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Review

Coumarin-induced Hepatotoxicity: A Narrative Review

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Abstract: Coumarin is an effective treatment for primary lymphoedema, as well as lymphoedema related to breast cancer radiotherapy or surgery. However, its clinical use is limited in several countries due to the possible occurrence of hepatotoxicity, mainly in the form of mild to moderate transaminase elevation. Noteworthy, only few cases of severe hepatotoxicity have been described in literature, with no reported cases of liver failure. Data available on coumarin absorption, distribution, metabolism and excretion have been reviewed, focusing on hepatotoxicity studies carried out in vitro and in vivo. Finally, safety and tolerability data from clinical trials have been thoroughly discussed. On the basis of these data, coumarin-induced hepatotoxicity seems to be restricted to a small subset of patients, probably due to the expression of specific alleles of CYP450 isoform not yet well characterized. In summary, more research is needed in order to identify patients at risk of developing hepatotoxicity following coumarin treatment, in order to improve the risk/benefit ratio of the product and allow more patients to benefit from its therapeutic properties.

Keywords: coumarin; 1,2-benzopyrone; Melilotus officinalis; narrative review; primary lymphoedema; secondary lymphoedema; hepatotoxicity

1. Historical background

By the end of the nineteenth century, breeders in North America began to sow Melilotus officinalis and Melilotus alba (Sweet clover) imported from Europe to feed their livestock [1]. Soon after, cattle began to develop a new and lethal disease characterised by profuse bleeding [2]. Francis Schofield, an English veterinarian who emigrated to Canada, guessed that the disease was linked to the consumption of spoiled hay, since fresh hay caused no disease. He demonstrated that the elimination of spoiled hay from the diet, as well as blood transfusion from healthy animals, significantly improved the health condition of the affected animals [3].

The etiopathogenesis of the new disease remained a puzzling question, until Karl Paul Link and his colleagues at the University of Wisconsin discovered that spoiled hay was contaminated by several species of Aspergillus [2]. These fungi oxidize the coumarin naturally present in Melilotus into 4-hydroxycoumarin, which in turn reacts with formaldehyde and another molecule of coumarin leading to the production of dicumarol [4]. Link also demonstrated that bleeding induced by spoiled Melilotus, as well as dicumarol, was antagonized by the administration of vitamin K, which promotes blood clotting [5]. Patent rights on dicumarol were transferred to the Wisconsin Alumni Research Foundation, which in turn licensed the patent to Lilly, Squibb and Abbott for the treatment of thrombosis and myocardial infarction [6].

In 1945 Link was hospitalized in a sanatorium, where he spent some time trying to identify an efficient rat poison [7]. He thought that an ideal substance should induce a slow death, otherwise rodents would associate product consumption with its lethal effects. Bearing in mind the haemorrhagic disease in livestock, Link began to test

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dicumarol in rodents. However, dicumarol turned out to be less toxic in rodents compared to cattle. For this reason, he examined a series of compounds synthesized a few years before by a group of Japanese researchers, focusing his attention on a specific compound named 3-phenyl-acetyl ethyl-4-hydroxycoumarin [8]. The product turned out to be an effective rat poison and Link transferred again the patent rights to the Wisconsin Alumni Research Foundation, calling the new product "warfarin" from the foundation's acronym [6].

Following its launch on the market as a rat poison, Link convinced some clinicians to test warfarin also as a therapeutic agent in humans [9]. Clinical studies showed that warfarin was superior to dicumarol as anticoagulant, and when President Eisenhower suffered a myocardial infarction in 1954 he was successfully treated with warfarin [10]. Still now, the product is marketed under the trade name "Coumadin", generating confusion between dicumarol derivatives and coumarin. However, while the formers are potent anticoagulants, the latter is completely devoid of anticoagulant activity.

2. Introduction

Natural coumarins are generally unsaturated lactones, with an oxygenated substituent in position 7, biosynthesized from phenylalanine via the shikimic acid [11,12]. They are classified in six main classes based on their structure: simple coumarins, furocoumarins, dihydro-furocoumarins, pyranocoumarins, phenyl-coumarins, and bis-coumarins [13,14].

The name “coumarin” refers exclusively to the simplest representative of these compounds, the 1,2-benzopyrone (or 5,6-benzo- [α] -pyrone).

From a chemical point of view, coumarins are aromatic heterocyclic compounds belonging to the family of benzopyrones; their structure consists of a benzene ring fused to an α-pyrone ring [13,15]. Coumarins are secondary metabolites of numerous species of higher pants, including Melilotus officinalis (sweet clover) and other different species (spp.), Angelica keiskei (ashitaba), Angelica pubescens (pubescent angelica), Artemisia scoparia (yin-chen wormwood), Citrus spp. (orange), Glycyrrhiza uralensis (licorice), Justicia pectoralis (chambá), Mikania glomerata (guaco), Pelargonium sidoides (African geranium), Leonurus heterophyllus (Chinese motherwort), Cinnamomum aromaticum (cassia) and Cinnamomum zeylanicum (true cinnamon) [16]. Coumarins are also produced by some species of bacteria, fungi and sponges [17,18] and can be obtained by synthetic processes [19].

Coumarin (1,2-benzopyrone) is a phytochemical known to exert diverse biological and pharmacological activities [20] that render this molecule very promising in a wide spectrum of applications, including medical and agrochemical fields as well as the cosmetic industry [21–24]. Coumarin is characterized by anti-inflammatory [25], antioxidant [26], hepatoprotective [27], anxiolytic [28], antimicrobial and antiproliferative properties [29]. Contrary to some natural coumarin family members, such as dicumarol, and synthetic coumarins (i.e., warfarin) that are vitamin K antagonists [30,31], coumarin is completely devoid of anticoagulant effects [11,31].

The first studies addressing the use of coumarin in the treatment of lymphoedema, a high-protein oedema caused by a failure of the lymphatic system, dates back to the seventies [32,33]. The effectiveness of coumarin in the treatment of both primary and secondary lymphoedema was also confirmed by more recent studies that showed its role in significantly increasing the reabsorption rate [34]. Most of these studies suggested that this activity is mainly due to the coumarin metabolite 7-hydroxycoumarin [35]. Furthermore, data suggest coumarin-mediate macrophages activation and recruitment at the level of target tissues as a putative mechanism of action. Two different mechanisms have been hypothesized: (1) macrophages activation leads to phagocytosis of coumarin and coumarin-bound plasma proteins at the level of microvascular vessels, resulting in a decrease of the colloid pressure in the intercellular spaces, and consequently in a decrease of lymphoedema volume; (2) coumarin-activated macrophages stimulate the release of

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lysosomal enzymes increasing proteolysis [36]. These data have been the basis for several clinical trials carried out in the past years on primary lymphoedema, as well as on lymphoedema due to radiotherapy and surgery in cancer patients.

In addition to lymphoedema, the health-promoting actions of coumarin have been demonstrated in other diseases including chronic venous insufficiency, asthma, and breast cancer [37,38]. These results, together with coumarin bioavailability and low cost, make this compound very attractive as a therapeutic agent: its great versatility could be exploited in diverse fields including pharmacology but also medicinal chemistry [39], and food science [40].

Despite its promising features, early after its first isolation (by Vogel in 1820, from the seeds of Dipteryx odorata) [41], coumarin was noted to cause hepatic damage in animal models [42] and, later, also to induce long-term tumour formation in rodents [43]. Coumarin-induced carcinogenesis was demonstrated not to be related to genotoxicity [44,45], but to its sub-acute and chronic toxic effects, especially hepatotoxicity, confirming previous studies [46]. In light of this, coumarin hepatotoxic effects were the most extensively studied not only in rodents but also in other mammalian species.

In humans, the onset of coumarin-induced hepatotoxicity is extremely rare. However, several clinical studies demonstrated a possible correlation between coumarin treatment and hepatotoxicity, usually in the form of a significant increase in transaminase level, in a very small subgroup (single-digit percentage) of patients [47].

Studies demonstrated that genetic variability, especially in the expression of CYP2A6, an enzyme involved in the metabolism of coumarin [48], and environmental factors can significantly induce inter-individual variations in the metabolism of coumarin and modulate the individual response to the drug [41]. Due to this, further studies to determine coumarin safety are needed. Importantly, research should mainly focus on the individuation of the susceptibility factors that make some individuals vulnerable to coumarin toxicity. The identification of idiosyncrasies can be crucial not only to protect vulnerable patients but also to fully exploit coumarin as a pharmaceutical for patients that are not at risk of developing hepatotoxicity and that could greatly benefit from this treatment. Among the diverse possible strategies to reach this aim, pharmacogenetics, together with new integrated methodologies, have recently been considered very promising approaches, as reported by Hu et al. [34].

In the last years, literature specifically focused on coumarin addressed hepatotoxicity only marginally, and in most cases referred to outdated studies. Several papers have been recently published on the use of coumarin in the treatment of diverse pathologies; these include most of the available data on hepatotoxicity. However, none of the recent clinical studies addressed hepatotoxicity specifically [13,49–56].

The purpose of this narrative review is to summarize what is known to date about coumarin hepatotoxicity, with a focus on the possible strategies to identify subjects at risk of developing this complication. This work aims to stimulate research in this field, opening the possibility to reconsider coumarin as a therapeutic agent

3. Materials and Methods

The research was carried out on two different databases, i.e. Medline (Pubmed) and EMBASE (Elsevier), using the following search strings:

- “coumarin” OR “1,2-benzopyrone” OR “5,6-benzo-[+]-pyrone” AND “hepatotoxicity”
- “coumarin” OR “1,2-benzopyrone” OR “5,6-benzo-[+]-pyrone” AND “ADME”
- “coumarin” OR “1,2-benzopyrone” OR “5,6-benzo-[+]-pyrone” AND “metabolism” AND “human” (last 10 years)
- “coumarin” OR “1,2-benzopyrone” OR “5,6-benzo-[+]-pyrone” AND “human cell line”

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- "coumarin" OR "1,2-benzopyrone" OR "5,6-benzo-[+]-pyrone" AND "clinical trial"
- "coumarin" OR "1,2-benzopyrone" OR "5,6-benzo-[+]-pyrone" AND "case report"
- "coumarin" OR "1,2-benzopyrone" OR "5,6-benzo-[+]-pyrone" AND "observational trial"
- "coumarin" OR "1,2-benzopyrone" OR "5,6-benzo-[+]-pyrone" AND "observational study"

Paper selection was carried out by three authors. For each search engine, two authors carried out the preliminary selection, involving the third author in case of disagreement. Additional papers were selected from the bibliography of previously selected papers.

Finally, the paper was sent to an independent reviewer (Prof. Neil Piller, Director of the Lymphoedema Clinical Research Unit at the Department of Surgery, College of Medicine and Public Health of the Flinders University and Medical Centre, South Australia) who kindly revised the manuscript, provided his valuable advice and suggested some articles not included in our selection.

4. Results

4.1. Absorption and Distribution

Coumarin is completely absorbed following oral administration. However only approximately 2-6% of coumarin reaches systemic circulation in its native form, while plasma levels of its main metabolite, 7-hydroxycoumarin-glucuronide (7-HCG), rise significantly and proportionally after administration [57]. Coumarin is also absorbed through the skin: in a 70% aqueous ethanol solution the overall amount of absorbed product reaches 60% of the applied quantity in humans after 72h, a percentage that increases if the skin is immediately occluded after exposure [58]. Coumarin half-life in the blood ranges between 1 and 1.5 hours. The biological half-life of both coumarin and 7-HCG does not vary between oral or intravenous administration [59]. Coumarin is considered a prodrug since the active form is 7-hydroxycoumarin.

Coumarin distribution in the body follows a two-compartment kinetics. The high distribution volume (1.7 times the body weight) is explained by the fact that coumarin and its metabolites are found not only in organs with high blood flow, but also in extracellular and intracellular compartments [60]. In early studies it was hypothesized that cells could store coumarin, but pharmacokinetic studies showed that this is not the case [48].

4.2. Metabolism and Excretion

Following intestinal absorption, coumarin reaches the liver through the portal circulation. About 97% of the coumarin absorbed is metabolized by the cytochrome P450-linked mono-oxygenase enzyme system (CYP2A6) in liver microsomes, which performs hydroxylation [49,61–63]. Although hydroxylation could potentially occur on each carbon (i.e., 3, 4, 5, 6, 7 and 8), 7-hydroxycoumarin is the main metabolite. The 7-hydroxycoumarin is conjugated in the gut and other tissues to glucuronic acid (and to a lesser extent to sulphate) producing 7-hydroxycoumarin-glucuronide. In a separate and rarely occurring metabolic pathway, coumarin is metabolized by other cytochrome P450 isoforms (namely CYP1A1, CYP1A2 and CYP2E1) via ring splitting pathway into a highly unstable compound called 3,4-epoxycoumarin (CE). CE can either rearrange spontaneously to o-hydroxyphenylacetaldehyde (o-HPA) or be conjugated with glutathione (GSH) [20,41]. o-HPA is a hepatotoxic aldehyde and can be further detoxified by oxidation to o-hydroxyphenylacetic acid (o-HPAA) [64]. Lewis et al. [65] reviewed studies on the metabolism catalysed by human P450 enzymes and reported that P450 isoforms CYP1A1, CYP1A2, CYP2B6, CYP2E1 and CYP3A4 could all catalyse the metabolism of coumarin to the 3,4-

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coumarin epoxide pathway, whereas CYP2A6 catalyses exclusively the formation of 7-hydroxycoumarin.

The main metabolite of coumarin, 7-hydroxycoumarin-glucuronide, is actively secreted in the renal tubules and accounts for approximately 60% of the ingested dose of coumarin. Coumarin is however also present in its free and sulphated forms, as o-HPAA is. The latter is a minor metabolite in humans but is found in greater amounts in mice (41% of the administered dose) and rat (12% of the administered dose) urines [66].

4.3. In vitro and in vivo studies

The first reports on coumarin toxicity in rats and dogs date back to the 1950s and prompted several studies on coumarin metabolism in the liver. While coumarin-induced hepatotoxicity has been observed in rats [43], coumarin is not toxic in other rodents, such as mice, hamsters, and gerbils [67,68]. Differences observed among species, regarding coumarin hepatotoxicity, have been demonstrated to be metabolism mediated. In most species coumarin is hydroxylated to 7-hydroxycoumarin (7-HC), a nontoxic metabolite [69–71]. In rats, instead, the formation of 7-HC is extremely low [72,73], and this is thought to make rats more susceptible to hepatotoxicity [43], since 3-4 epoxidation pathway is prevalent. Importantly, coumarin metabolism differs significantly in rats and humans. For this reason, it has been questioned rat's suitability for studying risk associated with coumarin intake in humans [74]. Indeed, several clinical trials carried out in humans indicate that coumarin hepatotoxicity develops only in a small subset of treated patients [75–79].

In humans the formation of 7-HC by CYP2A6 is the predominant metabolic pathway, while only a minor amount of coumarin follows the alternative pathway that leads to the activation of the epoxide intermediate CE. CE can rearrange to form o-HPA; it is hypothesized that this is the prevailing metabolic pathway in patients which develop hepatotoxicity. In this context it has been hypothesized that CYP2A6 polymorphisms decreasing its enzymatic activity [80–82] could shift coumarin metabolism towards the production of CE and o-HPA and could represent a possible risk factor for coumarin-induced hepatotoxicity [81,83]. Farinola and Piller suggested that a reduction in coumarin 7-hydroxylation could indeed lead to its toxicity and that subjects with low levels of CYP2A6 activity are more likely to metabolize coumarin via the cytotoxic pathway [84]. To date, 34 CYP2A6 polymorphisms have been identified. Most of the mutations significantly decrease enzymatic activity, however the variants CYP2A6 * 28 and CYP2A6 * 31 show the same activity of the "wild type" enzyme. On the other hand, variants CYP2A6 * 14 and CYP2A6 * 15 are characterized by a higher enzymatic activity. These data suggest that not all CYP2A6 polymorphisms lead to a decrease in enzymatic activity [85], and therefore to a putative higher susceptibility to coumarin hepatotoxicity.

A study carried out by Van Iersel et al. [86] using a panel of human liver microsomal samples of known P450 isoenzyme profile demonstrated a 30- to 2250-fold variation in coumarin metabolism to total polar products (i.e., all metabolites except products covalently bound to microsomal proteins) and 7-HC. The authors observed that a marked interindividual difference exists in coumarin metabolism by human liver microsomes, hypothesizing that subjects with low levels of CYP2A6 activity may metabolize coumarin by the 3-hydroxylation and other pathways. To test this hypothesis, a clinical trial on 231 patients treated with coumarin or placebo was carried out in Germany [87]. In this study patients were genotyped for the two allelic variants encoding the defective proteins CYP2A6*2 and CYP2A6*3. The authors determined that susceptibility to coumarin-associated liver dysfunction is not genetically determined by polymorphism in CYP2A6 concluding that the studied polymorphisms are not the primary reason for coumarin hepatotoxicity. In addition, Rietjens et al. noted that both the peak concentration and the area under the curve of o-HPA in the human liver after 24 hours is always significantly lower than that observed in the rat [88]. This observation is valid in subjects with normal (“wild type”), as well as decreased CYP2A6 activity. In other words, even in the case of CYP2A6 deficiency (a phenomenon that occurs in less than 1% of Caucasian populations and about

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20% of Asian populations) [89] the production of o-HPA in human liver is lower than in rat liver.

These data suggest that a reduction in CYP2A6 metabolic pathway should not be responsible alone for the observed cases of hepatotoxicity; however, this does not exclude that different factors could shift coumarin metabolism leading to the CE-derived hepatotoxic compound accumulation. Indeed, metabolic activation of coumarin to CE through the heterocyclic ring-splitting pathway is an important prerequisite of toxicity. Dihydrocoumarin, which lacks the 3,4-double bond, is not hepatotoxic, as well as the analogues of coumarin with substitutions on the 3,4 double-bond [90,91].

The hypothesis that epoxidation alone is responsible for hepatotoxicity is nevertheless partially disproved by in vitro analysis carried out on the kinetics of coumarin epoxidation in rat and mouse liver microsomes. These analyses indicated that hepatic clearance of coumarin through the epoxide intermediate is about four times greater in mice than in rats [92]. However, mice show little or no hepatotoxicity after coumarin treatment. The lack of a direct correlation between coumarin epoxidation and species sensitivity to hepatotoxicity suggests that factors other than metabolic activation to CE are important determinants of hepatotoxic outcome. Studies suggested that the CE detoxification process could play a role in this outcome. Indeed, CE can be conjugated with GSH or it can rearrange to o-HPA, toxic compound, that can be detoxified by further oxidation to o-HPAA or reduction to o-HPE [73,93–95]. Vassallo et al. showed that the oxidation of o-HPA to o-HPAA seems to be crucial in determining hepatotoxicity of coumarin. Major differences in this reaction among species were observed. The clearance of o-HPA through this pathway proceeds more than 20 times faster in mice than in rats. This is consistent with the observation that in mice, all the o-HPA formed was oxidized to o-HPAA, whereas in rats, o-HPA remained as a major component detected in the microsomal reaction mixture. The slower hepatic clearance of the toxic aldehyde appears to be responsible for coumarin-induced hepatotoxicity in rats [96]. However, authors did not investigate the potential shift of the metabolic pathways in humans with polymorphisms in which 7- hydroxycoumarin formation is blocked, and whether this might be linked to hepatotoxicity in humans.

Regarding human hepatic metabolism of coumarin, oxidation of o-HPA to o-HPAA was higher compared to GSH conjugation of CE, and clearance of o-HPA through the oxidation pathway was considerably faster (more than 50 times) compared to rats [96]. This means that, in the human liver, not only the conversion to CE is likely to be very low, but also that the oxidation of o-HPA to o-HPAA occurs efficiently leading to the low hepatotoxicity of coumarin in humans.

4.4. Clinical trials

Coumarin has been used since the seventies for the treatment of various pathologies including lymphoedema, varicose veins, lung and kidney carcinoma, melanoma, infections and chronic fatigue syndrome [97–104]. Thus, thousands of individuals have been exposed to therapeutic doses of coumarin for periods ranging from 2 weeks to over 2 years. Recommended doses range from 8 mg for the treatment of venous constriction to 7000 mg/day in antineoplastic therapies [37]. These are doses up to 2000 times higher than the estimated maximum daily intake of coumarin, calculated considering oral and dermal exposure [105].

Several clinical studies have investigated the occurrence of hepatotoxicity in patients treated with therapeutic doses of coumarin [75–79,106]. Overall adverse reactions linked to hepatotoxicity, such as elevated liver enzymes in serum and clinical hepatitis, were addressed only in a small proportion of patients. Those patients usually displayed liver alterations that reverted to normal after cessation of treatment, while liver failure occurred only in extremely rare cases [43]. However, for this reason, coumarin has been withdrawn from the market in France and other countries [107].

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Marshall et al. have published three papers on the use of coumarin in combination with cimetidine for the treatment of several metastatic cancers, such as non-small cell lung cancer, renal cell carcinoma and melanoma [102,103,108]. These studies were subsequently grouped into a single publication on the effects of coumarin for the treatment of advanced malignancies. Overall, 91 patients (24 with non-small cell lung cancer, 45 with renal carcinoma and 22 with melanoma) were treated with 100 mg of coumarin together with 300 mg of cimetidine daily. No cases of hepatotoxicity were reported by the authors [37].

Mohler et al. carried out a clinical trial in the United States on 48 patients with prostate cancer treated with 3 g per day of coumarin [109]. The authors reported limited treatment hepatotoxicity, with 3 patients developing asymptomatic transaminase increase; only mild adverse reactions (such as nausea with vomiting) were assessed in additional 4 patients, without any sign of hepatotoxicity.

Casley-Smith et al. carried out a randomized, crossover, double-blind clinical trial in Australia in 31 patients with upper limb lymphoedema secondary to breast cancer and 21 patients with primary lower limb lymphoedema treated for the first six months with placebo and for the following six months with 400 mg per day of coumarin [110]. Apart from a few cases of transient gastrointestinal discomfort (nausea and diarrhoea), the authors did not report any case of transaminase elevation.

An additional clinical trial on 104 patients with chronic lymphatic filariasis enrolled in the Shandong province of China was carried out by Casley-Smith et al. [111]. Of these, 45 patients were randomized to receive 400 mg per day of coumarin and 38 to receive placebos for 367 days. In this study about 60% of coumarin treated patients experienced mild symptomatology as dizziness or drowsiness that, however, disappeared after the first month of treatment. No correlation between these symptoms and hepatotoxicity was revealed. Blood and urine tests were found to be normal and, in particular, there were no elevations in transaminase levels or, more generally, alterations in liver function parameters.

In a total of five clinical trials including 1106 lymphoedema patients treated with a daily dose of 400 mg coumarin for a mean duration of 14.6 months, Casley-Smith et al. [76] reported two cases of hepatotoxicity (incidence 0.18%). In one case symptoms regressed immediately after stopping treatment, while the symptomatology of the second patient was successively related to other causes.

In another clinical trial performed on 2173 with cancer or chronic infections treated with a daily dose of coumarin ranging from 25 to 2000 mg, with a majority receiving 100 mg per day for one month and then 50 mg per day for two years, eight patients (0.37%) developed elevated liver enzymes (serum transaminases) after total doses of between 1 and 15 g coumarin [100].

Morrison and Welsby [77] were the first to report a severe hepatic reaction to coumarin (characterized by high transaminases levels, malaise and icterus) in a lymphoedema patient treated with 400 mg of coumarin daily for five months. All abnormalities however resolved five weeks after the treatment when coumarin was discontinued. Later, Koch et al [79] reported two cases of acute hepatitis in patients treated with 90 mg/d of coumarin for 5 months. Authors observed a marked increase in serum aminotransferases (ALT: 30 and 100 times higher than the upper limit of the physiological range) in conjunction with clinical features including jaundice, pruritus and diarrhoea. Coumarin withdrawal was rapidly followed by a favourable outcome in both cases.

Burgos et al. carried out a double-blind clinical trial in Spain on 77 women aged 35 to 65 with upper limb lymphoedema secondary to radiotherapy or surgery for breast cancer [112]. Patients were randomized to receive coumarin at a dose of 90 mg per day (38 women) or 135 mg per day (39 women) for 12 months. A patient treated with 90 mg per day of coumarin (2.63%) with normal SGPT levels at baseline (15 U/l) showed a significant increase after 6 months (107 U/l). Similarly, a patient treated with 135 mg per day of coumarin (2.56%) with normal SGPT levels at baseline (47 U/l) showed a significant increase after 6 months (82 U/l). The authors reported that, in both cases, SGPT levels decreased

 doi:10.20944/preprints202209.0330.v1

after 12 months of treatment (respectively to 43 and 56 U/l). In all these cases symptoms were reversible and ceased after termination of coumarin treatment.

In other trials performed on 50 [101] and 17 [113] cancer patients treated with 100 mg per day of coumarin in association with 1 g per day of cimetidine no evidence of liver toxicity was observed.

Jamal et al. carried out a double-blind, placebo-controlled, randomized clinical trial in India on 169 patients with chronic lymphoedema secondary to filariasis [97]. Of these, 42 patients were treated with 400 mg of coumarin and 6 mg/kg of carbamazine per day; 39 patients were treated with placebo and 6 mg/kg of carbamazine per day; 47 were treated with 400 mg per day of coumarin and placebo; finally, 41 patients were treated with placebo of both products. An interim analysis conducted when the average duration of treatment was 9.3 months and 20 patients had completed the two years of treatment reported no cases of hepatotoxicity. Only mild adverse reactions were recorded, mainly abdominal pain, diarrhoea, constipation and dizziness, with no significant difference between the four study groups.

In Ireland Thornes et al. recruited 29 patients with melanoma undergoing surgical resection. Among these, 13 patients were treated with 50 mg per day of coumarin until disease progression. Treatment tolerability was good in all patients and no adverse reactions were recorded, including a patient that assumed coumarin during pregnancy [99].

Kokron et al. carried out a clinical trial in Austria on 38 patients with metastatic renal cell carcinoma and one patient with a second primary renal cell carcinoma treated with 100 mg of coumarin and 400 mg of cimetidine per day until disease progression. One patient discontinued treatment due to the onset of nausea correlated to coumarin intake by the authors. No hepatotoxicity was registered [114].

More recently, Grötz et al. investigated the efficacy of coumarin in combination with troxerutin for the protection of salivary glands and mucosa during radiotherapy in 48 patients with head and neck cancer recruited in Germany in a randomized, double-blind, placebo-controlled clinical trial. The treatment schedule included the administration of 90 mg of coumarin and 540 mg of troxerutin per day for five weeks. No adverse events were correlated to the combination of the two products [115].

Vanscheidt et al. recruited 231 patients with lower limb oedema secondary to chronic venous insufficiency in a randomized, double-blind, placebo-controlled clinical trial in Germany. Of these, 114 were treated with 90 mg of coumarin and 540 mg of troxerutin daily for 16 weeks. Liver function parameters (ALT, AST, alkaline phosphatase and gamma-GT) were monitored at baseline and after 4, 6, 8, 12 and 16 weeks of treatment. No hepatotoxicity was reported, while minor reactions were found in 25 cases (21.9%) in the coumarin and troxerutin group versus 14 cases (12.0%) in the placebo group. The relative risk of developing elevations greater than 1.25 times the physiological range of major liver function parameters (GGT, ALT and AST) was 4.9% in the active treatment group while 2.1% in the placebo one. The values returned to normal, sometimes during the treatment, in other cases after treatment discontinuation. Overall, authors reported a high drug tolerability both in the active treatment group and in the placebo group [116]. These results were confirmed by a separate publication focused on safety assessment which concluded that coumarin treatment is safe and well tolerated [117].

Lessiani et al. carried out an open-label clinical trial in Italy on 60 patients suffering from lymphoedema of the lower limbs secondary to surgery. Of these, 36 were randomized to receive one tablet per day of a product containing 50 mg of Melilotus officinalis extract (corresponding to 10 mg of coumarin), 50 mg of Vitis vinifera and 200 mg of troxerutin for 30 days in addition to prophylaxis with heparin and the use of elastic stockings. No hepatotoxic effects were reported, and the number of other adverse reactions in the active treatment group did not differ significantly from those recorded in the control group [118].

Although the above discussed studies agree in reporting that coumarin causes hepatotoxic effects in fewer than 1% of patients, and an association between coumarin and

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hepatotoxicity cases was not clearly documented, the only discording and unexpected result was obtained by Loprinzi et al. In their study the authors recruited 140 patients in the United States with lymphoedema secondary to radiotherapy or surgery for breast cancer [119]. Patients received either 400 mg per day of coumarin or placebo for six months, followed by six months with the alternative treatment in a randomized, cross-over clinical trial. There were no significant differences in the incidence of nausea, vomiting or diarrhoea during treatment with coumarin or placebo, but the incidence of hepatotoxicity was higher during treatment with coumarin. Nine women (corresponding to 6%) experienced significant transaminase increase ($p = 0.006$) which promptly regressed upon treatment discontinuation. One woman developed jaundice with bilirubin levels reaching 19.3 mg/dL.

Table 1. summarizes the main safety and tolerability data of coumarin in the clinical studies described above.

Table 1. Coumarin safety and tolerability in clinical trials.

Number of patients	Disease	Coumarin dose	Cotreatment with other drugs	Hepatotoxic effects (number of patients)	References
7	Melanoma	100 mg/day	No	No	Zanker et al. (1984) [98]
17	Cancer	100 mg/day	Cimetidine 1 g/day	No	Nolte et al. (1987) [113]
13	Melanoma	50 mg/day	No	No	Thornes et al. (1989) [99]
42	Lymphoedema secondary to filiriasis	400 mg/day	Carbamazine 6 mg/Kg per day	No	Jamal et al. (1989) [97]
39	Lymphoedema secondary to filariasis	No	Carbamazine 6 mg/Kg per day	No	
47	Lymphoedema secondary to filariasis	400 mg/day	No	No	
50	Cancer	100 mg/day	Cimetidine 1.2 g/day	No	Dexeus et al. (1990) [101]
38	Renal cell carcinoma	100 mg/day	Cimetidine 400 mg/day	No	Kokron et al. (1991) [114]
48	Prostate cancer	3 g/day	No	Asymptomatic transaminase elevation (3)	Mohler et al. (1992) [109]
31	Lymphoedema secondary to breast cancer	400 mg/day	No	No	Casley-Smith et al. (1993a) [110]
45	Chronic lymphatic filariosis	400 mg/day	No	No	Casley-Smith et al. (1993) [111]
91	Cancer	100 mg/day	Cimetidine 300 mg/day	No	Marshall et al. (1994) [37]
1106	Lymphoedema	400 mg/day	No	Mild hepatotoxicity regressed after ceased treatment (1)	Casley-Smith et al. (1995) [76]
1 (Case report)	Lymphoedema	400 mg/day	No	Severe hepatotoxicity (1)	Morrison and Welsby (1995) [77]
30	Chronic lymphoedema	400 mg/day	No	No	Chang et al. (1996) [120]
2	Lymphoedema	90 mg/day	No	Hepatitis with favourable outcome (2)	Koch et al. (1997) [79]
2173	Cancer/chronic infections	100-300 mg/day	No	Serum transaminase elevation at 1 to 15 g of total dose (8)	Cox et al. (1989) [100]
38	Lymphoedema secondary to breast cancer	90 mg/day	No	SGPT elevation (1)	Burgos et al. (1999) [112]

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39	Lymphoedema secondary to breast cancer	135 mg/day	No	SGPT elevation (1)	Burgos et al. (1999) [112]
48	Cancer	90 mg/day	Troxerutin 540 mg/day	No	Grötz et al. (2001) [115]
114	Chronic venous insufficiency	90 mg/day	No	Mild changes in liver function parameters (4.9%) compared to controls (2.1%)	Vanscheidt et al. (2002) [116]; Schmeck-Lindenau et al. (2003) [117]
60	Lymphatic oedema of lower limbs	10 mg/day	No	No statistical differences compared to controls	Lessiani et al. (2015) [118]

Based on these studies, coumarin induced hepatotoxicity can be considered rare; most of the cases have been reported as idiosyncratic or due to an unpredictable adverse drug reaction affecting a small subgroup of the population. Except for Loprinzi et al. [119], most of the above-mentioned clinical studies report that there is no clear relationship between coumarin and hepatotoxicity and that any changes in liver function parameters are transient. Also, a relationship between coumarin dose and hepatotoxicity has not been clearly demonstrated. Indeed, the time to onset of hepatotoxicity varied from 1 to 6 months, with the lowest dose observed to cause adverse effects of 87 mg/kg bw for male patients and of 30 mg/kg bw for female patients, both with oral dosing [100]. In patients treated with less than 25 mg per day no liver toxicity has been reported [47]. Overall, coumarin can be considered safe and well tolerated in the majority of the treated patients.

5. Discussion

Clinical trials clearly demonstrated that coumarin-induced hepatotoxicity is restricted to a very small subgroup of subjects, and it mostly occurs in a mild to moderate form. In most cases, patients showed transient elevation of transaminase levels which returned to normal during the treatment or immediately after; furthermore, to our knowledge no cases of liver failure have been reported and only one case of severe hepatotoxicity was assessed. Coumarin-induced hepatotoxicity, even if isolated and in most cases not severe, cannot be neglected and many countries, including the United States and European Union, have banned, or strictly limited, the use of coumarin, not only for therapeutic purposes but also as a food additive. Indeed, several National and International Scientific authorities performed a safety assessment of coumarin. In 2004 the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food of the European Food Safety Authority (EFSA) adopted an opinion in which it was concluded that coumarin was not genotoxic in experimental animals allowing the derivation of a Tolerable Daily Intake (TDI) [106]. Taking into consideration that the most sensitive animal species were rats and dogs, based on a two-year dog study, the overall No Observed Adverse Effect Level (NOAEL) for liver toxicity was found to be of 10 mg/kg bw per day. Applying a total safety factor of 100 to this NOAEL (a factor of 10 for potential interspecies variation, together with a factor of 10 for potential interindividual differences in humans), it was concluded that a TDI of 0 – 0.1 mg coumarin/kg bw could be established [106,121]. This conclusion was further supported by the German Federal Institute for Risk Assessment (BfR) [122,123]. Based on the data of Bergmann et al. [105], the Bfr considered 25 mg to be the lowest dose capable of inducing an hepatotoxic response and applied an extrapolation factor of 5 (assuming a typical slope of the dose-response curve) resulting in a level of 5 mg coumarin per day, which is expected to cause no adverse effects, even in sensitive people, confirming a TDI of 0.1 mg/kg bw. Considering the toxicity data of coumarin, including the timing to onset of liver effects, recovery of these effects after cessation of treatment and the elimination half-life, international committees have concluded that exposure to coumarin resulting in an intake 3 times higher than the TDI for one to two weeks is not of safety concern. Even if the coumarin TDI is considerably lower than

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the therapeutic doses, coumarin beneficial properties should not be ignored. Indeed, it should be considered that TDI determination has been based on the potential continuous intake of coumarin as a food additive. Furthermore, these data have been obtained from animal models, and even if interspecies variations have been considered, it should be stressed that we still lack a deep knowledge of coumarin metabolism in humans, especially for the subgroup of patients at risk of developing hepatotoxicity. In this context, the current state of advancements in the field of genetic screening and pharmacogenetics, and the advent of new methodologies that can be nowadays efficiently used to target risk subjects, could open the possibility to reintroduce coumarin as a therapeutic agent.

As previously discussed, members of the cytochrome p450 enzymes (CYPs) family are involved in the metabolism of coumarin. In humans, coumarin is predominantly eliminated via 7-hydroxylation by p450 CYP2A6. Conversely, the coumarin-induced liver toxicity is related to the coumarin metabolite o-HPA deriving from CE via the alternative heterocyclic ring-splitting pathway. This seems to be the predominant pathway in patients that develop hepatotoxicity.

Several studies, confirmed by recent experiments performed on rat and human liver microsomes, showed that the enzymes CYP1A2 and CYP2E1 are involved in catalysing the ring-splitting route [124]. Interestingly, Miura et al. demonstrated that the inhibition of human CYP2E by furafylline results in a marked reduction of o-HPA plasma levels, compared to the control [125].

Interesting data come from computational studies that are thoroughly discussed in a recent review. These studies regarding the interaction of coumarin with CYPs enzymes show some of the possible mechanisms involved in the activation/inhibition of coumarin metabolic pathways that can help clarify its significance in toxicology [126].

Understanding the role of the enzymes involved in the coumarin metabolism and detoxification, and identifying alterations in these enzymes can be useful to clarify the mechanisms underlying the metabolic activation of CE pathway and to assess the risk in the population.

Analysis of CYP2A6 polymorphism, as previously reported, did not reveal a correlation with coumarin-induced hepatotoxicity: a study carried out to determine whether coumarin-associated liver dysfunction is genetically determined by polymorphism in CYP2A6 and impairment of the 7-hydroxylation of coumarin [87] demonstrated that here was no significant difference in the incidence of liver dysfunction between heterozygotes with CYP2A6*2, CYP2A6*3 and wild-type homozygotes. However, it is not to be excluded that polymorphism in other genes such as those coding for CYP2E1, CYP1A2 enzymes, as suggested by the results obtained by Miuru et al., or those coding for ALDH, that is involved in the oxidative detoxification of o-HPA, could be involved in hepatotoxicity [34]. Indeed, an ALDH2 polymorphism is known to be related to a marked sensitivity to acetaldehyde, mainly in Asiatic population [127]. Notably, it is not to be excluded a contingent combination of these. Studies demonstrated that the expression of p450 genes is influenced by a combination of different factors including genetic polymorphisms, sex, age, ethnicity, health conditions, and induction by xenobiotics [128]. Thus, other factors such as the concomitant use of other drugs that could interfere with coumarin metabolism, previous liver injuries, smoke habits [124] and alcohol consumption could contribute to increase coumarin toxicological risk and must be considered. Indeed, it has been demonstrated that human CYP2A6 expression is induced by alcohol [129].

As coumarin-induced hepatotoxicity is a multifactorial outcome the identification of risk factors should follow a multidisciplinary approach. For this purpose, current advances in biotechnology and computational models could be exploited to improve pharmacogenetics and genetic screening data obtained in the past studies. Furthermore, these could be joined into a new approach methodologies system (NAMs). An interesting and innovative approach, currently limited to cosmetic ingredients toxicity assessment, is the Next Generation Risk Assessment (NGRA). Interestingly, a recent Next-Generation Risk Assessment Case study has been reported specifically for coumarin in cosmetic products [130].

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In association with data obtained from conventional experimental methods, extending NGRA to the risk assessment of products intended for therapeutic use could be a possible future strategy to improve our knowledge on coumarin toxicity.

6. Conclusions

Coumarin, used for different purposes, in monotherapy as well as in combination with other products, is safe and well tolerated in most patients, with only a small subgroup of subjects showing signs of mild to moderate hepatotoxicity. However, the rare occurrence of these outcomes led to limitations and/or bans to the use of coumarin as therapeutic in several countries. Further studies are needed to identify individuals at risk of developing hepatotoxicity. In this context, two areas of particular interest are the analysis of polymorphisms of genes coding for enzymes involved in the metabolism and/or detoxification of coumarin, including ALDH and cytochrome P450 isoforms (also different from CYP2A6), and the identification of the environmental factors involved. In addition, a useful parameter could be the evaluation of the type and concentration of coumarin metabolites in urine during the first weeks of treatment, that could indicate possible variations in metabolic pathways. Importantly, due to the multifactorial nature of coumarin-induced hepatotoxicity, an integrated approach (e.g., NAMs) should be applied to improve the current knowledge on this topic.

In conclusion, the aim of this work is to stimulate research and advancements on coumarin hepatotoxicity knowledge since recent literature in this field is very limited and should be implemented in light of current biotechnological advancement. Early identification of patients at risk of hepatotoxicity could allow the possibility of safely exploiting the health-promoting effects of the product.

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