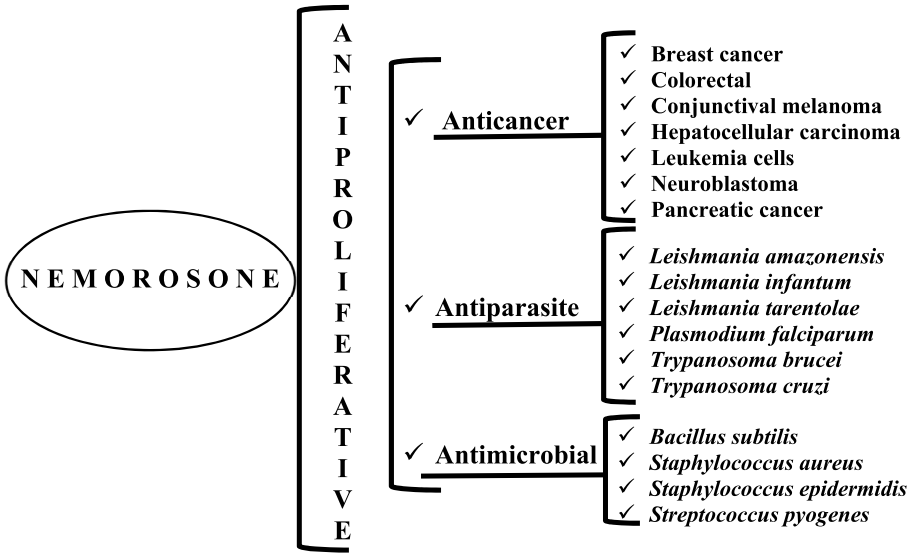


Fig. 2. Main biological targets of nemorosone related with its antiproliferative activity by importance/diversity order.

total cell division rate that exceeds the total cell death rate, leading to uncontrolled growth within the host tissue (Anttila et al., 2019). Consequently, the search for antitumor agents that act at different phases of the cell cycle has been explored as a central strategy in cancer therapy.

Nemorosone was recognized as both a cytostatic (decreases cell division rate) and cytotoxic (increases cell death rate) compound since early investigations. The cytocidal effect was reported for the first time by Díaz-Carballo et al. in two works published in 2008. Nemorosone was shown to control cell cycle progression from G1 to S phase, i.e., it induced an accumulation of human neuroblastoma (LAN-1) cells in G0/G1 phase with a parallel reduction in S phase (Díaz-Carballo et al., 2008b). Nemorosone-induced tumor cell cycle progression arrest was associated with increased expression of p21, a cyclin-dependent kinase inhibitor that is capable of inhibiting all cyclin/CDK complexes required for cell proliferation (Xiong et al., 1993). In this regard, nemorosone was found to modulate the MAPK/ERK pathway (also known as the Ras-Raf-MEK-ERK pathway), a signal transduction pathway that controls cellular processes such as proliferation, differentiation, development, stress response, and apoptosis (Rubinfeld and Seger, 2005). Specifically, nemorosone inhibited the kinase activity of MEK1/2 and reduced the phosphorylation status of ERK1/2, while it worked synergistically with BAY 43–9006, an inhibitor of Raf-1 which acts upstream of ERK1/2. Furthermore, it inhibited Akt/PKB kinase activity. This protein can phosphorylate p21 and attenuate its inhibitory activity, which in turn can result in uncontrolled cell proliferation (Rössig et al., 2001). As a possible result of nemorosone-induced ERK1/2 and Akt/PKB modulation, N-myc protein levels were reduced. N-myc has been identified as a limiting factor of cell cycle progression, as it accelerates progression into S phase (Lutz et al., 1996).

Similar results were obtained in the second study (Díaz-Carballo et al., 2008a). A G1 arrest was detected in Jurkat and K-562 leukemia cells, while a significant depletion of Jurkat cells in S phase was also found. The cell cycle arrest was explained by the downregulation of protein levels of cyclins (A, B1, D1, and E) and downregulation, as well as dephosphorylation, of CDC2, a nuclear protein essential for mitosis that acts as a regulatory element of cyclins A, B and E (Aleem et al., 2005; Ducommun et al., 1991). The turn-off of the transcription of cyclins was associated with downregulation of ERK1/2, Stat3 and p38 MAPK, protein elements of the MAPK/ERK pathway.

A third independent study (Dal Piaz et al., 2010) proposed a link between nemorosone-induced cell cycle arrest and its capacity to interact with histone acetyltransferase p300 (p300/HAT). p300/HAT is an enzyme that functions as histone acetyltransferase acetylating histone and non-histone substrates to regulate a plethora of fundamental biological processes including cell growth, development, oncogenesis and apoptosis (Zhang et al., 2014). In this regard, nemorosone was shown to increase the population of HeLa cells in G1 phase and decrease it in S phase, while it efficiently interacted with p300 acting as an enzyme activator and enhancing histone acetylation in the cells (Dal Piaz et al., 2010). Surprisingly, nemorosone was the only type A BPAP derivative (among the eight tested) without secondary cyclization and with a free enol function (no compromise of the hydroxyl group), and the only one that interacted with p300, indicating a critical role for the core hydroxylated enol involving C_4 in the interaction with p300. The results of Dal Piaz et al. (2010), regarding the structure-activity relationship, are consistent with those obtained in the first investigation on the biological properties of nemorosone, which indicated that the lack of tautomeric equilibrium, induced by methylation of the hydroxyl group, severely affects the antioxidant and cytotoxic activity of nemorosone (Cuesta-Rubio et al., 2002a,b).

Nemorosone was also reported to increase the population of several pancreatic cell lines in the G1 phase, while at higher concentrations a slight G2 arrest was instead observed (Holtrup et al., 2011). In this study, microarray experiments were performed to identify the molecular action pathways underlying nemorosone-induced growth inhibition. A general repression of cyclins and cyclin-dependent kinases (CDKs), involved in all phases of the cell cycle, was observed. Particularly, the upregulation of *CDKN1A* (gene encoding p21 protein) and the repression of *E2F* (a group of genes involved in the cell cycle regulation and synthesis of DNA), as well as cyclin D1 and *CDK4* downregulation, were linked to the impact of nemorosone on G1/S phase check point. Likewise, nemorosone induced the expression of *GADD45a* (growth arrest and DNA damage inducible alpha protein coding gene) and the repression of *CDC2*, *CDC25B* and cyclin B, which may block cell progression through G2 phase.

Other studies have also established the capacity of nemorosone to inhibit cell cycle progression, as well as the molecular mechanisms involved in this effect. Nemorosone-induced accumulation of MCF7 cells (from ATCC) in the G0/G1 phase and reduction of the S phase fraction was also associated with downregulation of the phosphorylated form of ERK1/2 (p-ERK1/2) and inhibition of the expression of phosphorylated Akt/PKB, which may correspond to a decrease in its kinase activity (Popolo et al., 2011). Another study carried out on MCF7 BUS cells [BUS refers to the stock from which the cells come (Villalobos et al., 1995)] showed that nemorosone induced a discrete arrest of the cell cycle in the

Table 2
Nemorosone as a natural cytotoxic compound against cancer cells. IC_{50} values correspond to 24 h of treatment unless otherwise indicated.

Cancer type	Cell lines_ in *vitro* (animal models _*in vivo*)	IC_{50} [μM] (assay type)	Effects	References
Larynx carcinoma	HEp-2	3.1 ± 0.17[a] (SRB assay)	Cytotoxicity	Cuesta-Rubio et al. (2002a,b)
Prostate carcinoma	PC-3	7.2 ± 1.30[a] (SRB assay)		
Central nervous system carcinoma	U251	3.9 ± 1.40[a] (SRB assay)		
Cervix carcinoma	HeLa	3.3 ± 0.17[a] (SRB assay)	Cytotoxicity	Cuesta-Rubio et al. (2002a,b)
		5.2 ± 0.5 (trypan blue exclusion test)	Cell cycle arrest (increase of cells in G1 phase and decrease in S-phase) associated with p300 activation	Dal Piaz et al. (2010)
		2.9 ± 1.1[b] (resazurin proliferation assay)	Cytotoxicity similar to other nemorosone isomers	Simpkins et al. (2012)
Neuroblastoma	LAN-1 MDR1-	4.10 ± 0.28 (SRB assay)	Apoptosis induction (DNA damage, increase of caspase-3 activity, and increase of cells in sub-G1 phase); G0/G1 arrest and decrease of cells in S phase; inhibition of topoisomerases activity (100 μM); inhibition of telomerase activity (10-fold the IC_{50}); upregulation of p21; inhibition of Akt/PKB and MEK1/2 kinase activities; dephosphorylation of ERK1/2; synergistic effect with BAY 43–9006 (Raf-1 inhibitor); downregulation of N-myc oncoprotein.	Díaz-Carballo et al. (2008b)
	LAN-1 CP	4.22 ± 0.26 (SRB assay)	Cytotoxicity	
	LAN-1 ETO MDR1+	4.99 ± 0.22 (SRB assay)	Cytotoxicity; downregulation of N-myc oncoprotein.	
	LAN-1 ADR MDR1+	4.92 ± 0.36 (SRB assay)	Cytotoxicity	
	LAN-1 5FU	4.12 ± 0.31 (SRB assay)	Cytotoxicity	
	NB69	3.10 ± 0.15 (SRB assay)	Cytotoxicity	
	Kelly	5.2 ± 0.42 (SRB assay)	Cytotoxicity	
	SK-N-AS	6.3 ± 0.21 (SRB assay)	Cytotoxicity	
	IMR32	-(fluorometric method based on SYTOX Green dye)	Ferroptosis associated with glutathione depletion	Fernández-Acosta et al. (2023)
Leukemia	HL60 WT	2.66 ± 0.06 (MTT assay)	Cytotoxicity	(Díaz-Carballo et al., 2008a)
	HL60 ADR MDR1+	2.56 ± 0.05 (MTT assay)	Cytotoxicity	
	CEM WT	3.10 ± 0.11 (MTT assay)	Cytotoxicity	
	CEM VBL MDR1+	3.00 ± 0.06 (MTT assay)	Cytotoxicity	
	Jurkat WT	2.30 ± 0.08 (MTT assay)	G0/G1 arrest and decrease of cells in S phase; downregulation of cyclins A, B1, D1, and E; downregulation and dephosphorylation of cdc2; downregulation of GSK-3b (slight effect), ERK1/2, Stat3, and p38 MAPK; downregulation of c-Myb (leukemia-specific oncoprotein); inhibition of Akt/PKB kinase activity.	
	Tanoue WT	2.10 ± 0.05 (MTT assay)	Cytotoxicity	
	Kasumi WT	2.60 ± 0.25 (MTT assay)	Cytotoxicity	
	K-562 WT	2.10 ± 0.06 (MTT assay)	Apoptosis induction (phosphatidyl serine translocation and increase of cells in sub-G1 phase); G0/G1 arrest; downregulation of cyclins A, B1, D1, and E; downregulation and dephosphorylation of cdc2; downregulation of p38 MAPK; downregulation of BCR/ABL (leukemia-specific oncoprotein).	
	(NMRI nu/nu mice) 100 mg/kg of nemorosone per day. Administered i.p. over 15 days in a single dose per day regimen		Reversible monocytosis and thrombocytosis induced by sub-chronic nemorosone treatment in mouse model.	
	NB4	4.8 ± 0.5 (trypan blue exclusion test)	Cytotoxicity associated with p300 activation	(Dal Piaz et al., 2010)
Colorectal cancer	HCT-116	6.8 ± 0.3 (trypan blue exclusion test)	Cytotoxicity associated with p300 activation	
	LoVo Dox	65.8 ± 10.7 (MTT assay)	Nem-Dox combination (synergistic effect): Apoptosis (phosphatidyl serine translocation); G0/G1 arrest; ROS production increase; ΔΨm increase.	Frión-Herrera et al. (2019b)
	LoVo WT	62.7 ± 7.5 (MTT assay)		
	HT-29	64.3 ± 4.7 (MTT assay) 57.1 ± 3.7 (MTT assay)	Apoptosis (phosphatidyl serine translocation and activation of caspase 3/7); inhibition of clonogenic capacity; G0/G1 arrest; downregulation of *BCL2* and upregulation of *TP53* and *BAX*; attenuation of cell migration and invasion; inhibition of MMP9 activity; increase of E-cadherin and decrease of β−catenin and vimentin expression.	Frión-Herrera et al. (2020)
Non-small cell lung cancer	NCI-H460	5.0 ± 0.3 (trypan blue exclusion test)	Cytotoxicity associated with p300 activation	(Dal Piaz et al., 2010)
Pancreatic cancer	AsPC-1	16 ± 1.5 (resazurin proliferation assay)	Apoptosis (phosphatidyl serine translocation, DNA fragmentation, cytochrome *c* release, and caspase activation); extrinsic apoptosis (involving caspase-8 activation) was detected only in AsPC-1 and Capan-1 cell	Holtrup et al. (2011)
	Capan-1	25 ± 2.0 (resazurin proliferation assay)		

(*continued on next page*)

Table 2 (*continued*)

Cancer type	Cell lines_ in *vitro* (animal models _*in vivo*)	IC_{50} [μM] (assay type)	Effects	References
	MIA-PaCa-2	16 ± 1.0 (resazurin proliferation assay)	lines; G0/G1 arrest and slight G2 arrest at higher concentrations; ΔΨm decrease; increase of cytosolic calcium concentrations; regulation of genes associated with ER stress, UPR, apoptosis, and cell cycle (*ATF4*, *DDIT3*, *ATF3*, *HSPA5* …).	
		2.4 ± 1.1[b] (resazurin proliferation assay)	Cytotoxicity similar to other nemorosone isomers	Simpkins et al. (2012)
	(MIA-PaCa-2 xenograft mice) 50 mg/kg of nemorosone per day. Administered i.p. over 28 days in a single dose per day regimen.	–	No expression of *CYP3A4* in primary human hepatocytes; well tolerated (hyperventilation shortly after injection, normal respiration was restored again 5–10 min after i.p. application); inhibition of xenograft tumors growth; apoptosis (caspase-3 activation); rapid absorption into the bloodstream; half-life of approximately 30 min.	Wolf et al. (2013)
Hepatocellular carcinoma	HepG2	-(MTT assay)	Apoptosis (phosphatidyl serine translocation); ΔΨm and ATP levels decrease.	Pardo-Andreu et al. (2011); Reis et al. (2014); Andreu et al. (2015)
		-(MTT assay)	Repression of HepG2-induced THP-1 cell recruitment and differentiation; inhibition of the proliferation, colony formation and migratory capacity; G0/G1 arrest.	(Frión-Herrera et al., 2019a)
–	Mitochondria isolated from rat liver	–	Protonophoric mitochondrial uncoupling; ΔΨm decrease; induction of mitochondrial Ca^{2+} release; prevention of mitochondrial Ca^{2+} uptake; ATP levels decrease; mitochondrial swelling; slight reduction of H_2O_2 levels; depletion/oxidation of NAD(P)H.	Pardo-Andreu et al. (2011); Reis et al. (2014); Andreu et al. (2015)
Breast cancer	MCF-7	-(MTT assay)	Cytotoxicity: reduced by E2 and enhanced by ICI 182,780; G0/G1 arrest and decrease of cells in S phase (enhanced by ICI 182,780 and reverted by E2); downregulation of pAkt and pERK1/2 (potentiated by ICI 182,780).	Popolo et al. (2011)
		5.2 ± 1.1[b] (resazurin proliferation assay)	Cytotoxicity similar to other nemorosone isomers	(Simpkins et al., 2012)
	MCF-7 BUS[b]	–	Discrete arrest in G0/G1 phase and decrease of cells in G2/M phase; regulation of genes associated with cell cycle, apoptosis and hormone receptors (*TP53*, *MLH1*, *MYC*, *CCND1*, *CCNE1*, *ESR1*).	Camargo et al. (2015)
Conjunctival malignant melanoma	CRMM-1	25.3 ± 1.7 (SRB assay)	Cytotoxicity	Westekemper et al. (2013)
	CRMM-2	12.9 ± 3.1 (SRB assay)	Cytotoxicity	
Fibrosarcoma	HT1080	16.7 ± 0.2 (fluorometric method based on SYTOX Green dye)	Ferroptosis: decrease in glutathione levels and increase in labile intracellular Fe^{2+} pool via heme oxygenase-1	Fernández-Acosta et al. (2023)

Abbreviations: ADR (Dox): Adriamycin (doxorubicin); ICI 182,780: Fulvestrant (estrogen receptor antagonist); E2: 17β-estradiol; ER: Endoplasmic reticulum; MDR: Multidrug resistance; MMP9: Matrix metalloproteinase 9; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; Nem: Nemorosone; ROS: Reactive oxygen species; SRB: Sulforhodamine B; UPR: Unfolded protein response; VBL: Vinblastine; WT: Wildtype (parental); ΔΨm: Mitochondrial membrane potential. "Between parenthesis are the reported animal models in column 2, and the type of *in vitro* assay in column 3"

[a] 48 h of incubation with nemorosone.

[b] 72 h of incubation with nemorosone.

G0/G1 phase alongside a significant depletion of the G2/M phase population (Camargo et al., 2015). In this case, nemorosone-induced upregulation of *TP53,* gene encoding tumor suppressor p53 protein, and *MLH1*, which cooperates with *TP53* to promote cell cycle arrest and cell death, was associated with the effect of nemorosone on cell progression. This is consistent with the observations described above, as p53-mediated cell cycle arrest is primarily mediated by transcriptional activation of p21(Cheng et al., 1993). Regulatory genes encoding cyclin D (*CCND1*) and cyclin E1 (*CCNE1*) were also downregulated following nemorosone treatment. More recent studies have also reported the capacity of nemorosone to induce cell cycle arrest in the G0/G1 phase in colorectal carcinoma (LoVo and HT29) and hepatocarcinoma (HepG2) cells (Frión-herrera et al., 2020; Frión-Herrera et al., 2019a; Frión--Herrera et al., 2019b).

7.3. Induction of apoptotic cell death

The cytotoxic effect of nemorosone on several cancer cell lines was published in 2002 and 2003 by independent researchers. Cuesta-Rubio et al. (2002a,b) reported the effect on cervix, larynx, prostate, and central nervous system carcinoma cell lines, while Díaz-Carballo et al. (2003) showed the cytotoxic activity against breast, colon, ovary, liver and lung carcinoma cell lines (Cuesta-Rubio et al., 2002a,b; Díaz-Carballo et al., 2003). However, the first description of a cell death mechanism occurred in 2008 with the two works, previously addressed, published by Díaz-Carballo.

Both studies concluded that nemorosone induced an apoptotic pattern of cell death (Díaz-Carballo et al., 2008a; Díaz-Carballo et al., 2008b). In addition to the induction of DNA fragmentation and caspase-3 activity, the apoptotic effect of nemorosone on neuroblastoma cells was associated with a drastic decrease in N-myc protein levels (Díaz-Carballo et al., 2008b), oncoprotein whose downregulation, in addition to its effect on cell cycle arrest, has also been associated with caspase-3 activation and apoptosis induction (Cheng et al., 1993; Kang et al., 2006). In leukemia cell lines, nemorosone induced downregulation of BCR/ABL fusion protein (K-562 cells) and c-Myb protein (Jurkat cells) (Díaz-Carballo et al., 2008a), both oncoproteins that mediate antiapoptotic responses, conferring cells increased aggressiveness and resistance to chemotherapy (Lauder et al., 2001; McGahon et al., 1997; Ramsay et al., 2003). Overall, these two studies proposed that nemorosone cytotoxicity is possibly mediated by inhibition of the kinase activity of one or more of the upstream elements of ERK1/2 and

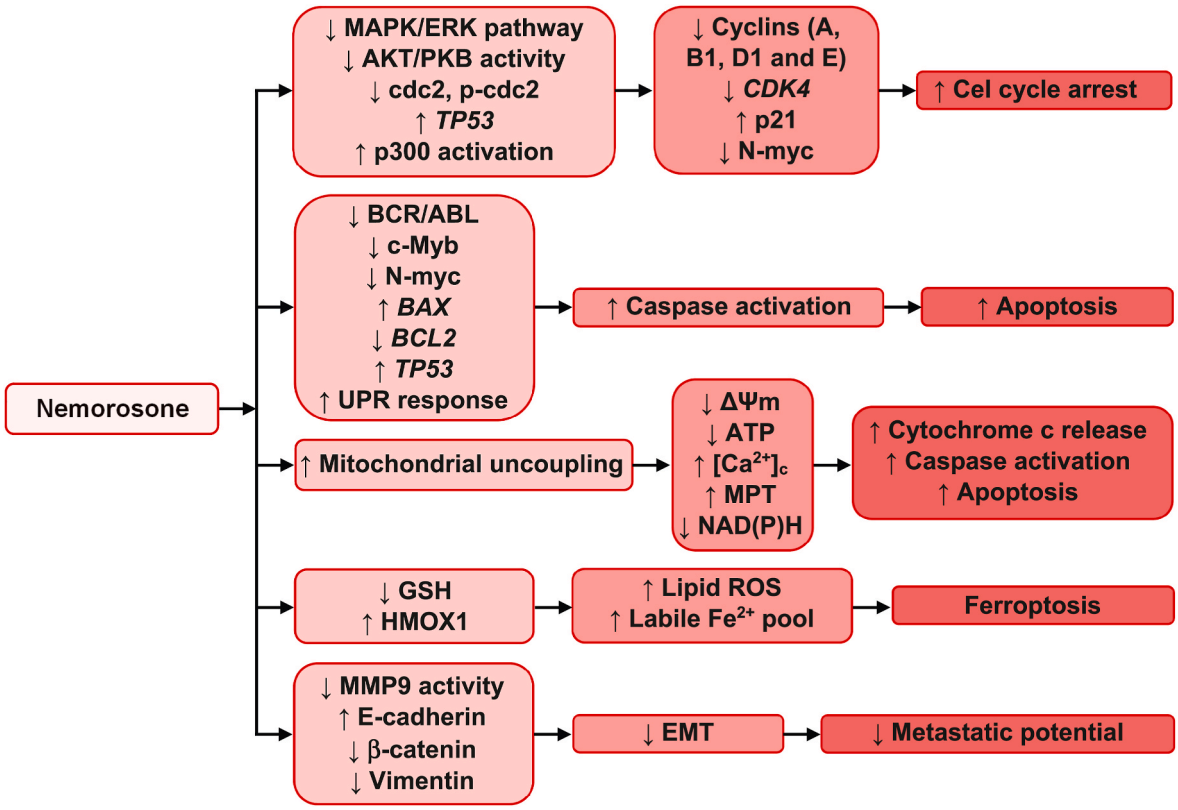


Fig. 3. Schematic representation of the molecular pathways modulated by nemorosone in cancer cells. The color gradation indicates the transition from cause to effect, that is, it relates the sequence of molecular events that occur from the start of treatment with nemorosone to the appearance of antiproliferative effects (cell cycle arrest, apoptosis or ferroptosis) and the antimetastatic potential. $[Ca^{2+}]_c$: cytosolic calcium concentration; ΔΨm: mitochondrial membrane potential; EMT: epithelial-mesenchymal transition; GSH: glutathione; MPT: mitochondrial permeability transition. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Cytotoxicity of nemorosone in nontumorigenic cells. IC_{50} values correspond to 24 h of treatment.

Cell type (acronym)	IC_{50} [μM] (assay type)	References
Mouse fibroblasts (NIH-3T3)	>21.0 (SRB assay)	(Díaz-Carballo et al. (2008b))
Human embryonic fibroblasts (MRC-5)	>40.0 (SRB assay)	
Human dermal fibroblasts (HDF)	80.0 ± 4.5 (resazurin proliferation assay)	Holtrup et al. (2011)
Human foreskin fibroblasts (HFF)	73.0 ± 4.0 (resazurin proliferation assay)	
Human embryonic kidney 293 cells (HEK293)	>25.0 (MTT assay)	Pardo-Andreu et al. (2011)
Mouse hippocampal cells (HT22)	>100.0 (fluorometric method based on SYTOX Green dye)	Fernández-Acosta et al. (2023)

the Akt/PKB cascade (Díaz-Carballo et al., 2008a; Díaz-Carballo et al., 2008b).

The induction of apoptosis by nemorosone in pancreatic cancer cells was evidenced by phosphatidylserine translocation, DNA fragmentation, mitochondrial cytochrome *c* release, and caspase activation (Holtrup et al., 2011). Specifically, nemorosone induced both intrinsic (involving release of cytochrome *c* and caspase 9) and extrinsic (involving caspase 8) apoptosis, depending on the cellular context. It was also found that nemorosone triggered a rapid and complete abolition of the mitochondrial membrane potential (ΔΨm), which was hypothesized to be a direct cause of the nemorosone-induced increase in cytosolic calcium concentration due to release from intracellular stores, mainly from the endoplasmic reticulum (ER). This proposition was based on the fact that nemorosone activated the unfolded protein response (UPR), an ER stress response pathway that can be triggered by alterations in calcium levels. Consistent with this, nemorosone treatment differentially regulated many genes involved in UPR signaling, such as *DDIT3* (DNA damage-inducible transcript 3), which accumulates in the cell and initiates the proapoptotic branch of the UPR (Kim et al., 2008). To validate the observation of UPR-induced apoptosis, *DDIT3* knockdown cells were treated with nemorosone, which resulted in a partial inhibition of caspase 9 and 3/7 activation and a reduction in DNA fragmentation.

Interestingly, almost at the same time, other research addressing related topics was published. Nemorosone was shown to act as a potent protonophoric mitochondrial uncoupler, both in HepG2 cells and in isolated rat liver mitochondria (RLM), which was associated with dissipation of ΔΨm, release of calcium from calcium-laden mitochondria and decreased calcium uptake by the mitochondria (Pardo-Andreu et al., 2011). Considering both studies, it can be suggested that nemorosone disrupts calcium signaling by targeting both cytoplasmic compartments (ER and mitochondria) and perhaps by interfering with the interorganellar Ca^{2+} signaling, also known as ER-mitochondrial Ca^{2+} communication (White, 2017). Through the experimental approach in RLM it was also shown that nemorosone reduces ATP levels, induces NAD(P)H depletion/oxidation and triggers mitochondrial swelling, sensitive to cyclosporin A, which may be associated with modulation of the mitochondrial permeability transition pore and induction of apoptotic cell death (Andreu et al., 2015; Pardo-Andreu et al., 2011; Pestana et al., 2009; Reis et al., 2014).

As a continuation to the analysis of the anticancer activity of nemorosone in pancreatic cancer cells, the effect on pancreatic cancer xenografts (NMRI nu/nu mice) was evaluated. Daily injection via i.p. of 50

mg/kg of nemorosone resulted in a significant and complete abrogation of xenograft tumor and, at the same time, treatment was well tolerated as body weight remained stable over the 28-day treatment period (Wolf et al., 2013). Histological analysis showed extensive apoptotic and necrotic sections in the nemorosone-treated tumors, while immunohistochemical staining revealed caspase-3 activation. As part of this study, a pharmacokinetic analysis was performed, which showed that nemorosone is rapidly absorbed and oxidatively metabolized independently of CYP3A4 with a half-life of approximately 30 min. Altogether, this study confirmed that nemorosone is a potential anti-cancer lead compound with good bioavailability, and promising growth-inhibitory effects. Nevertheless, its potential toxics side-effects related with its mitochondrial bioenergetics-interfering actions should be addressed at early stages of the drug development process.

In line with this, several studies have reported that nemorosone may have therapeutic application in the treatment of breast cancer because of its activity on estrogen receptor (ERα) (Camargo et al., 2013, 2015; Popolo et al., 2011) which has been associated with the promotion of cell proliferation and the appearance of breast and endometrial cancer. In (ERα+) MCF7 cells, nemorosone-induced cell cycle arrest was reverted by 17β-estradiol (E2) and enhanced by ICI 182,780 (a high-affinity agonist of the membrane estrogen receptor) (Popolo et al., 2011). The cytotoxic effect of nemorosone on MCF7 cells was also significantly reduced by E2 and significantly enhanced by ICI 182,780, while it was completely absent in Erα-cells (MDA-MD-231 and LNCaP cancer cells). In addition, nemorosone-induced reduction of the expression of key proteins of the estrogen signaling pathway (pERK1/2 and pAkt) was potentiated by ICI 182,780. Later work showed that nemorosone possesses antiestrogenic activity (it reduced E2-induced cell proliferation), and does not have genotoxic properties, suggesting that nemorosone could be a promising adjuvant to estrogen receptor antagonists in the prevention or treatment of ERα+ breast cancer (Camargo et al., 2013). Finally, nemorosone was found to downregulate the gene that encodes the estrogen receptor α, while it upregulated *ESR2* gene (Camargo et al., 2015), which encodes ERβ, associated with inhibition of ERα-dependent cell proliferation and prevention of cancer development (Visser et al., 2013).

More recently, three consecutive works published by Frión-Herrera et al.(2019a, 2019b, 2020) have expanded the knowledge about the anticancer properties of nemorosone as an inducer of apoptosis. First, nemorosone was shown to act synergistically with doxorubicin in cell cycle arrest, cytotoxicity through induction of apoptosis, ROS production, and ΔΨm increase (Frión-Herrera et al., 2019b). Notably, this chemosensitizing activity was observed in both doxorubicin-resistant (LoVo Dox) and sensitive (LoVo) human colon carcinoma cells. It is notable that LoVo Dox cells treated with nemorosone alone, unlike LoVo cells, showed an increase in ΔΨm, which was also observed in co-treatment of both cell lines with doxorubicin. How nemorosone, a natural protonophoric mitochondrial uncoupling compound (Pardo-Andreu et al., 2011), induces hyperpolarization instead of depolarization is a question that needs to be addressed in further studies. However, the fact that nemorosone treatment alone induced apoptotic cell death in LoVo Dox cells shows that nemorosone-induced hyperpolarization may play a role in inducing cytotoxicity.

The second work, showed the role of nemorosone in preventing tumor-associated macrophages (TAMs)-induced tumor growth (Frión-Herrera et al., 2019a). Tumors can establish an almost symbiotic relationship with their host that can lead, among other effects, to the recruitment of monocytes and the induction of TAMs, which in turn stimulates cancer cell proliferation (Prenen and Mazzone, 2019). Nemorosone counteracted the recruitment and differentiation to macrophages of THP-1 (human monocytic leukemia cell line) induced by HepG2 cells. Furthermore, nemorosone inhibited colony formation and migratory capacity of HepG2 cells, driving a high percentage of cells to the G0/G1 phase (Frión-Herrera et al., 2019a).

Finally, the effect of nemorosone on human colorectal cancer cells was addressed again, obtaining additional results (Frión-Herrera et al., 2020). Regarding the induction of apoptosis, nemorosone negatively regulated the expression of the antiapoptotic gene *BCL2* and increased the expression of the proapoptotic genes *TP53* and *BAX*, with the consequent activation of caspase 3/7. However, nemorosone not only exhibited antiproliferative activity in HT29 and LoVo cells through cell cycle arrest and apoptosis induction, but also decreased their angiogenic and metastatic potential by modulating epithelial mesenchymal transition (EMT)-related molecules such as E-cadherin, cell membrane β-catenin and vimentin, as well as it affected the expression of matrix metallopeptidase 9 (MMP-9), involved in the breakdown of the extracellular matrix.

In addition to the effect of nemorosone as a pure compound, the anticancer activity of propolis, a bee-processed product of plant origin harvested across the globe, has also been analyzed. Depending on the botanical sources and the geographical origin, biological activities of propolis vary; however, in general, nemorosone is considered one of the predominant bioactive components (Patel, 2016). Indeed, various investigations have shown that the anticancer activity of propolis is closely related to the content of nemorosone, even when nemorosone is not the only anticancer compound present in this natural matrix. Consistent with this, propolis has been associated with antiproliferative effects in, for example, hepatocellular, colorectal, and breast cancer cells (Andreu et al., 2015; Frión-herrera et al., 2020; Popolo et al., 2009). Furthermore, also similar to nemorosone, propolis extracts have been shown to induce apoptosis and act as chemosensitizing agents against drug-resistant human cancer cells (Frión-herrera et al., 2020; Frión-Herrera et al., 2019b; Popolo et al., 2009).

7.4. Induction in ferroptotic cell death

A recent study expanded the anticancer potential of nemorosone by showing that this compound is also capable of inducing non-apoptotic cell death, namely ferroptosis (Fernández-Acosta et al., 2023). Ferroptosis is an iron dependent cell death driven by excessive peroxidation of poly unsaturated fatty acids of membranes (Dixon et al., 2012; Wiernicki et al., 2020). Ferroptosis induction has been repeatedly suggested as a cutting-edge strategy in cancer treatment due, for example, to the possibility of treating tumor cells resistant to apoptotic cell death (Zhang et al., 2022). As mentioned, the induction of ferroptosis by nemorosone has just been reported (Fernández-Acosta et al., 2023). Nemorosone triggered a double-edged mechanism of ferroptosis in fibrosarcoma (HT1080) and *MYCN*-amplified neuroblastoma (IMR32) cancer cells consisting of a decrease in glutathione levels (GSH) and an increase in labile intracellular Fe^{2+} pool via heme oxygenase-1 (HMOX1), all of which were suggested to be associated with the induction of mitochondrial uncoupling (Fernández-Acosta et al., 2023).

Greco et al. (2021) added nemorosone to a considerably large list of antitumor compounds derived from natural sources capable of triggering non-apoptotic cell death (Greco et al., 2021). Additionally, it shows the lack of a complete characterization of the antitumor activity of nemorosone and uncoupler compounds in general. Given the progress made in elucidating the different mechanisms of regulated cell death and understanding the crosstalk between them, nemorosone-induced cell death should be studied in the light of these emerging experimental approaches. In fact, the conclusions of previous investigations that relate the cytotoxic activity of nemorosone and uncouplers only with the induction of apoptosis, should be reviewed.

On the other hand, this capacity to trigger both apoptosis and ferroptosis has also been found for erastin and cisplatin, suggesting that cell death mechanisms depend on cell type and, in general, on the cellular context (Guo et al., 2018; Huo et al., 2016; Wang et al., 2020). The study of the different cell death subroutines that nemorosone could induce would make possible to expand its applications as a therapeutic agent against cancer.

7.5. Nemorosone as a potential tumor-selective cancer treatment

After reviewing the anticancer activity of nemorosone (Table 2 and Fig. 3), it is essential to evaluate the feasibility of using this compound as a cancer therapy. Traditional cancer treatments, based on the proliferative advantage of tumor cells over normal cells, are often limited by undesirable damages of normal cells (side-effects). On the contrary, tumor-selective cancer treatment can be achieved by targeting pathways essential for cancer cells survival, but not for normal cells. To date, nemorosone has been shown to be a selective cytotoxic agent against malignant cells.

Potency values (IC_{50}) determined for nemorosone in cell lines from healthy tissue, such as human dermal fibroblasts (HDF), human foreskin fibroblasts (HFF), embryonic fibroblasts (MRC-5) and human embryonic kidney cells (HEK293) (Table 2) have been shown to be 7–10 times higher than potencies measured in human neuroblastoma (LAN-1, SK-N-AS, Kelly and NB69), pancreatic (MIA-PaCa-2, AsPC-1 and Capan-1) and hepatocarcinoma (HepG2) cancer cell lines (Andreu et al., 2015; Díaz-Carballo et al., 2008b; Holtrup et al., 2011; Pardo-Andreu et al., 2011). In line with this, several molecular effects induced by nemorosone, such as growth inhibition, cytochrome *c* release, caspase activation, abolition of $\Delta\Psi m$, and increase of cytosolic calcium concentration, were minor or absent in normal fibroblast cells compared to cancer cells (Holtrup et al., 2011).

Interestingly, the selectivity observed in nemorosone, also typical of other mitochondrial uncouplers, has been explained on the basis of the inherent differences that exist between the mitochondria of cancer cells with respect to those of normal cell lines. First, uncouplers seem to preferentially accumulate in the mitochondrial matrix of malignant cells due to differences in the negative membrane potential of cancer cells (around −220 mV) compared to normal cells (around −140 mV). The more aggressive the cancer, the more hyperpolarized the membrane potential, and greater selectivity can be achieved through the use of uncoupler compounds (Fernández-Acosta et al., 2019; Chen et al., 2019; Heerdt et al., 2005, 2006; Shrestha et al., 2021). On the other hand, cancer cells acidify the extracellular medium. However, these cells, like normal cells, require a neutral cytoplasm (Casey et al., 2010). Therefore, cancer cells have to maintain a neutral intracellular pH versus an acidic extracellular pH (Gerweck et al., 2006). Then, it is possible that the action of protonophoric uncouplers, which exchange protons and are weak acids, affects this homeostasis in its transit towards the mitochondria. Furthermore, extracellular acidification has been proposed as a fundamental physiological feature (of cancer cells) to account for the selectivity and preferential accumulation of nemorosone (Pardo-Andreu et al., 2011). This acid context could favor the sequestration and protonated state of weak acids such as nemorosone and thus facilitate their passage through the inner mitochondrial membrane.

In addition to the low cytotoxicity towards healthy cells, it was also found that nemorosone can reduce the mutagenic damage of mitomycin C, which is a chemotherapeutic agent to treat, among others, bladder and intraperitoneal cancer. This means that nemorosone could decrease the probability of secondary tumors being induced in cancer patients treated with mitomycin C (Camargo et al., 2011). Absence of genotoxic and mutagenic activity after treatment with nemorosone has been also reported (Camargo et al., 2011, 2013). These findings show the potential for nemorosone to be used in cancer therapy to increase the efficacy of current chemotherapy drugs with minimal side effects.

On top of that, cancer cells are characterized by their acquired resistance to the induction of cell death, such as apoptosis; therefore, selective cancer treatments are also required to be able to circumvent the adaptive survival mechanisms of cancer cells. Nemorosone-induced cytotoxicity has been observed not only in parental cell lines, but also in derived sublines refractory to the conventional chemotherapy drugs cisplatin, etoposide, adriamycin (doxorubicin), vinblastine, and 5-fluorouracil (Díaz-Carballo et al., 2008a; Díaz-Carballo et al., 2008b; Frión-Herrera et al., 2019b). Moreover, a link between mitochondrial uncoupling, specifically through dysregulation of calcium homeostasis, and P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) has been suggested (Fernández-Acosta et al., 2019). This protein induces an ATP-dependent efflux of several anticancer drugs, and both its synthesis and function have been linked to calcium signaling (Sulová et al., 2009). Therefore, compounds that interfere with intracellular calcium homeostasis could have reversal effects on MDR, as is the case with 1,4-dihydropyridine calcium antagonists such as nifedipine (Philip et al., 1992; Vilpo et al., 2000). Recently, the induction of mitochondrial uncoupling triggered by VE-3N, a novel 1,4-dihydropyridine derivative (structurally close to nifedipine), was reported (Marín-Prida et al., 2017), reinforcing the idea of a possible link between reversion of P-gp-mediated MDR and mitochondrial uncoupling. Nemorosone, an uncoupling compound that affects calcium homeostasis, could induce cytotoxicity in the aforementioned resistant cells by targeting P-gp.

As already mentioned, pharmacokinetic studies in mice have shown that nemorosone is well tolerated and rapidly absorbed into the bloodstream (Wolf et al., 2013). Likewise, as part of a research on the effect of nemorosone on leukemic cells, a subchronic toxicology study was performed to analyze the influence of nemorosone on hematopoiesis. Unlike several cytostatic agents that induce bone marrow depletion and drastic disorders in blood cell formation, nemorosone did not affect hematopoiesis globally, and all nemorosone-modified hematologic parameters returned to normal values within 30 days (Díaz-Carballo et al., 2008a).

To conclude this point, it is possible to note from the studies discussed above that nemorosone not only exhibits selectivity towards cancer cells, but also that its controlled administration does not endanger the life of the treated animals, while it can be effective in tumor suppression. Furthermore, the antimutagenic activity without genotoxic effect and the possibility of circumventing multidrug resistance led to the conclusion that nemorosone can be used both as single and combined therapy, thereby increasing the efficacy of conventional drugs and reducing undesirable side effects.

7.6. Antiparasitic activity

Parasites cause different types of tropical illnesses like malaria, trypanosomiasis and leishmaniasis constitute a public health problem, especially in developing countries where millions of people are affected every year (Hotez et al., 2020). Despite the great advances in modern medicine, parasitic infections continue to burden the world with huge costs in terms of human and animal health, and national economies directly and indirectly (Torgerson, 2013). Currently, no vaccines are presently available against any of the major parasitic infections of humans and chemotherapy remains the only option for both clinical and control management. However, conventional drugs have several drawbacks that limit their utility, such as high cost, poor compliance, drug resistance, low efficacy and poor safety (Monzote, 2014; Soto-Sánchez and Ospina-Villa, 2021). For these reasons, there is a clear need for new therapeutic agents against parasite infections and the Program of Tropical Diseases of WHO has regarded the investigation of natural products as an essential priority for the treatment of parasitic diseases (Monzote, 2014).

Nemorosone showed interesting results against protozoan parasites. The highest activity was found for *Plasmodium falciparum* (IC_{50} = 0.4 μM) after 72 h of incubation. This value was similar to the reference drug Chloroquine®, which showed an IC_{50} value of 0.3 μM (Mozirandi et al., 2019).

In parallel, the compound also displayed activity against kinetoplastid parasites at same IC_{50} values. In particular, against amastigotes of *Trypanosoma cruzi* and trypomastigotes of *Trypanosoma brucei* caused IC_{50} values of 12.5 and 17.5 μM after 72 h and 7 days, respectively (Monzote et al., 2011). Finally, *in vitro* activity against *Leishmania* spp. was appreciated, showing an IC_{50} of 0.67 μM against promastigotes of *L. tarentolae* (Monzote et al., 2015) after 48 h of incubation, 11.2 μM

against amastigotes of *L. amazonensis* after 48 h of incubation, and 32.9 μM against amastigotes of *L. infantum* after 120 h of incubation (Monzote et al., 2011). Nevertheless, activity was inferior compared with respective reference drugs (Benznidazol®: IC_{50} = 2.2 μM for *T. cruzi*, Suramine®: IC_{50} = 0.05 μM for *T. brucei*, Amphotericin B®: IC_{50} = 0.03 μM for *L. amazonensis*, miltefosine: IC_{50} = 7.7 μM for *L. infantum*; (Monzote et al., 2011), except for *L. tarentolae* (Pentamidine®: IC_{50} = 8.0 μM; (Monzote et al., 2015).

In a general overlook of some propolis, antiparasitic activity in presence of nemorosone have been highlighted. For example, the American brown propolis (mainly in Cuba and Venezuela) is famous for its high content of nemorosone. This propolis from Cuba has been shown to exhibit potent inhibitory action on *P. falciparum* (IC_{50} = 0.2–12.5 μg/mL; (Monzote et al., 2012), *L. infantum* (IC_{50} = 8.4–22.2 μg/mL; (Monzote et al., 2012), *L. amazonensis* (IC_{50} = 7.8–33.9 μg/mL; (Fidalgo et al., 2011), *T. cruzi* (IC_{50} = 4.5–8.0 μg/mL; (Monzote et al., 2012), *T. brucei* (IC_{50} = 8.2–16.3 μg/mL (Monzote et al., 2012); and *Trichomonas vaginalis* (IC_{50} = 6.2 – >200 μg/mL; (Fidalgo et al., 2011).

In the mentioned studies, in *vitro* cytotoxicity assay of nemorosone revealed CC_{50} values of 15.5 μM on human fetal lung fibroblast MRC-5 cells (Monzote et al., 2011) and 29.5 μM on peritoneal macrophages from BALB/c mice (Monzote et al., 2015). In general, a low selectivity was reported, except for *P. falciparum* with a selectivity index (SI) value of 39 (Monzote et al., 2011) and *L. tarentolae* with a SI of 44 (Monzote et al., 2015). Cuban brown propolis also showed high cytotoxicity on peritoneal macrophages from BALB/c mice (Fidalgo et al., 2011) and MRC-5 cells (Monzote et al., 2012), with CC_{50} values between 9.6 and 70 μg/mL.

Few studies have explored the cellular and molecular mechanisms of nemorosone antiprotozoal action. One of them explored the possible role of mitochondria in the antileishmanial activity, in comparison with the action on mammalian mitochondria (Monzote et al., 2015). Interestingly, inhibition of oxygen consumption was observed in promastigotes of *L. tarentolae* treated with nemorosone, which produced significant inhibition of oxygen consumption measured using Clark-type oxygen electrode equipment. In this model, an IC_{50} of 40.3 μM was obtained after 30 min of incubation of increasing concentration (25–400 μM) of nemorosone (Monzote et al., 2015). Moreover, assays on individual electron transfer complexes were also performed and nemorosone displayed a versatile activity. On mitochondrial complex II from *L. tarentolae*, a specific inhibition was observed. The IC_{50} value results in 36.5 μM, which was in same range that value for oxygen consumption; while no activity (IC_{50} > 100 μM) was observed against mitochondrial complex of mammalian model (Monzote et al., 2015). In parallel, unspecific inhibition of complex III was also observed, with a similar activity on mitochondrial complex from *L. tarentolae* (IC_{50} = 39.6 μM) and mammalian (IC_{50} = 30.4 μM) (Monzote et al., 2015).

Therefore, since mitochondria is the main site for generation of cellular ATP and *Leishmania* presents a single mitochondrion, this organelle is vital for cell survival (Monzote and Gille, 2010). The reported study suggests that nemorosone caused almost its antileishmanial action due to inhibition of complexes II and III of mitochondrial electron transport chain of *Leishmania* parasites, which could cause an increase in ROS production and triggers parasites death (Monzote et al., 2015).

7.7. Antimicrobial activity

The incidence, as well as the severity of microbial infectious diseases, continue to increase. This is greatly associated to the rise of antimicrobial resistant pathogens which cannot be adequately treated with the currently existing repertoire of approved antibiotics (Mozirandi et al., 2019). In this scenario, innovative strategies to combat microorganisms have been demanded and natural-derived antimicrobials have been established to be one of the most auspicious sources (Mickymaray, 2019).

The *in vitro* antimicrobial profile of nemorosone against bacteria and fungi has been assessed. A study against both Gram-negative and Gram-positive bacteria, reported that better activity was observed against Gram-positive bacteria. In particular, *Bacilus subtilis* and *Staphylococcus aureus* showed higher susceptibility, with an inhibition halo of 30 mm and a Minimal Inhibitory Concentration (MIC) of 0.2–0.5 mg/mL by agar diffusion plate method (Rojas et al., 2001). Another study, using microtitration method, corroborated the reported sensibility of *S. aureus* with IC_{50} value of 16.1 μM (Monzote et al., 2011). On the other hand, nemorosone has shown inhibitory activity against *Staphylococcus epidermitis* with a MIC of 5 mg/mL; while lower effect was appreciated on *Streptococcus pyogenes* with MIC of 10 mg/mL and *Alcaligenes faecalis*, *Proteus mirabilis*, *Serratia marcescens* and *Shigella flexneri* with MIC of 20 mg/mL (Rojas et al., 2001). Against *Escherichia coli* a MIC of 20 mg/mL was obtained in agar diffusion plate method (Rojas et al., 2001); while by microtitration method no activity was reported at 64 μM (Monzote et al., 2011). In concordance with mentioned studies, Cuban brown propolis only showed activity on *S. aureus* (IC_{50} = 4.7–13.7 μg/mL), while no activity was observed on *E. coli* at 64 μg/mL (Monzote et al., 2012).

Related with antifungal activity, only one study was found in reviewed scientific literature, in which no effect was reported against *Trichophyton rubrum* and *Candida albicans*. In this case, IC_{50} values were >64 μM (Monzote et al., 2011). However, Cuban brown propolis showed inhibitory activity almost on *T. rubrum* (IC_{50} = 6.7–16.7 μg/mL); although continues to be inactive against *C. albicans* (Monzote et al., 2012).

8. Conclusions and perspective

The fascinating chemical structure of nemorosone and its promising antiproliferative action have attracted the attention of both chemists and biomedical researchers. The relative high abundance of this molecule at its natural sources and the advent of new procedures for its chemical synthesis predict growth in nemorosone-based research from the drug discovery point of view. In this sense, with the goal of developing new drug candidates, nemorosone bicyclo[3.3.1]nonane scaffold functionalization would facilitate structure–activity relationship studies, with the potential of discovering new antitumor and antiparasitic derivatives with higher efficacy, lower mitochondrial toxicity and improved ADME properties. The efficacy studies of nemorosone and its derivative in combination with other chemotherapeutic agents also need to be established in relevant pre-clinical models. This review forms the basis for further nemorosone's pre-clinical research towards its translation to the clinic.

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Compound	R_1	R_2	R_3	R_4
Nemorosone	Benzoyl	Prenyl	Prenyl	Prenyl (endo)
7-*epi*-nemorosone	Benzoyl	Prenyl	Prenyl	Prenyl (exo)
Clusianone	Prenyl	Benzoyl	Prenyl	Prenyl (endo)
7-*epi*-clusianone	Prenyl	Benzoyl	Prenyl	Prenyl (exo)

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.

Data availability

No data was used for the research described in the article.

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