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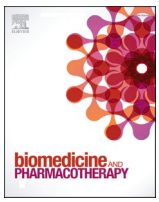

Review

Clofazimine: A journey of a drug

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ABSTRACT

Among different strategies to develop novel therapies, drug repositioning (aka repurposing) aims at identifying new uses of an already approved or investigational drug. This approach has the advantages of availability of the extensive pre-existing knowledge of the drug's safety, pharmacology and toxicology, manufacturing and formulation. It provides advantages to the risk-*versus*-rewards trade-off as compared to the costly and time-consuming *de novo* drug discovery process. Clofazimine, a red-colored synthetic derivative of riminophenazines initially isolated from lichens, was first synthesized in the 1950 s, and passed through several phases of repositioning in its history as a drug. Being initially developed as an anti-tuberculosis treatment, it was repurposed for the treatment of leprosy, prior to re-repositioning for the treatment of multidrug-resistant tuberculosis and other infections. Since 1990 s, reports on the anticancer properties of clofazimine, both *in vitro* and *in vivo*, started to appear. Among the diverse mechanisms of action proposed, the activity of clofazimine as a specific inhibitor of the oncogenic Wnt signaling pathway has recently emerged as the promising targeting mechanism of the drug against breast, colon, liver, and other forms of cancer. Seventy years after the initial discovery, clofazimine's journey as a drug finding new applications continues, serving as a colorful illustration of drug repurposing in modern pharmacology.

1. History of clofazimine: from tuberculosis to leprosy

Clofazimine (Fig. 1), also named B663, belongs to a large series of riminophenazine derivatives synthesized by Vincent Barry and co-workers as part of a project to find treatment for tuberculosis (TB). The synthesis and chemistry of this chemical series have been described in a series of papers in the 1950 s, starting from isolating diploicin from extracts of the lichen *Buellia canescens*. This natural chlorinated compound showed inhibition against *Mycobacterium tuberculosis in vitro*, and chemical modifications of diploicin led to a series of phenazine dyes with improved anti-TB activity [1–3]. The forerunner of these compounds, clofazimine was shown to inhibit the growth of *Mycobacterium in vivo* in mice and guinea pigs [1,2]. However, early studies in animal models (hamsters, guinea pigs, rabbits, non-human primates) of TB demonstrated inconsistent therapeutic activity of clofazimine [4]. Further development of clofazimine against TB was restrained by the emergence of more potent anti-TB agents isoniazid and pyrazinamide in the early 1950 s and rifampicin and ethambutol in the early 1960 s [5]. Clinical application of clofazimine also has setbacks due to the fact that clofazimine, as a potent phenazine dye, turns the patients' skin red. Although after the termination of treatment, the skin discoloration is reversed with time, this cosmetic side effect provides a psychological discomfort to the patients.

The new breath in clofazimine development was brought thanks to its repositioning as an anti-leprotic agent (Fig. 2). In 1962, the use of clofazimine to treat human patients with leprosy was first reported, describing clinical and bacteriological improvement [6]. Since then, clofazimine has significantly contributed to the global control of leprosy. Clofazimine demonstrated an inhibitory effect in experiments injecting *M. leprae* in mouse foot pads [7]. Subsequently, small-scale clinical studies conducted in Brazil suggested that clofazimine as a monotherapy could be as efficient as dapsone, the first drug proven to be effective against *M. leprae* and the standard treatment at the time [8]. As an anti-leprosy agent, clofazimine was initially launched by Novartis in 1969 as Lamprene®. Around the 1960 s, dapsone resistance and treatment failures began to be reported, and clofazimine started to become a major component of multidrug therapy studies [9]. Eventually, in 1977, this led to the inclusion of clofazimine into the World Health

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Organization (WHO)'s recommendation for the standard treatment of leprosy with the multidrug therapy combining dapsone, rifampin and clofazimine [10].

2. Clofazimine in various disease contexts

2.1. Broad spectrum antibiotic

The number of non-tuberculous mycobacterial (NTM) infections has been rising in the past decades, linked with the population ageing and the human immunodeficiency virus (HIV) epidemic. NTM infections are especially prevalent in those predisposed to impaired host immunity or structural lung diseases [12,13]. Activities of clofazimine against rapidly growing NTMs were explored since the 1980 s [14]. Clofazimine was reconsidered as an alternative drug in NTM infections thanks to large *in vitro* studies followed by several clinical studies comparing the efficacy and safety of clofazimine with the recommended regimens. Despite the initial promise, further studies suggested some inconsistency in the efficacy of clofazimine [11]. The British Thoracic Society has recently released new guidelines on NTM treatment, mentioning clofazimine as an alternative drug to consider in difficult-to-treat *M. abscessus* infections [15]. In general, there is a potential benefit for the use of clofazimine against rapidly growing mycobacteria, especially of the *M. abscessus* group [16].

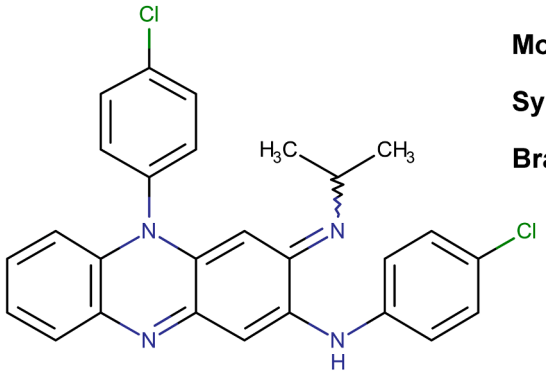
The widespread dissemination of drug-resistant *M. tuberculosis* strains has been a continuous threat to the global effort of ending TB. Current first-line anti-TB antibiotics are not sufficiently effective against drug-resistant TB (DR-TB) [17]. The renaissance of interest in exploring clofazimine as an anti-TB agent was revived in the 2010 s [18,19]. In these studies, the so-called 'Bangladesh regimen' was developed, which includes clofazimine for the treatment of multidrug-resistant TB (MDR-TB, defined as resistance to at least isoniazid and rifampin), demonstrating the shortening of the treatment duration for the difficult-to-treat cases. And in 2019, WHO published their guideline with a recommendation to incorporate clofazimine as a second-line compound for the use in combination with other drugs for the treatment of MDR-TB [20].

In addition to its anti-mycobacterial activities, clofazimine has been reported to have a broad spectrum of antimicrobial, anti-parasitic, and anti-fungal activities [21]. Among the bacteria, clofazimine is active against diverse Gram-positive organisms, whereas Gram-negative bacteria are uniformly resistant to the drug. Parasites that are susceptible to clofazimine include *Plasmodium falciparum*, *Leishmania donovani*, *Trypanosoma cruzi*, *Babesia*, *Theileria*, and *Schistosoma* species; the yeast *Candida albicans* is also susceptible [21].

The exact mechanism of clofazimine-mediated antimicrobial activity remains unclear, but the site of action of the drug is suspected to be the bacterial outer membrane. Upon initial discovery and description of clofazimine, the lipophilic nature of the drug was suggested to mediate its high redox potential [2]. Oxidation of reduced clofazimine was then suggested to generate reactive oxygen species (ROS), superoxide and H_2O_2, contributing to the antimicrobial activity of clofazimine [22]. Disruption of the membrane structure and function was proposed as the mechanism of its selective antimicrobial activity towards Gram-positive bacteria (including mycobacteria), suggesting that Gram-negative bacteria are insensitive to clofazimine due to the poor penetration of the outer membrane by this agent [23]. A more elaborated theory was later developed [5], suggesting that clofazimine interacts with bacterial membrane phospholipids to generate antimicrobial lysophospholipids, with the bactericidal efficacy arising from the combined membrane-destabilizing effects of both clofazimine and lysophospholipids, which interfere with the K^+ uptake and, ultimately, the ATP production.

2.2. Clofazimine as an anti-inflammatory/immunosuppressive agent

Since the 1970 s, when clofazimine began to be used to treat leprosy patients, the drug demonstrated effects on different aspects of the immune system [3]. During leprosy treatment, clofazimine shows anti-inflammatory activities, controlling the occurrence of potentially destructive immune-mediated reactions [8,11], typical in leprosy patients and including episodes of acute inflammation intervening the chronic infection course [24]. Of the types of destructive immune-mediated reactions, leprosy type I (or reversal) reactions represent prolonged hypersensitivity, caused by infiltration of T-helper cells leading to exacerbation of old lesions and appearance of local erythemas. Meanwhile, type II reactions (aka erythema nodosum leprosum) represent systemic inflammation mediated by immunocomplexes, whereas high levels of proinflammatory cytokines cause systemic symptoms such as fever along with new subcutaneous erythematous nodules. The anti-inflammatory and immunosuppressive properties of clofazimine have been suggested to contribute to its reported efficacy in various off-label uses against non-microbial chronic inflammatory disorders, such as lupus erythematosus, pustular psoriasis, and non-cutaneous autoimmune disorders including multiple sclerosis and type I diabetes mellitus [5,9]. The anti-inflammatory effects, pro-oxidative activities, and immunopharmacological properties of clofazimine have been demonstrated in a series of observations studying its effects on the immune system. In these studies, clofazimine was found to enhance production of ROS, phagocytosis, and antimicrobial activities of human and murine neutrophils and macrophages [25–27], the effects proposed to be mediated by phospholipase A2 [28]. Clofazimine



Chemical formula: $C_{27}H_{22}Cl_2N_4$

Molecular weight: 473.396

Synomyms: Clofazimine, B663

Brand names: Lamprene (Novartis)
Clofozine (AstraZeneca)
Hansepran (Abbott)
Lamcoin (Pond's Chemical)

Chemical name: N,5-Bis(4-chlorophenyl)-3,5-dihydro-3-[(1-methyethyl)imino]-2-phenazinamide

Fig. 1. Clofazimine. Structure, chemical formula, synonyms and brand names.

was also found to suppress lymphocyte transformation and to inhibit mitogen and antigen-driven proliferative responses of isolated T-lymphocytes [29–31]. The anti-inflammatory effects of the drug on T-cells could be indirect, such as through clofazimine-stimulated prostaglandin synthesis and ROS production in neutrophils that suppress lymphocyte proliferation [32]. Alternatively, inhibition of lymphocyte Na^+, K^+ adenosine triphosphatase (ATPase) was proposed as a direct mechanism of immunosuppression [30].

Another mechanism of clofazimine's anti-inflammatory effects was described by Ren and co-authors [31]. They identified clofazimine as an inhibitor of the T-cell receptor antigen-mediated intracellular calcineurin-NFAT signaling pathway, which mediates the transcriptional activation of the gene encoding the cytokine interleukin-2 (IL-2). To identify the molecular target of clofazimine, the authors elucidated that clofazimine interferes with Ca^{2+} signaling in T-cells through inhibition of the Kv1.3 potassium channel [31]. The authors further demonstrated that clofazimine is selective towards Kv1.3 among the Kv channel species, and provided evidence for the direct interaction between purified Kv1.3 protein and clofazimine *via* the electrophoretic mobility shift assay.

Thus, investigations in immune cells proposed multiple potential targets of clofazimine in its immunosuppressive activities, two of which (Na^+, K^+-ATPase and Kv1.3) being suggested in later studies as responsible for the anti-cancer effects of clofazimine, which is elaborated in Section 5 below.

2.3. Conflicting evidence on the antiviral potential of clofazimine against SARS-CoV-2

When severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged at the beginning of 2020, drug repurposing became a hot topic again. COVID-19 caused by SARS-CoV-2 has claimed over 6.9 million lives in just 3.5 years (who.int/publications/m/item/weekly-epidemiological-update-on-covid-19–-20-july-2023). When faced with the need to urgently identify potential treatments against the unrolling pandemic, the conventional drug discovery and development process is by far too long as compared to the pace potentially offered by drug repositioning.

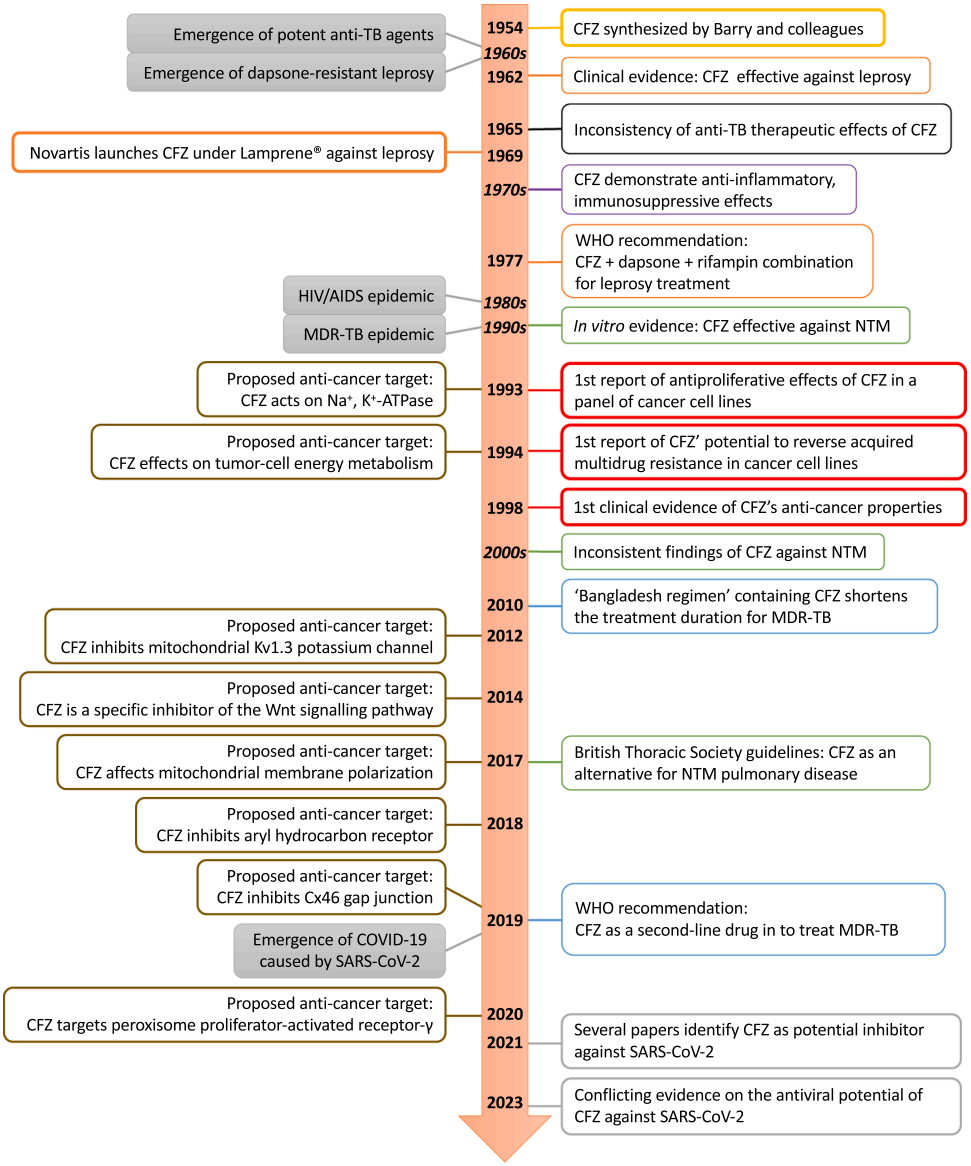


Fig. 2. Timeline of clofazimine discovery and applications. The discovery of clofazimine (CFZ) and its applications in leprosy, tuberculosis (TB), multidrug-resistant TB (MDR-TB), non-tuberculous mycobacterial (NTM) infections, viral infections, and cancer, accompanied by historical timelines and information on the associated diseases.

High-throughput screening identified clofazimine among several other drugs as potential inhibitors of arenavirus lymphocytic choriomeningitis virus and SARS-CoV-2 [33]. Similarly, hierarchical *in silico / in vitro* screening pinpointed clofazimine among other drugs as having an activity against the cellular entry of SARS-CoV-2 [34]. Clofazimine has also been identified in a transcriptomics-based drug repositioning pipeline for SARS-CoV-2 [35]. Another *in silico* high-throughput screening study has suggested that clofazimine is a potential inhibitor of SARS-CoV-2 PL^{PRO} and $3CL^{PRO}$ proteases, critical for the life cycle of SARS-CoV-2 [36]. In terms of clofazimine's antiviral activities, Yuan and co-authors presented the most compelling body of work suggesting that clofazimine is effective in a number of SARS-CoV-2 assays, both *in vitro* and in animal models, showing that the drug blocks several steps of the coronavirus infection, such as the initial viral entry, spike-mediated cell fusion, viral RNA synthesis, and the unwinding activity of the SARS-CoV-2 helicase [37].

Clofazimine has also been proposed to possess activities against the rabies virus, inhibiting the g-glycoprotein-mediated viral membrane fusion process [38]. A follow-up study screening a panel of clofazimine derivatives against rabies and SARS-CoV-2 confirmed the antiviral activities of clofazimine and multiple derivatives [39]. Interestingly, the proposed mechanism of action of the best clofazimine derivative 15 f was, for SARS-CoV-2, the targeting of G or S protein to block membrane fusion while for the rabies virus – binding to L protein (nsp13) leading to inhibition of intracellular biosynthesis [39].

As detailed below (Section 5), we identified clofazimine is a potent inhibitor of the Wnt signaling pathway [40–42]. As the Wnt signaling and its major component β-catenin have been reported to play an important role in the SARS-CoV-2 infection [43], we hypothesized that the broadness of the anti-SARS-CoV-2 activities ascribed to clofazimine might indicate that clofazimine acts not on a given viral component, but on a host cell component repeatedly used by the coronavirus [44]. Using clofazimine and its derivative [45], as well as several other Wnt pathway inhibitors, we have unexpectedly found that a diverse set of Wnt inhibitors, including clofazimine, efficiently block Wnt signaling but do not result in any suppression of the SARS-CoV-2 infection in lung epithelial cells [44]. We suspect that the discrepancy is due to the fact that the earlier studies used Vero-E6 cells, the African green monkey kidney cell line, less relevant to modelling the pulmonary SARS-CoV-2 infection [37,43]. Thus, clofazimine and, in general, pharmacological modulation of the Wnt pathway is unlikely to be a promising strategy to control the SARS-CoV-2 infection [44], calling for caution with the choice of relevant models to investigate the potential antiviral effects of clofazimine.

3. Pharmacokinetics, pharmacodynamics, and safety of clofazimine

Absorption of clofazimine varies from 45% to 62% following oral administration in leprosy patients, depending on whether the drug is taken with or without food. Co-administration of 200 mg clofazimine with food resulted in a C_{max} of 0.41 mg/L with a T_{max} of 8 h; while administered in a fasting state, the corresponding C_{max} was 30% lower with a T_{max} of 12 h [5].

Clofazimine is highly lipophilic, therefore it deposits primarily in fatty tissues and cells of the reticuloendothelial system. In the serum, clofazimine is bound primarily to beta-lipoproteins (saturates at ~10 μg/mL) and to a lesser extent, alpha-lipoproteins, whereas binding to gamma-globulin and albumin is negligible. In leprosy patients, daily oral administration of 100, 300 or 400 mg clofazimine resulted in average plasma levels of 0.7, 1.0 and 1.41 mg/L, respectively. Daily intake of 600 mg results in peak serum levels of 4 mg/L. In leprosy patients, the concentration of clofazimine in the fat has been reported to be as high as 5.3 mg/g, while concentrations of 0.1 mg/g were found in bile, gall bladder, kidney, pancreas, skin, liver, spleen, lymph nodes, eyes and lung. Autopsies have revealed crystalline deposits of clofazimine in various tissues, including the intestinal mucosa, liver, spleen, and mesenteric lymph nodes. Upon administration of clofazimine to mice at a dose of 25 mg/kg body weight for 28 days, the average concentrations of this agent in the lungs, spleen, fat and plasma were ~800, 4000, 80 mg/kg and 3 mg/L, respectively. Clofazimine can cross the placenta, but very poorly – the blood-brain barrier [46]. The metabolism of clofazimine is hepatic. Three clofazimine metabolites with unclear pharmacological activities have been detected in the urine. Clofazimine has been described as a CYP3A4 inhibitor with an IC_{50} of 1.69 μM in human liver microsomes [47]. Interestingly, in HepaRG cells (an *ex vivo* model compliant with FDA and EMA guidelines), at > 0.25 μM clofazimine exhibited weak inductive effects on CYP3A4 [47]. The mean elimination half-life of the drug is *ca.* 25 days. Small amounts of clofazimine are excreted in the sputum, sebum, and sweat. Part of an ingested dose of clofazimine is found in the feces, which may represent the bile excretion; urinary excretion is neglectable.

As an important, cosmetic side effect, clofazimine results in pigmentation in 75–100% of patients within a few weeks of treatment, from pink to brownish-black color. Clofazimine has additional side effects like ichthyosis and dryness (8–28%); rash and pruritus (1–5%). Skin discoloration disappears within a few months after termination of the therapy. The side effects rarely warrant discontinuation of the treatment. However, some patients develop depression because of the skin discoloration. Because clofazimine is secreted into the body fluids, it can stain the cornea and conjunctiva, without affecting vision. Like the skin discoloration, the ocular staining is gradually reversible upon cessation of the therapy. Mild gastrointestinal side effects like abdominal pain, diarrhea, nausea, vomiting or gastrointestinal intolerance may occur in 40–50% of patients. When administered in a high dose (>100 mg daily) for a prolonged time (several months or more), rare but more severe gastrointestinal side effects (splenic infarction, bowel obstruction, bleeding) may occur, and occasionally be fatal [5,9].

As clofazimine has been routinely used to treat leprosy, there is extensive data on the pharmacokinetics, toxicity and side effects of the drug. The fact that clofazimine has been taken by leprosy patients in a daily regimen with a treatment period that lasts for years and still demonstrates mild to moderate side effects, makes clofazimine a promising candidate for drug repurposing. The lipophilic property of clofazimine led to its tissue accumulation and slow elimination, which might in turn become beneficial for the drug accumulation in tumors during cancer treatment (see below).

4. Exploration of clofazimine as an anti-cancer drug

In 1993, Van Rensburg and co-authors were the first to report antiproliferative effects of clofazimine against a panel of cancer cell lines [48]. In the 30 years that passed, there have been many observations of the anti-cancer potential of clofazimine. The initial *in vitro* studies on cancer cell proliferation were followed by reports that clofazimine can reverse the acquired multidrug resistance in cancer cell lines [49–52], encouraging further exploration of the anti-cancer potential of the drug. These investigations were followed by studies in animal models, and, ultimately, clinical studies [53,54]. We detail these developments in the following sections.

4.1. Broad evidence of clofazimine's anti-cancer activities in vitro and in vivo

Clofazimine has been shown as a promising cytotoxic drug targeting cancer cells while sparing healthy cells across different types of cancers *in vitro*. A summary of the published works employing *in vitro* or *in vivo* models to address clofazimine's anti-cancer activities is provided in Table 1. Some examples of these studies are as follows.

In the first studies reporting the anti-cancer properties of clofazimine published 30 years ago, Van Rensburg and colleagues investigated the effects of the drug *in vitro* and *in vivo* [48,55]. In the former, clofazimine

Table 1
Cell line and animal model studies of anticancer properties of clofazimine.

Year	Study type	Cancer type (cell lines) / Animal models	Ref.
1993	*in vitro*	human pharynx squamous carcinoma (FaDu) human cervix epithelioid carcinoma (HeLa) T24 human transitional cell bladder carcinoma (HTB4) human hepatocellular carcinoma (PLC), primary culture	[48]
1993	*in vivo*	benzo(a)pyrene-induced sarcomas of mice dimethylbenz-anthracene (DMBA)-induced rat mammary tumors	[55]
1994	*in vitro*	human pharynx squamous carcinoma (FaDu/HTB 43) human cervix epithelioid carcinoma (HeLa) T24 human transitional cell bladder carcinoma (HTB 4)	[49]
1994	*in vitro* *in vivo*	non-small cell bronchial carcinoma non-small cell bronchial carcinoma (subcutaneous xenograft, athymic mice)	[56]
1994	*in vitro*	human lung cancer cells (H69/LX4), doxorubicin-resistance selection	[49]
1996	*in vitro*	human myeloid leukemia (K562), doxorubicin-resistance selection	[52]
1996	*in vitro*	human hepatocellular carcinoma cells (HepG2, PLC, Mahlavu) human colorectal carcinoma (CaCo2) human cervix epithelioid carcinoma cells (HeLa)	[60]
2000	*in vitro*	human colon cancer cells (CaCo2, COLO 32 DM, HT-29)	[51]
2012	*in vitro*	human leukemic T lymphocytes (Jurkat) human osteosarcoma (SAOS2) mouse melanoma (B16F10) human chronic myelogenous leukemia (K562) human skin fibroblasts, primary culture	[57]
	in vivo	melanoma mouse model (B16F10 subcutaneous injection, syngeneic C57BL/6 mice)	
2013	*in vitro*	chronic lymphocytic leukemia (B-CLL)	[61]
2014	*in vitro*	human myeloid leukemia (OCIAML3, HL-60, K562, mL-1, MOLM-13) human breast adenocarcinoma (MCF-7, MDA-MB-231) human colon carcinoma (DLD-1, Colo205) human neuroblastoma (SHSY5Y)	[62]
2014	*in vitro*	TNBC (BT-20)	[40]
2017	*in vitro*	pancreatic ductal adenocarcinoma (As PC-1, Bx PC-3, Capan-1, Capan-2, Mia PaCa 2, Panc-1, Panc-89, Panc-TUI, PT-45)	[58]
	in vivo	orthotopic pancreatic ductal adenocarcinoma xenotransplantation model (Colo35, SCID beige mouse)	
2017	*in vitro*	murine glioblastoma (GL261) human glioblastoma (A172, LN308)	[59]
	in vivo	mouse orthotopic glioma model (GL261)	
2017	*in vitro*	human multiple myeloma (U266) hematological cell lines (Jurkat, Namalwa, K562, HL60)	[63]
2018	*in vitro*	human multiple myeloma (MM .1 S, RPMI-8226, U266, ARH-77, KMS-11) mouse hepatoma (Hepa 1c1c7)	[64]
	in vivo	multiple myeloma model (MM .1 S or RPMI-8226 xenograft, SCID mice) Vk*Myc orthotopic model (immunocompetent transgenic model of spontaneously arising myeloma)	
2019	*in vitro*	glioblastoma cancer stem cell population isolated from patient-derived glioblastoma xenografts	[65]
2019	*in vitro*	TNBC cancer (BT-20, MDA-MB 231, MDA-MB 468, HCC 1395, HCC 1806, HCC 38, IOWA-1 T)	[41]
	in vivo	TNBC cancer mouse model (IOWA-1 T or BT-20 transmammary graft, NOD-SCID-gamma mouse)	
2020	*in vitro*	human myeloid leukemia (K562) peripheral blood samples from chronic myeloid leukemia patients, primary culture	[66]
	in vivo	myeloid leukemia mouse model (K562 xenograft, athymic nude (nu/nu) mice)	
2020	*in vitro*	colorectal cancer (SW48, HT29, DLD1, HCT116, SW620, LS174T) hepatocellular carcinoma (Hep3B, HepG2, SNU398, Huh7) ovarian cancer (OVCAR3, PEO1, OVSAHO, KURAMOCHI) glioblastoma (U87, U118, U251)	[42]

was found cytotoxic against a panel of squamous carcinoma cell lines, at concentrations achieved in plasma upon ingestion of clofazimine in leprosy-treated patients [48]. In the latter, the anti-tumor activity of clofazimine (supplied at 30 mg/kg/day for 4 weeks) was found against carcinogen-induced sarcomas of mice and mammary tumors in rats, accompanied by the markedly improved survival of the experimental animals [55]. These pioneering findings were followed by multiple investigations (Table 1), such as those reporting the efficiency of clofazimine in suppressing tumor growth in xenograft mouse models of human non-small cell bronchial carcinoma [56], melanoma [57], pancreatic ductal adenocarcinoma [58], or triple-negative breast cancer (TNBC) [41,45]. In these studies, diverse routes of clofazimine administration all resulted in a strong reduction in tumor growth rate and size, without any noticeable adverse effects. A detailed toxicity investigation reported no detectable changes in the brain, heart, lungs, small intestine, kidney, liver and spleen [57]. In opposition to these success stories, intraperitoneal administration of clofazimine was found ineffective in an orthotopic mouse glioma model [59]. This inefficiency could be attributed to the poor permeability of the undamaged blood-brain barrier by clofazimine [46], but also to the low sensitivity of glioma cells to the drug [42].

4.2. Clofazimine reverses acquired cancer multidrug resistance

Cancer treatment with chemotherapy faces challenges of resistance [67]. In many such incidences MDR1/P-glycoprotein/ABCB1 is overexpressed, which is an energy-dependent multidrug efflux pump extruding a variety of unrelated anti-tumor drugs from the cancer cells [68]. Certain resistance-modifying agents can reverse the drug resistance by allowing cytotoxic drugs to accumulate in tumor cells. For example, cyclosporin A (CsA) was one of the first compounds shown to produce chemo-sensitizing effects [69]. The chemo-sensitizing activity of clofazimine, in comparison to CsA, was tested on the human lung cancer cell line (H69/LX4) that had been evolved by progressive exposure to increasing concentrations of doxorubicin, gaining chemoresistance due to overexpression of MDR1 [49]. Clofazimine, at concentrations lower than those of CsA, was found to efficiently neutralize chemoresistance to vinblastine, doxorubicin, daunorubicin and mitomycin in the H69/LX4 cells. In contrast, the resistance to methotrexate and cyclophosphamide not associated with MDR1 overexpression was not abrogated by clofazimine [49].

In a similar study, resistance to doxorubicin, vinblastine and daunorubicin that had been evolved in human myeloid leukemia K562 cells through increased MDR1 expression was efficiently reverted by clofazimine [52]. Further investigations by the team of Van Rensburg studied the potential of clofazimine to oppose MDR1-mediated resistance in intrinsically multidrug-resistant cell lines. Using three human hepatocellular carcinoma cell lines (HepG2, PLC and Mahlavu) and three human colorectal carcinoma cell lines (CaCo2, COLO 32 DM, HT-29), the efficiency of clofazimine in combination treatments was found to correlate with the initial levels of MDR1 expression by the lines [51,60]. These studies identify MDR1 as the primary target of clofazimine's activity as a chemo-sensitizing agent. Interestingly, MDR1 has been revealed as a target gene of the oncogenic Wnt signaling pathway [70], and the pathway-driven overexpression of MDR1 has emerged as

one of the primary molecular mechanisms of Wnt-dependent acquired chemoresistance in cancer [71]. Our studies have identified clofazimine as a specific inhibitor of Wnt signaling in cancer (see more on that below) and highlighted Wnt pathway-dependent suppression of MDR1 expression as the key mechanism in the chemo-sensitization of cancer cells to clofazimine [40–42].

4.3. Clinical evidence of clofazimine's anti-cancer properties

Clofazimine has been tested in clinical settings for hepatocellular carcinoma (HCC) as a monotherapy [53] or in combination with doxorubicin [54]. In the monotherapy phase II study [53], 30 patients (of which 26 males) were enrolled, 25 of them having unresectable HCC and 5 having metastasis HCC. The patients received clofazimine treatment with an initial dose of 600 mg daily for 2 weeks, followed by 400 mg daily until progression or death. Three patients (10%) responded to the treatment. Fourteen patients (46.7%) had early disease progression, and 13 patients (43.3%) achieved disease stabilization lasting between 3 and 20 months. The overall medium survival was 13 weeks, representing a small improvement over the medium survival of 6–8 weeks from diagnosis of the advanced unresectable disease, which is normally low in these patients due to the limited response to conventional chemotherapies.

In the combination treatment phase II clinical trial [54], 28 patients (of which 23 males) with primary HCC were enrolled. Seven patients received oral clofazimine alone at a dose of 600 mg daily for 2 weeks, followed by 400 mg daily thereafter. The other twenty patients received the same schedule of clofazimine plus doxorubicin 50 mg/m^2 intravenous injection every 21 days. Of these patients, 9 had disease stabilization, and the remaining 18 had disease progression. The medium survival time of both groups of patients receiving clofazimine with or without doxorubicin was 7 weeks; the co-admission of clofazimine with doxorubicin did not show an additional therapeutic effect as had been expected from the pre-clinical studies.

Looking retrospectively at these early clinical trials, we may expect that their limited effectiveness could have been due to the lack of patient stratification for inclusion into the trial, that was due to the poor understanding of the mechanism of action of the drug in cancer at the time. Nowadays, with a better understanding of this mechanism(s) of action, re-initiation of the clinical trials of clofazimine on cancer patients with molecularly defined inclusion criteria is desired [41,42].

5. Potential mechanisms of action of clofazimine as an anticancer agent

Clofazimine has demonstrated promising anti-cancer properties in numerous models. However, the mechanism of action of clofazimine in terms of its anti-cancer activities is still controversial. As mentioned above, the anti-inflammatory properties of clofazimine have been attributed to the inhibition of Na^+, K^+-ATPase [30] and Kv1.3 potassium channel [31]. Interestingly, both proteins were later proposed as the targets of clofazimine's anticancer effects as well. Other works have suggested diverse additional mechanisms of action and targets of clofazimine in cancer. A summary of these investigations on the drug's potential targets and mechanisms of action is provided in Table 2.

5.1. Clofazimine may act on Na^+, K^+-ATPase in cancer cells

Na^+, K^+-ATPase as a potential target of the anti-cancer effects of clofazimine was addressed in the works of Van Rensburg and co-workers [48,72]. Following the initial study highlighting this enzyme as a target of clofazimine in lymphocyte proliferation [30], the team verified that clofazimine promotes the activity of phospholipase A2 (PLA2) and increases the release of lysophosphatidylcholine. They then demonstrated that clofazimine inhibits the activity of Na^+, K^+-ATPase in both intact cells and in purified membranes. Noteworthy, the three reports [30,48,

Table 2
Targets and mechanisms of action of clofazimine in cancer.

Year	Paper title	Clofazimine (CFZ) mode of action summary	CFZ target	Ref.
1993	Clofazimine and B669 inhibit the proliferative responses and Na^+, K^+-adenosine triphosphatase activity of human lymphocytes by a lysophospholipid-dependent mechanism	Authors investigated the anti-inflammatory mechanism of action of clofazimine. They identified that the Na^+, K^+-adenosine triphosphatase (ATPase) as the primary target of clofazimine, responsible for its lysophosphatidylcholine-mediated inhibition of lymphocyte proliferation.	Na^+, K^+-ATPase	[30]
1993	The riminophenazine agents clofazimine and B669 inhibit the proliferation of cancer cell lines *in vitro* by phospholipase A2-mediated oxidative and nonoxidative mechanisms	Clofazimine was shown to have anti-proliferative effects on cancer cell lines. The authors verified that clofazimine promotes the activity of phospholipase A2 (PLA2) and increased the release of LPC.	(Na^+, K^+-ATPase)	[48]
1993	Evaluation of the antineoplastic activities of the riminophenazine agents clofazimine and B669 in tumour-bearing rats and mice	Clofazimine treatment before the appearance of the tumor (from carcinogen induction), does not have protective effects on tumor load. The authors suspected this may due to the immunosuppressive properties of the drug.	?	[55]
1994	The antiproliferative riminophenazine agents clofazimine and b669 promote lysophospholipid-mediated inhibition of Na+ , K+ -adenosine triphosphatase-activity in cancer cell-lines *in-vitro*	The authors support the earlier finding that Na^+, K^+-ATPase is the primary target of clofazimine. Suggesting the effects of clofazimine on Na^+, K^+-ATPase activity of tumor cells is similar to those described for synthetic anti-neoplastic alkyllysophospholipids's antiproliferative effects on cancers.	Na^+, K^+-ATPase	[72]
1994	Clofazimine alters the energy metabolism and inhibits the growth rate of a human lung cancer cell line *in vitro* and *in vivo*	The authors demonstrated that clofazimine has a direct effect on tumor-cell energy metabolism, suggesting that further investigation is needed to identify its target.	(Tumor-cell energy metabolism)	[56]
2008	Clofazimine Inhibits Human Kv1.3 Potassium Channel by Perturbing Calcium Oscillation in T Lymphocytes	Clofazimine was identified in the screening of the FDA-approved drugs for Kv1.3 inhibitors as a novel immunosuppressant. The authors investigated the anti-inflammatory mechanism of action of clofazimine. Clofazimine demonstrated to inhibit the intracellular T cell receptor antigen-mediated intracellular calcineurin-NFAT	Kv1.3	[31]

(*continued on next page*)

Table 2 (*continued*)

Year	Paper title	Clofazimine (CFZ) mode of action summary	CFZ target	Ref.
		signaling pathway, which mediates the transcriptional activation of the gene encoding for IL-2.		
2012	Inhibitors of mitochondrial Kv1.3 channels induce Bax/Bak-independent death of cancer cells	The authors support earlier findings of Kv1.3 as target of clofazimine. They demonstrated that clofazimine targets the mitochondrial Kv1.3 (mtKv1.3) and activation of Bax/Bak-independent intrinsic apoptotic pathway in cancer cells. They propose clofazimine could act by inducing cytochrome c release and mitochondrial membrane depolarization resulting in apoptosis.	mtKv1.3	[57]
2013	Clofazimine, Psora-4 and PAP-1, inhibitors of the potassium channel Kv1.3, as a new and selective therapeutic strategy in chronic lymphocytic leukaemia	Another demonstration that clofazimine targets mtKv1.3, induces mitochondrial ROS, and subsequent apoptosis. B-CLL cells are characterized by a higher expression of the antiapoptotic Bcl-2 proteins. The efficacy of clofazimine in B-CLL cells, therefore, indicates it acts on a Bax/Bak-independent pathway. They also suggest the specificity of cancer cells could due to a chronic increase in mtROS levels that have been associated with cancer cells.	mtKv1.3	[61]
2014	Correlation between Potassium Channel Expression and Sensitivity to Drug-induced Cell Death in Tumour Cell Lines	Inverse correlation between Kv1.3 expression and susceptibility to clofazimine-induced cell death is reported. Negative correlation is found between Bcl-2 expression and sensitivity to clofazimine; Bcl-2 overexpression is known to occur in myeloid cancer cells.	(mtKv1.3)	[62]
2014	Anti-leprosy drug clofazimine inhibits growth of TNBC cells *via* inhibition of canonical Wnt signalling	Clofazimine is identified in an *in silico* screening of FDA-approved drugs as potential inhibitors of Wnt signaling. Clofazimine is validated *in vitro* to inhibit growth of TNBC cells, whereas non-cancerous mammary epithelial cells are much less affected by clofazimine. Clofazimine inhibits the Wnt signaling below the level of β-catenin nuclear translocation.	Wnt signaling pathway	[40]
2017	Tumour-reducing effect of the clinically used drug clofazimine in a SCID mouse model of pancreatic ductal adenocarcinoma	The study supports earlier findings that clofazimine inhibits mtKv1.3 thus increasing mitochondrial ROS production. They propose that the clofazimine-induced ROS release drives PDACs over a critical threshold of oxidative stress, causing cell death, and affecting cancer cells while sparing normal cells.	mtKv1.3	[58]
2017	Targeting the Potassium Channel Kv1.3 Kills Glioblastoma Cells	The authors support earlier findings that clofazimine inhibits mtKv1.3, activating the intrinsic apoptotic pathway.	mtKv1.3	[59]
2017	Anti-cancer effect of clofazimine as a single agent and in combination with cisplatin on U266 multiple myeloma cell line	Clofazimine 's antiproliferation effect in U226 cells is caused by apoptosis, not due to changes in the progression of the cell cycle. Clofazimine is reported to cause depolarization of the mitochondrial membrane.	(mitochondrial membrane polarization)	[63]
2018	Inhibition of the aryl hydrocarbon receptor/ polyamine biosynthesis axis suppresses multiple myeloma	Clofazimine emerges from the NextBio Pharmaco-Atlas Gene Expression Database as a compound downregulating AZIN1 and ODC1 expression. AHR is known to positively regulate intracellular polyamine production *via* direct transcriptional activation of ODC1 and AZIN1. Clofazimine is shown to prevent AHR nucleus translocation, suppress ligand-induced AHR-dependent transcription, and inhibit AHR binding to DNA.	AHR	[64]
2019	Development of a Cx46 Targeting Strategy for Cancer Stem Cells	Clofazimine is identified in a screening of FDA-approved drugs as a candidate to inhibit Cx46 gap junction intercellular communication, without affecting hemichannel activity. Glioblastoma cancer stem cells (CSC) are known to express higher levels of Cx46 compared to non-stem tumor cells. In patient-derived xenografts, clofazimine inhibits CSC growth and self-renewal, accompanied by apoptosis in the CSC population, with minimal induction of apoptosis in non-CSCs.	Cx46	[65]
2019	Towards the first targeted therapy for triple-negative breast cancer: Repositioning of clofazimine as a chemotherapy-compatible	The authors validate *in vitro* and *in vivo* that clofazimine is a specific inhibitor of the canonical Wnt signaling pathway; further studies are required to elucidate the exact Wnt pathway	Wnt signaling	[41]

(*continued on next page*)

Table 2 (*continued*)

Year	Paper title	Clofazimine (CFZ) mode of action summary	CFZ target	Ref.
	selective Wnt pathway inhibitor	component as the molecular target of clofazimine.		
2020	Leprosy drug clofazimine activates peroxisome proliferator-activated receptor-γ and synergizes with imatinib to inhibit chronic myeloid leukaemia cells	Clofazimine is identified in a screening of FDA-approved drugs for inhibiting the viability of chronic myeloid leukaemia cell line K562. The mechanistic evaluation reveals that clofazimine, *via* physical interaction with PPARγ, induces nuclear factor kB-p65 proteasomal degradation, leading to sequential myeloblastoma oncoprotein and peroxiredoxin 1 downregulation and induction of ROS-mediated apoptosis. Clofazimine also suppresses STAT5 expression and downregulates stem cell maintenance factors hypoxia-inducible factor-1α and − 2α and CBP/P300 interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (CITED2).	PPARγ	[66]
2020	Beyond TNBC: Repositioning of Clofazimine Against a Broad Range of Wnt-Dependent Cancers	Continued exploration of clofazimine as a Wnt pathway inhibitor and anti-proliferative agent against a broad panel of cancer cell lines. The study reveals correlations between Wnt signaling levels of cell lines and the susceptibility to clofazimine, proposing molecular readouts for future patient stratification in clinical studies of the drug.	Wnt signaling	[42]

72] specify that no effect of clofazimine on the purified Na^+, K^+-ATPase could be revealed, which weakens the claim that this enzyme is the direct target of clofazimine. Instead, indirect effects of clofazimine on Na^+, K^+-ATPase, *e.g.*, through regulation of expression, could be suspected.

5.2. Clofazimine may act on Kv1.3 potassium channel in cancer cells

Kv1.3, playing important roles in the activation and function of human T-cells, first emerged as a possible target of clofazimine through a screening of FDA-approved drugs to identify novel immunosuppressants [31]. Clofazimine could directly interact with the purified Kv1.3 protein in an electrophoretic mobility shift assay and inhibit Kv1.3 in cells, disturbing the oscillation frequency of the calcium-release activated calcium channel. This effect dysregulated the T-cell receptor antigen-mediated calcineurin-NFAT signaling pathway, which mediates the transcriptional activation of the gene encoding for the cytokine IL-2. Ultimately, clofazimine was found to inhibit T-cell proliferation, agreeing with the earlier studies of the immunosuppressive activities of the drug [29–32].

As Kv1.3 has been associated with the induction of apoptosis, Szabo and co-workers investigated the anti-cancer properties of clofazimine and other Kv1.3 inhibitors (Psora-4, PAP-1) [57]. All tested inhibitors produced pro-apoptotic effects, blunted when Kv1.3 expression was suppressed with siRNA. The authors next confirmed the clofazimine's anti-cancer effects *in vitro* and *in* vivo and suggested that clofazimine could target the mitochondrial Kv1.3 (mtKv1.3), activating Bax/Bak-independent intrinsic apoptotic pathway in cancer cells and inducing cytochrome c release and mitochondrial membrane depolarization, all contributing to apoptosis [57]. These initial findings were corroborated by follow-up studies by the team [58,59,61,62], demonstrating clofazimine's effects against various cancer types and revealing an inverse correlation between the Kv1.3 expression and the susceptibility to clofazimine-induced cell death.

Despite this convincing body of evidence, opposing findings have been reported. Thus, anticancer effects of clofazimine against multiple myeloma [64], chronic myeloid leukemia [66], glioblastoma [65], or TNBC [40] were reported to occur in cells having low or no expression of Kv1.3, suggesting that clofazimine acted on another target in these cancer cell types. These studies proposed other molecular targets of clofazimine, as elaborated below.

5.3. Clofazimine may target the aryl hydrocarbon receptor

Elevated polyamine levels have been associated with malignancies, contributing to tumor growth and metastasis [73,74]. Aryl hydrocarbon receptor (AHR) positively regulates intracellular polyamine production *via* direct transcriptional activation of 2 genes, ODC1 and AZIN1, which are involved in polyamine biosynthesis and control, respectively. The study identifying AHR as a potential target of clofazimine [64] started with a query in the NextBio Pharmaco-Atlas Gene Expression database for compounds that downregulate AZIN1 or ODC1 expression. Clofazimine emerged from this query, and was validated to reduce ODC1 and AZIN1 levels in a dose-dependent manner in normal human fibroblast WI-38 cells. The drug was further suggested to act on AHR, preventing nuclear translocation of the receptor, its binding to DNA *in vitro*, and ultimately suppressing ligand-induced AHR-dependent transcription. Using an immunocompetent transgenic mouse model of spontaneously arising myeloma, the authors further reported clofazimine to be as effective as bortezomib (first-line treatment for multiple myeloma) in reducing the disease burden in mice [64].

5.4. Clofazimine may target the gap junction protein Cx46

The connexin family proteins assemble into hemichannels on the plasma membrane and can exchange small molecules between the cytoplasm and the extracellular space. When these channels between neighboring cells dock, gap junction (GJ) channels are formed, permitting GJ intercellular communication (GJIC) [75]. In glioblastoma, cancer stem cells (CSCs) express higher levels of connexin 46 (Cx46) than non-CSCs, and their GJIC mediated by Cx46 is essential to the maintenance of GBM CSC proliferation, survival, and self-renewal [76]. Mulkearns-Hubert and coauthors identified clofazimine as an inhibitor of the Cx46 gap junction protein [65]. GJIC was blocked by clofazimine in HeLa cells expressing Cx46, but not Cx43, Cx37, or Cx45. In patient-derived xenografts, clofazimine inhibited growth and self-renewal of GBM CSCs, which was accompanied by a concentration-dependent increase in CSC apoptosis, with minimal apoptosis in the non-CSCs. The standard of care chemotherapy for GBM is temozolomide; hence, a combination of temozolomide and clofazimine was explored, suggesting that clofazimine sensitizes CSCs to chemotherapy [65]. As clofazimine has a low penetration through the blood-brain barrier [46], subcutaneous flank tumors generated by implantation of glioblastoma CSCs were generated and shown to be sensitive to clofazimine [65].

5.5. Clofazimine may target peroxisome proliferator-activated receptor-γ

Clofazimine emerged from a screening of FDA-approved drugs for inhibiting the viability of chronic myeloid leukemia cell line K562 by Kumar and co-authors [66]. The authors suggested that the drug induced mitochondrial-mediated apoptosis through cytochrome c release and activated caspase-3 and − 9, but not − 8; clofazimine also decreased Bcl-2 and increased Bax expression. Noteworthy, this mechanism of action opposes earlier findings [31,57] that highlighted the Bax/Bak-independent apoptotic pathway involving mitochondrial ROS release is caused by clofazimine inhibiting the Kv1.3 potassium channel. In leukemia cells, clofazimine was further found to suppress the mRNA levels of antioxidant enzyme peroxiredoxin 1 (PRDX1) [66]. As the mechanism of this downregulation, clofazimine was proposed to act as an agonist to peroxisome proliferator-activated receptor γ (PPARγ), which induces nuclear factor κB (NFκB)-p65 proteasomal degradation. NFκB-p65 degradation in turn leads to transcriptional downregulation of myeloblastoma oncoprotein and PRDX1. The authors also demonstrated the physical interaction of clofazimine with PPARγ, in a cell-free time-resolved fluorescence resonance energy transfer assay, further confirmed by isothermal titration calorimetry [66]. This mechanism of action, however, appears restricted to leukemia or to the exact cell type studied, as our investigation in TNBC cells where clofazimine inhibited the canonical Wnt signaling pathway, failed to detect any effects of the drug on NFκB pathway signaling [41].

Imatinib and other tyrosine kinase inhibitors have become the standard treatment for patients with BCR-ABL1-positive chronic myeloid leukemia (CML). However, treatment resistance highlights the need for new targeted therapies. The PPARγ agonist pioglitazone is reported to erode quiescent leukemia stem cells by targeting and downregulating the signal transducer and activator of transcription 5 (STAT5). However, a population-based study suggests pioglitazone is associated with the risk of bladder cancer and cardiac and hepatic safety concerns [77]. Kumar and co-authors identified the superior anti-CML efficacy of clofazimine as compared with PPARγ agonist including pioglitazone. In the combination treatment of blood mononuclear cells derived from patients with CML with imatinib, as well as in a mouse K562 xenograft study, clofazimine demonstrated superior synergy than pioglitazone, opening the opportunity to CML clinical trials [66].

5.6. Clofazimine targets the canonical Wnt signaling pathway in cancer

The Wnt signaling pathways play critical roles in cancer initiation, growth and metastasis, therapy resistance, and tumor microenvironment in tissues such as colon, breast, liver, and many others [78,79]. Despite this oncogenic importance of Wnt signaling, no drugs targeting it currently exist, and many drug candidates have difficulties in progressing through preclinical or early clinical trials [80–83]. This delay in development of drugs targeting the Wnt pathway calls for inclusion of other approaches of drug discovery, such as fishing from natural products or drug repositioning [84,85]. Clofazimine, originating from a lichen-derived compound and having passed through several rounds of drug repurposing, fulfills both.

We initially came upon clofazimine when conducting an *in silico* screening of FDA-approved drugs in a search for novel inhibitors of Wnt signaling in cancer [40]. The anti-Wnt pathway activity of clofazimine was later confirmed using the Wnt-specific reporter assay [86], as well as through analysis of stabilization and nuclear translocation of β-catenin, the key transducer of the canonical Wnt pathway, in breast, colon, liver, and ovarian cancer cells [40–42]. Clofazimine emerged as a specific inhibitor of the Wnt/β-catenin pathway as a broad panel of reporters to other signaling pathways (including the non-canonical, β-catenin-independent Wnt signaling) were not affected by the drug [41]. Investigations aiming to elucidate the mode of action of clofazimine within the Wnt/β-catenin pathway revealed that in the acute setting, clofazimine treatment inhibited the signaling without reducing the levels or nuclear translocation of β-catenin [40]. However, prolonged clofazimine treatment *in vitro* and *in vivo* strongly reduced the cytoplasmic and nuclear β-catenin [41,45]. These findings suggest that the as of yet unidentified molecular target of clofazimine may engage a positive feedback loop within the Wnt pathway, so that the initial pathway inhibition by the drug is followed by the later decrease in the cytonuclear β-catenin levels. Our studies also excluded the conventional known components of the Wnt signaling as clofazimine targets [40,41,45], highlighting the urgency of clofazimine's target deconvolution for fundamental research on oncogenic Wnt signaling, as well as for the drug development of clofazimine and its derivatives for targeted anticancer treatments. We have attempted to elucidate the exact target of clofazimine through a proteomics-based approach. Using a pull-down assay with clofazimine analogs we synthesized to permit the drug's linkage to beads, we narrowed down the list of potential clofazimine targets to 15 clofazimine-interacting proteins, among them some ubiquitin ligases and RNA-binding proteins. Validation of these candidates as components of the Wnt pathway in TNBC cells is under way. We hope to be able to clarify the identity of the molecular target of clofazimine within the oncogenic Wnt pathway, which will be important for basic cancer biology and to the prospects of clofazimine's future development as an anticancer agent.

We have found strong *in vitro* effects of clofazimine against a panel of TNBC cell lines representing the clinical diversity of the disease including the luminal-like ductal (BT-20), squamous (HCC-1806), adenocarcinoma (MDA-MB-468), basal-like adenocarcinoma (MDA-MB-231), and tumor initiating cells (TNBC "stem cells") (IOWA-1 T) in cell proliferation, cell motility, and colony formation assays, while non-cancerous breast epithelia were considerably more resistant to the drug [40,41]. Further exploration of clofazimine's *in vitro* efficacy against cancers of other origins demonstrated the high activity of the drug against Wnt-dependent colorectal cancer lines (DLD1, HCT116, HT29, LS174T, SW48, SW620) and subsets of hepatocellular carcinomas (HEP3B and SUN398, but not HepG2 and Huh7 lines) and ovarian cancer (OVCAR3, OVSAHO and PEO1, but not KURAMOCHI cell lines). In contrast, we found a poor sensitivity of glioblastoma cell lines (U87, U118, and U251) to clofazimine. This study revealed a correlation between the efficacy of clofazimine and the levels of Wnt pathway activation in a given cell line, forming the basis for future patient stratification in prospective clinical trials [42].

In vivo models of TNBC using NOD-SCID-gamma (NGS) mice with intramammary grafts were established using 3 different types of cells for xenograft: IOWA-1 T, BT-20, and a patient-derived TNBC xenograft [41,45]. Mice were given oral clofazimine at 50 and 100 mg/kg daily, which corresponds to the moderate (100–300 mg/day) human doses of clofazimine [87,88]. The 50 mg/kg regimen was expected to result in tissue levels around 50–100 μM, well above the typical *in vitro* IC_{50} of 3–8 μM. Clofazimine treatment resulted in a profound suppression of the tumor growth, without adverse effects apart from the skin reddening due to the accumulation of this colored lipophilic drug in subcutaneous fat [45]. An additional trial with a decreased clofazimine dose of 25 mg/kg also resulted in a similar inhibition to tumor growth. The decreased proliferation of tumor xenografts was due to the on-target suppression of Wnt signaling, as monitored by following the tumor β-catenin and MDR1 (Wnt pathway target gene [89]) biomarkers; pro-apoptotic and cell cycle-related effects of clofazimine were also seen in the tumors [41]. As described above, the multidrug resistance mediator MDR1 is the key player in acquired chemoresistance in cancer, responsible for pumping from the cells of a number of chemotherapeutic agents, such as doxorubicin. We thus argued that inhibition of the Wnt pathway by clofazimine, leading to reduced MDR1 expression, would re-sensitize the tumors to chemotherapies. Indeed, strong additive effects of clofazimine and doxorubicin were identified *in vitro* and *in vivo*, while the combination did not aggravate the adverse effects of the single doxorubicin treatment. These studies represent the complete preclinical profiling of clofazimine in TNBC treatment, and serve as the basis for the planned

clinical trials of clofazimine+doxorubicin in TNBC patients [41].

Finally, we performed medicinal chemistry-based optimization of clofazimine in order to dispose of its main side effect – the cosmetic skin discoloration induced by prolonged exposure. This effect is due to the lipophilic nature of the drug, and through a series of modifications of the clofazimine core, we managed to markedly increase the water solubility of the compound, further enhancing its potency as a Wnt pathway inhibitor. *In vivo* experiments confirmed the potent and on-target anti-cancer effects of our best derivative, MU17 (5-(4-(chlorophenyl)− 3-((2-(piperazin-1-yl)ethyl)imino)-N-(pyridin-3-yl)− 3,5-dihydrophenazin-2-amine), accompanied by no adverse effects in mice and, importantly, absence of discoloration of the skin and internal organs in the animals [45]. These studies highlight the high promise of clofazimine-based drug development, which should be guided by structure-based optimization upon the hopeful elucidation of the molecular target of clofazimine within the Wnt pathway.

6. Conclusion and perspectives

Clofazimine was first developed with the aim of treatment of tuberculosis, but appeared to be less effective than other anti-TB agents that emerged in the 1950–60 s. Showing efficacy against *M. leprae*, clofazimine experienced its first repositioning against leprosy in the 1960 s, maintained until now as one of the key (and highly affordable) anti-leprosy treatments. However, the initial indication of clofazimine was revived in the 2010 s with the spread of drug-resistant *M. tuberculosis* strains, resulting in the second repositioning of the drug approved by the WHO, against multidrug-resistant TB. In parallel, developments of clofazimine as a general antibacterial, antifungal, antiparasitic, and antiviral compound continued.

Another wave of repositioning of clofazimine started thirty years ago, this time towards cancer indications. Several clofazimine targets and anti-cancer modes of action have been proposed, which may reflect the existence of several relevant molecular targets of the drug. Another level of complexity is the anti-inflammatory effects of clofazimine, possibly engaging additional target(s) in immune cells and providing potentially confounding effects of the drug's anti-cancer treatments. Noteworthy, the molecular target of the anti-mycobacterial activity of clofazimine is also still unclear. In our lab, the specific Wnt signaling inhibitory activity of clofazimine was discovered as the molecular mechanism of the drug's anti-cancer effects in the breast, colon, liver, and ovaries. The exact molecular target of clofazimine within the pathway still awaits to be discovered, which will contribute to the development of clofazimine or its derivatives for more efficient, targeted anti-cancer treatments.

Two small-scaled clinical trials of clofazimine against hepatocellular carcinoma were performed in the 1990 s. These trials led to inconclusive data and did not have a continuation. The newly discovered mechanism (s) of action of clofazimine in cancer open the avenue for relaunching the clinical trials, armed with molecularly defined patient inclusion criteria and biomarker assessment prior to and during the trials. We believe that clofazimine, celebrating 70 years since its synthesis and having passed through several waves of repurposing in its history, has a new colorful future in the anti-cancer repositioning, to be accompanied by discoveries of the molecular mechanisms underlying the disease as affected by this drug.

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Declaration of Competing Interest

The authors declare that they have no conflict of interests.

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