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Protective Role of Riboflavin and Carboxymethylflavin: Toxicological and Cytotoxic Evaluation

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Abstract

Riboflavin (vitamin B2) is a water-soluble vitamin that has antioxidant, anti-inflammatory, and mitochondrial-stabilizing properties, rendering it an attractive neuroprotective candidate. Its therapeutic potential has, however, been limited by low bioavailability and cellular uptake.

In this study, carboxymethylflavin (CMF), a riboflavin derivative with better solubility and membrane permeability, was synthesized and compared with riboflavin to evaluate their toxicity and neuroprotective effects. The toxicity was determined using brine shrimp lethality, and antiproliferative activity was determined using MTT assays.

Riboflavin was moderately cytotoxic and was more effective against cancer cell lines as compared to normal fibroblasts. CMF, on the other hand, showed significantly reduced cytotoxicity in all the cell lines examined, which implies that it could be safer compared to riboflavin. The improved biocompatibility and maintenance of the biological activity of CMF indicate the possibility of developing it into a biologically useful agent with fewer side effects without compromising its neuroprotective properties and having a safer profile in therapy.

KEYWORDS

Riboflavin; carboxymethylflavin; neuroprotection; toxicity; bioavailability; cytotoxicity

1.0 INTRODUCTION

The recent attention given to riboflavin (vitamin B2) and its analogues has highlighted the immense possibility of these molecules to combat oxidative stress and cellular damage, which is a major cause of numerous diseases, especially neurodegenerative disorders. The effectiveness of riboflavin as a strong antioxidant and mitochondrial stabilizer is well-reported, and studies have indicated that it can be used to alleviate oxidative stress in migraine and Parkinson's disease [1, 2]. Recent studies, such as the one by Plantone et al. (2021) indicate the possibilities of riboflavin in neurological diseases, particularly due to its capacity to regulate mitochondrial activity, reduce oxidative stress, and stabilize neuronal membranes [3]. Their work highlights the therapeutic benefits of riboflavin in diseases like epilepsy, multiple sclerosis and Parkinson's disease and its neuroprotective effects on the cellular and systemic levels.

To attain even more therapeutic advantages of riboflavin, its analogues, such as carboxymethylflavin, have been developed. These derivatives aim to increase the bioavailability and membrane permeability of riboflavin and its neuroprotective effect. Studies have shown that chemical modifications of riboflavin derivatives strongly influence cellular absorption, enhancing their ability to prevent neuronal damage. However, regardless of these positive results, the safety of these compounds is an extremely important factor. Their efficacy can be compromised due to toxicity at high concentrations, and that is why toxicological screening should be carried out extensively. Bioassays, such as the brine shrimp lethality test and the MTT test, are still key in determining the biocompatibility of riboflavin and its analogues, which should make them safe to use in clinical practice.

A significant trend in recent years has been moving towards the exploration of naturally occurring bioactive compounds, particularly those of antioxidant and cytoprotective properties, as therapeutic agents. Riboflavin is the water-soluble vitamin that is a precursor to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). It is the most important molecule in redox reactions, mitochondrial respiration, and cellular energy metabolism. There is a high susceptibility of the central nervous system to oxidative damage due to its high metabolic rate and lipid-rich composition; hence, riboflavin is an important component of neuronal homeostasis. The antioxidant, anti-inflammatory, and mitochondrial-stabilizing effects of riboflavin have been emphasized by numerous studies, which could be involved in its neuroprotective effects on diseases such as epilepsy [4–6]. The recent research also revealed that riboflavin also plays a role in controlling the mitochondrial activity and redox homeostasis of neurological diseases, which further confirmed its therapeutic significance [7].

Nevertheless, these favorable biological implications are typically restrained in the therapeutic use of riboflavin by its low bioavailability, rapid elimination, and low cellular uptake. To address these challenges, different derivatives of riboflavin have been synthesized in order to enhance the pharmacokinetic and biological characteristics of the drug. To address these challenges, different derivatives of riboflavin have been synthesized in order to enhance the pharmacokinetic and biological characteristics of the drug. Among these carboxymethylflavin is a modified derivative with chemical modifications on the ribityl side chain to enhance its solubility and membrane permeability. Such modifications enhance intracellular delivery, besides enhancing retention, which enhances the biological activity of riboflavin. Other flavin derivatives, including lumiflavin and phosphorylated flavin analogues like FMN derivatives, have been investigated to have the potential to regulate redox homeostasis, promote mitochondrial activity, and improve cellular resilience under stress [8,9].

Although the pharmacological and neuroprotective advantages of riboflavin and its derivatives are increasingly accepted, their toxicity and safety should be strictly assessed before they can be widely used in clinical practice. Toxicological screening allows obtaining the necessary information on safe concentrations and the possible adverse effects. Bioassays such as the brine shrimp lethality assay (Artemia salina) and the MTT assay are widely used as preliminary toxicity screening tools due to their simplicity, reliability, and correlation with more advanced biological systems [10]. The brine shrimp

assay is especially applicable in the measurement of cytotoxicity and prediction of pharmacological activity, whereas the MTT assay is a well-established method of measuring cell viability and cytotoxicity of a compound on the basis of mitochondrial metabolic activity [11].

Given the growing interest in flavin-based compounds and their derivatives, it is crucial to establish their toxicological profiles prior to exploring their therapeutic applications. Therefore, this study focuses on evaluating the toxicity of riboflavin and its derivatives, including carboxymethylflavin, using both the brine shrimp lethality and MTT assays. Through these complementary methods, the study aims to establish the foundation for the safety and biocompatibility of these compounds, preparing the ground for their possible development as pharmacologically relevant compounds.

2.0 MATERIALS AND METHODS

2.1 Synthesis and Characterization of Carboxymethylflavin

Carboxymethylflavin (CMF) was prepared by using riboflavin (RF) by alkaline oxidative photolysis. Riboflavin was in this process treated with sodium hydroxide and hydrogen peroxide and then allowed to be exposed to light over a long period to undergo the desired structural changes. The reaction mixture was then extracted with chloroform to remove photolytic by-products of the reaction, such as lumichrome and lumiflavin, and any unreacted riboflavin, resulting in purified CMF as a yellow crystalline product.

The synthesized compound was comprehensively characterized using multiple analytical techniques. Its absorption properties were estimated using UV- visible spectroscopy (200-700 nm), and spectrofluorimetric analysis was used to estimate fluorescence properties and electronic transitions. Fourier-transform infrared (FTIR) spectroscopy was performed over the range of 600–4000 cm^{-1} to analyze the functional groups. Moreover, the separation of the compounds was performed, and the molecular identification and purity evaluation of the compounds were conducted using high-performance liquid chromatography and time-of-flight mass spectrometry (HPLC-TOF-MS) with an electrospray ionization source in positive mode. All these analyses established the successful synthesis and structural integrity of CMF.

2.2 *In Vitro* Toxicity Test

The toxicity profile of riboflavin and its analogue, carboxymethylflavin, was tested through the brine shrimp lethality assay (BSLA) and MTT cytotoxicity assay.

2.2.1 Brine Shrimp Lethality Assay

The brine shrimp lethality assay was performed using Artemia salina nauplii as a simple and convenient model to provide a preliminary toxicity screening system [10,13,14]. Artificial seawater was prepared by dissolving 38 g of iodine-free sea salt in 1 L of distilled water and filtered before use. The brine shrimp eggs were incubated in a hatching chamber where aeration and illumination were maintained throughout a 24-48 h period to obtain active nauplii. Ten nauplii were moved to test tubes of 2.5 mL seawater.

Riboflavin and carboxymethylflavin stock solutions were prepared and diluted in series to get concentrations of 50 to 3.125 µg/mL. The test tubes had 2.5 mL of the

corresponding dilution of each compound and nauplii. Vincristine sulfate was taken as a positive control in lower concentrations (0.125-10 µg/mL), and seawater alone acted as a negative control. The surviving nauplii were then counted with the help of a magnifying lens after 24 h of incubation, and the percentage mortality was determined. Probit analysis was used to determine the median lethal concentration (LC50) by plotting the percentage mortality versus concentration [13].

2.2.2 MTT Assay

To measure in vitro cytotoxicity, the MTT assay was conducted as reported by Mosmann [11]. Three cell lines, HeLa (cervical cancer), PC3 (prostate cancer), and 3T3 (mouse fibroblast), were grown under the appropriate media under standard conditions (37°C, 5% CO_2). Minimum Essential Medium (MEM) was used to grow HeLa cells, and Dulbecco Modified Eagle Medium (DMEM) was used to grow PC3 and 3T3 cells, with additions of fetal bovine serum (5%), penicillin (100 IU/mL), and streptomycin (100 µg/mL).

Cells were seeded in 96-well plates at appropriate densities (HeLa: 6×10^4 cells/mL; 3T3: 5×10^4 cells/mL; PC3: 1×10^5 cells/mL) and incubated overnight. Then, cells were incubated with different concentrations of riboflavin and carboxymethylflavin (1-30 µM) over a period of 48 h. After treatment, MTT solution (0.5 mg/mL) was added and allowed to incubate for 4 hours, to form formazan crystals. Dimethyl sulfoxide (DMSO) was used to solubilize the crystals, and absorbance was taken at 570 nm (HeLa and PC3) and 540 nm (3T3) with the help of a microplate reader.

The cell viability was determined as a percentage of untreated control cells, and the IC_{50} was observed using the appropriate software. A combination of these assays provided preliminary data on the cytotoxic and safety profiles of the tested compounds.

3.0 RESULTS AND DISCUSSION

3.1 Synthesis and Characterization of CMF

Carboxymethylflavin (CMF) was obtained by alkaline oxidative photolysis of riboflavin, which was then extracted using chloroform to eliminate by-products and unreacted precursor. Structural characterization was used to identify and determine the purity of CMF. The UV-visible spectra showed absorption peaks of 223, 266, 376 and 445nm, which is also a characteristic of retention of flavin isoalloxazine core as reported in flavin derivatives [9]. Fluorescence showed excitation at 376 and 445nm and emission at approximately 530nm, which are characteristic of flavin compounds [8]. FTIR analysis revealed the presence of important functional groups (NH, C=O, C–N and OH), which also supported the successful modification. Mass spectrometry (LC-ESI-TOF-MS) was used to verify the molecular mass of CMF at m/z 301.0925, which aligns with theoretical values. These results confirm the successful synthesis of CMF with structural characteristics similar to other flavin analogues [8,9].

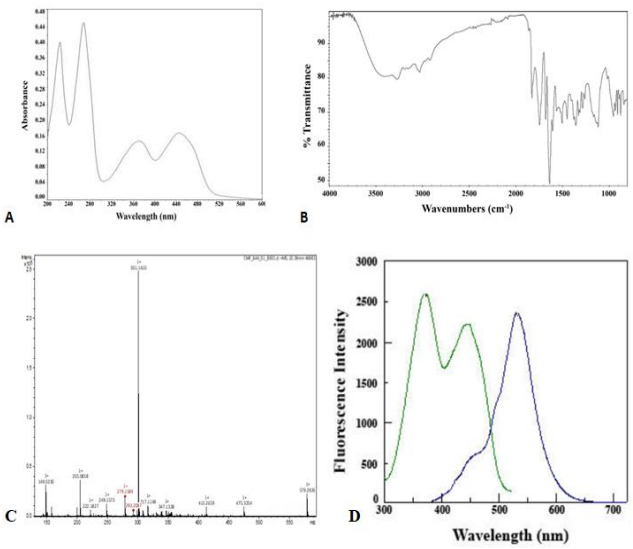

Figure 1: Spectroscopic analysis of Carboxymethylflavin: (A) UV-Visible absorption spectrum; (B) FTIR transmittance spectrum; (C) Mass spectrometry (MS) analysis; (D) Fluorescence emission spectrum.

3.2 Toxicological Studies

3.2.1 Brine Shrimp Lethality Assay

The brine shrimp lethality assay (BSLA) is widely used as a preliminary toxicity screen, offering a rapid prediction of overall cytotoxicity, which can be correlated with further biological systems [10,13]. In this assay, riboflavin (RF) showed an LC_{50} value of 11.072 µg/mL (Table 1, Figure 2A), indicating low to borderline non-toxic behavior based on standard classification criteria. CMF exhibited an LC_{50} value of 32.758 µg/mL (Table 1, Figure 2B), placing it within the low-toxicity range. Comparatively, vincristine sulfate exhibited a significantly stronger cytotoxic effect with an LC_{50} of 0.86 µg/mL (Table 1, Figure 2C), validating the sensitivity and reliability of the assay system.

These findings are consistent with past findings that flavin compounds tend to have low systemic toxicity in preliminary models [10]. The slightly greater LC50 of CMF than riboflavin suggests that carboxymethyl modification may cause a minor alteration in the organismal interactions but does not introduce an extra toxicity, further arguing in favor of the toxicity profile of flavin derivatives in preliminary toxicity tests.

Table 1: Percentage Mortality of RF and CMF, and Standard Vincristine Sulphate Determined using Brine Shrimp Lethality Test.

Test Sample	Concentration (µg/ml)	Log Concentration	% Mortality	Probit of Kill	LC_{50} (µg/ml)
RF	50	1.699	60	5.25	11.072
	25	1.398	60	5.25	
	12.5	1.097	50	5.00	
	6.25	0.796	40	4.75	
	3.125	0.495	50	5.00	
CMF	50	1.699	60	5.25	32.758
	25	1.398	50	5.00	
	12.5	1.097	20	4.16	
	6.25	0.796	20	4.16	
	3.125	0.495	10	3.72	
	1.562	1.699	60	5.25	
Vincristine Sulphate	10	1	100	8.09	0.86
	5	0.69	80	5.84	
	1	0	60	5.25	
	0.5	-0.30	40	4.75	
	0.25	-0.60	20	4.16	
	0.125	-0.90	20	4.16	

Compounds were categorized as highly toxic when $LC_{50} \geq 100$ µg/mL, moderately toxic when $50 \leq LC_{50} < 100$ µg/mL, low toxic when $10 \leq LC_{50} < 50$ µg/mL, and non-toxic when $LC_{50} \leq 10$ µg/mL.

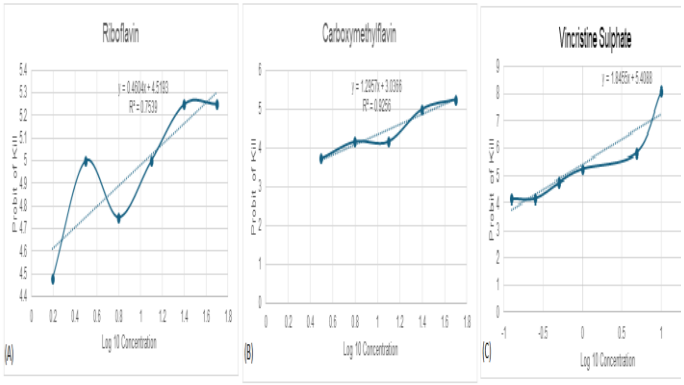

Figure 2: Dose–response relationship of the tested compounds. Probit analysis plots illustrate the relationship between log10 concentration and probit of % mortality (Kiwi) of (A) Riboflavin, (B) Carboxymethylflavin, and (C) Vincristine sulphate. Each of the compounds has linear regression lines with the corresponding equations and coefficient of determination (R^2) values that reveal the goodness of fit of the dose response model.

3.2.2 MTT Cytotoxicity Assay

The MTT assay determines cellular metabolic activity, which is used to estimate compound-induced cytotoxicity [11]. Riboflavin (RF) displayed a biological response that was cell line-dependent in the MTT cytotoxicity assay. RF had a moderate cytotoxicity of 54.9% at 100 µM and an IC_{50} of 62.42 ± 4.08 µM in normal fibroblast (3T3) cells, which implies moderate toxicity to normal cells. RF exhibited moderate anticancer activity with 66.47% inhibition at 100 µM and an IC_{50} of 29.1 ± 7.2 µM in HeLa cervical cancer cells. In PC3 prostate cancer cells, however, RF exhibited very low inhibitory activity (12.51% at 100 µM) and no measurable IC_{50}, reflecting a lack of efficacy in this cell line (Table 2, Figure 3) [12].

Carboxymethylflavin (CMF), on the other hand, demonstrated relatively low cytotoxic and antiproliferative activity in all cell lines tested. In 3T3 cells, CMF exhibited low inhibition (22.91% at 100 µM) and no measurable IC_{50}, indicating that CMF did not have toxicity in normal cells. The same pattern was found with HeLa cells, which showed 48.96% inhibition at 100 µM, and PC3 cells, which showed 20.16% inhibition at the same concentration. In both cases, a value of IC_{50} was not reached within the concentration range being tested, indicating that the bioactivity had significantly decreased after carboxymethylation (Table 2, Figure 3) [13].

Doxorubicin as a positive control showed high cytotoxic activity in all cell lines, with 95.8% inhibition in 3T3 cells (IC_{50}: 0.1 ± 0.04 µM), 93.66% in HeLa cells (IC_{50}: 0.86 ± 0.12 µM), and complete inhibition in PC3 cells at 100 µM, confirming the robustness and sensitivity of the assay system (Table 2, Figure 3) [14].

Table 2: Cytotoxic Activity of RF and CMF in 3T3 and HeLa Cell Lines with Doxorubicin

Compound	Concentration (µM)	3T3	HeLa	PC3	3T3	HeLa	PC3
		(% Inhibition)			IC_{50} (µM)		
RF	100	54.9	66.47	12.51	62.42 ± 4.08	29.1 ± 7.2	–
	10	33.78	36.90	9.15			
	1	2.64	22.36	6.85			
CMF	100	22.91	48.96	20.16	–	–	–
	10	17.15	21.51	8.92			
	1	8.11	15.06	1.14			
Doxorubicin	100	95.8	93.66	100	0.1 ± 0.04	0.86 ± 0.12	–

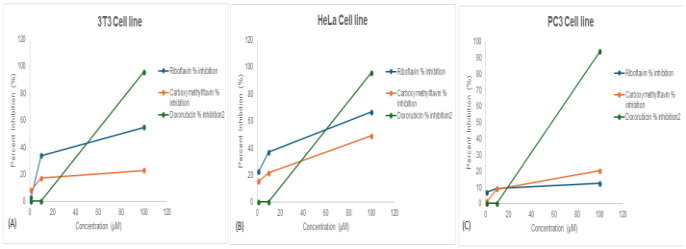

Figure 3: Dose-response curves of cytotoxic effects of riboflavin (RF), carboxymethylflavin (CMF), and doxorubicin (standard) against (A) 3T3, (B) HeLa, and (C) PC3 cell lines. The percentage inhibition was measured within a concentration of 1-100 µM.

Overall, the findings indicate that RF continues to have moderate cell line-dependent antiproliferative effects, particularly on HeLa cells, whereas CMF has a significantly less cytotoxic and anticancer effect in all models. This means that carboxymethyl modification considerably reduces biological activity but may increase biocompatibility.

4.0 CONCLUSION

The results of this study suggest that riboflavin and its analogue CMF could be promising bioactive compounds with effective cytoprotective activities. Despite the moderately high cytotoxicity of riboflavin, CMF was much less toxic; it is preferable in the context of additional therapeutic applications. Both compounds demonstrated low toxicity during initial assessments, implying that they can be considered safe and effective agents to be explored further. Further studies are needed to improve their pharmacokinetics and bioavailability in order to improve their clinical potential in different therapeutic settings.

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