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REVIEW ARTICLE

Repurposing of Acriflavine to Target Chronic Myeloid Leukemia Treatment

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Abstract: Drug repurposing has lately received increasing interest in several diseases especially in cancers, due to its advantages in facilitating the development of new therapeutic strategies, by adopting a cost-friendly approach and avoiding the strict Food and Drug Administration (FDA) regulations. Acriflavine (ACF) is an FDA approved molecule that has been extensively studied since 1912 with antiseptic, trypanocidal, anti-viral, anti-bacterial and anti-cancer effects. ACF has been shown to block the growth of solid and hematopoietic tumor cells. Indeed, ACF acts as an inhibitor of various proteins, including DNA-dependent protein kinases C (DNA-PKcs), topoisomerase I and II, hypoxia-inducible factor 1α (HIF-1α), in addition to its recent discovery as an inhibitor of the signal transducer and activator of transcription (STAT). Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by the expression of the constitutively active tyrosine kinase BCR-ABL. This protein allows the activation of several signaling pathways known for their role in cell proliferation and survival, such as the JAK/STAT pathway. CML therapy, based on tyrosine kinase inhibitors (TKIs), such as imatinib (IM), is highly effective. However, 15% of patients are refractory to IM, where in some cases, 20-30% of patients become resistant. Thus, we suggest the repurposing of ACF in CML after IM failure or in combination with IM to improve the anti-tumor effects of IM. In this review, we present the different pharmacological properties of ACF along with its anti-leukemic effects in the hope of its repurposing in CML therapy.

Keywords: Acriflavine, ACF, drug repurposing, chronic myeloid leukemia, leukemia, anti-tumoral, anti-leukemic.

1. INTRODUCTION

Drug repurposing, also known as "drug repositioning" or "drug reprofiling", has been lately receiving increased interest as a new therapeutic strategy for several diseases [1]. This strategy focuses on finding new possible therapeutic effects of a drug that already exists and which has been shown to establish good safety and pharmacokinetic profiles [2]. While drug research and development (R&D) represents a long process with high costs and complexity, drug repurposing has demonstrated many advantages facilitating the development of new therapeutic strategies in several diseases. Specifically, using available drugs as new anti-cancer agents has become popular [3]. For instance, retinoic acid or tretinoin was first introduced as a topical treatment for acne Vulgaris [4]. It was later largely described as an anti-leukemic agent for patients with acute promyelocytic leukemia (APL) who have previously relapsed from chemotherapy. In fact, the use of this repositioned drug has improved the survival of APL patients to more than 90% [5].

Drug repurposing could be an important therapeutic strategy for chronic myeloid leukemia (CML) treat-

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ment, where a previously used antiseptic agent, Acriflavine (ACF), has been reported to have beneficial effects. Hence, the aim of this review is to present ACF as a new repositioned drug, by providing an overview of its different uses and pharmacological properties, along with its new reported anti-leukemic effects in the hope of its repurposing in CML therapy.

2. DRUG REPOSITIONING APPROACHES AND RESOURCES

Since 1995, productivity of drug development has been decreased, despite the substantial increase of investments in pharmaceutical research and development [6, 7]. Although the development of a new drug requires between 13 to 15 years, the average success rate of developing a new molecule is ~2% only [8]. Thus, therapeutic innovation has become more challenging, and hence a new strategy for drug discovery is urgently needed.

To overcome the challenges of traditional *de novo* drug design, new approaches for drug discovery have been developed. With the highly evolving computational methods*, in silico* drug design has been widely applied in pharmaceutical drug discovery and optimization [9]. Such methods are defined by the creation of computational models or simulations of the therapeutic target that can be used to make predictions or to test the proposed hypothesis [10]. This approach presented a highly efficient, cost-friendly approach that required less time and allowed the parallel evaluation of a large number of drugs against one target, at the same time. Despite the promising results presented by the *in silico* drug design approach, challenges include the need to develop more complex computational technology as well as the high rate of false-positive results obtained during the experimental process [11].

Thereafter, drug repurposing has been introduced as a new approach for drug development, which will be our main focus in this review. In fact, the notion of 'old drugs for new uses' is a highly efficient, low-cost and riskless novel method to find new indications for existing drugs [12]. This strategy removes the steps of molecular origination, *in vivo* and *in vitro* screening, chemical optimization, pharmacokinetics, toxicology and possibly phase I clinical trials [13]. Lately, it has been widely used because of the simplicity of the drug approval procedure. Indeed, while only 10% of new drug applications gain FDA-approval, amongst the repositioned drugs, 30% gain the approval [14]. Drug repurposing cost is estimated at an average of 20-100 million dollars [13, 15], a fraction of the average cost for *de novo* drug development. Moreover, it requires only 6.5 years of development [12, 16], thus offering a great opportunity to develop drugs with lower investments and shorter time. In addition, drug repurposing involving the use of generic therapies already approved by the FDA can offer a better risk-versus-reward trade-off as compared with traditional drug development methods [17]. This is because repositioned drugs have already passed all clinical tests in the first three phases and their safety has been confirmed [18]. Historically, most of the repositioned drug candidates (e.g. sulfonamide, sildenafil, minoxidil, aspirin, valproic acid) have been discovered from serendipitous observations or from unorganized discovery processes such as off-target adverse effects [19, 20]. Thereafter, the detection of novel drug-disease relationships has been more organized and it is now possible due to the development of three kinds of approaches: computational, biological, and mixed [12].

Computational approaches have much lower costs and fewer barriers when compared to biological approaches. These approaches are based on the belief that similarity and shared properties between biological entities can help to identify their new mechanisms of action [6]. Computational drug repositioning methods include drug combination prediction, electronic health records (EHR), genome sequencing, structure-based repositioning, off-targeting data, protein abundance, protein-protein interactions (PPI), protein-small molecules interactions, pathways based repositioning, gene expression, and network-based repositioning [6, 12, 21-23]. These computational approaches have been rapidly developed and improved due to the availability of a vast amount of experimental data covering various diseases that led to establishing massive databases from chemical, medical, pharmacological and biological fields. More than 80 databases or resources are currently used for designing drug repositioning approaches [12]. For instance, DrugBank is a unique pharmacological database that combines detailed drug data with comprehensive drug target information [24]. Proteomic databases such as MIPS are also important because they can provide high-quality PPI data from scientific literature [25]. In addition, numerous other databases could be used, such as ChemBank, OMIM, KEGG, PubMed, PDB, and others [12].

Experimental approaches include target-focused screening and phenotype screening approaches. The first is carried out in the context of a study evaluating a specific drug, while the second is a consequence of a screening study on a disease and its physiopathology.

Phenotype screening includes all parts of the signaling pathways and cell behaviors; thus, it is considered more successful than the target-focused approach where the signaling pathways are not considered in the study [19]. Experimental methods may also include chemical genomics, chemical systems biology, serological markers, clinical side effects, genome-wide association studies (GWAS), network medicine and others [13].

The mixed approaches, which include both computational and experimental approaches, have been used in several studies [12, 21, 26]. In these mixed studies, preliminary outcomes of computational methods are confirmed by clinical tests and experiments.

Despite its efficiency, drug repurposing is still facing several challenges. In fact, it is highly dependent on the availability of information about the repurposed drug and the targeted molecule. The public access to certain types of data, such as clinical trials, is still limited despite the advanced open-source model of sharing information in the scientific community [18]. The different challenges and opportunities of traditional, *in silico* and repurposing drug development approaches, are presented in Table **1**.

3. ACRIFLAVINE

Acriflavine or ACF, a mixture of 3,6-diamino-10-methylacridinium chloride (Trypaflavin) and 3,6-diaminoacridine (Proflavine), is a byproduct of coal tar initially developed in 1912 by the German medical research worker Paul Ehrlich and his assistant Benda [27]. ACF is an acridine derivative and it is considered as an attractive compound in medicine, science and industry [28]. It has first been introduced as an antiseptic during the First World War and used extensively to kill the parasites that cause sleeping sickness [29, 30]. Besides its trypanocidal activity reported in 1912, antibacterial activity has been reported in the next year by Carl Browning, a student of Ehrlich [31]. Thus, ACF popularity increased by being one of the first antibacterial agents to be discovered and used, until it has been replaced by penicillin [31].

ACF has the form of odorless, reddish-brown powder used in dilute aqueous solutions primarily as topical antiseptics or given orally, mainly as a urinary antiseptic [32]. As a member of acridine's family, ACF possesses two characteristics that have led researchers to consider it as a potential pharmacophore: its structure that permits its entry into the nucleus, and the intense fluorescence of the agent [29]. The anti-microbial activity of ACF has always been attributed to the fact that it intercalates into nucleic acids, thereby inhibiting bacterial and viral replication. Due to this process, ACF has been an interesting subject for important chemical investigations that lead to a better understanding of its action [33]. The structure is characterized by the presence of a planar polycyclic system presenting three rings and two substituent groups (Fig. **1**). The biological effect of ACF has been referred to the planarity of these aromatic structures [34, 35], which can bind to double-stranded DNA through intercalation, thereby inhibiting a number of enzymes and interfering with many crucial cellular functions. In addition, the strong fluorescence properties of this compound allowed its use as a chemiluminescent agent [33, 36]. Its international nonproprietary name (INN) is Acriflavinium chloride, and it continues to be used to this day as a highly effective disinfectant [37]. It is approved by the FDA of the United States for human non-oncological uses topically for the healing of wounds [38].

4. ANTI-INFECTIOUS EFFECT OF ACF

The first clinical use of ACF as an anti-microbial agent occurred in 1917 [27]. Since 1919, the Council on Pharmacy and Chemistry, an advisory board created in 1905 by the American Medical Association, approved its use in therapy [30]. The trypanocidal activity has been reported by Ehrlich team, who found that ACF possesses some therapeutic roles in trypanosome infections (Human African trypanosomiasis also known as sleeping sickness); hence it has been called Trpaflavine [30, 39]. In the same context, being a strong DNA intercalating agent, ACF exhibited wide potent activities against various groups of microorganisms including Gonorrhea, HIV1 and malaria parasites [40].

Gonorrhea, a sexually transmitted infection (STI) caused by the *bacterium Neisseria gonorrhoeae* [41], has been treated by ACF for several years before it has been replaced by more targeted antibiotics [42]. ACF has also shown to be effective as a genito-urinary antiseptic. It occupied the main place in the treatment adopted in the venereal clinic [43]. In the nineties, Mathé *et al.,* decided to investigate the effect of ACF in the treatment of HIV1 infection, knowing that it inhibits the synthesis and functions of proviral DNA [44], which showed promising results [40]. In other studies, ACF has been revealed to inhibit the growth of the human malaria parasite *Plasmodium falciparum in vitro* and *in vivo,* and it has been proposed as a powerful therapeutic molecule against malaria, an infection often associated with drug-resistance, mainly to chloroquine [40]. Several other indications have been further dis-

Table 1. Advantages and disadvantages of drug design approaches.

Drug Design Approaches	Advantages	Disadvantages	References
Traditional drug design	- Established a very efficient approach for drug development -High specificity in targeted therapy -Low rate of false positives during experiments	-Time and labor consuming -Estimated time: 13-15 years -High costs -Estimated costs: 2-3 billion dollars -High failure rate in clinical trials -Strict FDA restrictions	Wong *et al.* (2014) [8] Grinnan *et al.* (2019) [13]
***In silico* drug design**	-Cost and time-efficient approach -Easy to evaluate large number of drugs at once -Alternative to animal use -Highly predictive	-Higher rate of false positives during the experiments -Complex, needs high computational technology	Ekins *et al.* (2007) [10] Shim *et al.* (2014) [11]
Drug repurposing	-Time and labor efficient -Estimated time: 6.5 years -Cost-efficient approach -Estimated costs: 20-100 million dollars -Bypass early stages of drug development -Faster approach to enter the advanced phases of clinical trials -Bypass FDA restrictions	-Higher rate of false positives related to the observations -Highly dependent on information's availability -Needs to develop efficient drug repurposing approaches	Grinnan *et al.* (2019) [13] Pushpakom *et al.* (2018) [15] Talevi *et al.* (2020) [18]

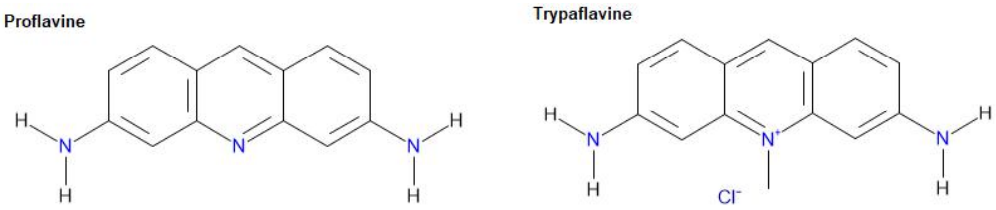

Fig. (1). Chemical structure of Proflavine and Trypaflavine. ACF is a mixture of (**A**) Proflavine ($C_{13}H_{11}N_3$, Molar Mass= 209.25 g·mol^{-1}) and (**B**) Trypaflavin ($C_{14}H_{14}ClN_3$, Molar Mass = 259.74g·mol^{-1}).

covered, including its use in 1930 to treat severe undulant fever and its complications [47]. Despite all of these trials, ACF is currently not approved for any of these indications and it has been out of use systemically for more than 60 years, probably, because we do not understand its exact mechanism of action [40]. The only approved indication has been reported by Maxwell Quackenbos, who found that ACF's main value was not its antiseptic properties, but its remarkable power to stimulate epithelial growth on healthy granulating surfaces. Thus, he recommended the use of ACF dressings and noticed that healing occurs with great rapidity [48]. ACF is still widely used for wound dressing by health professionals and remains a popular, topical antiseptic agent purchased over-the-counter [49].

Since 1959, ACF has grabbed the attention of scientists working in cancer research due to its versatile biological activities [50]. Subsequently, its anti-bacterial and anti-protozoal effects were neglected and recent research has been mainly focused on its anti-cancer effects.

5. POTENTIAL ROLE OF ACF IN CANCER TREATMENT

The first use of ACF on cancer cells was reported 60 years ago when ACF, administered intraperitoneally into mice, completely inhibited the proliferation of tumor cells without affecting normal tissues [51]. A recent study demonstrated that ACF inhibits osteosarcoma cell proliferation in a dose-dependent manner, causing both apoptosis and autophagy, which are associated with the decrease in BCL-2 expression, a pro-survival protein, and the increase of BAX levels, a pro-apoptotic protein [46]. ACF was also shown to target patient-derived cells with advanced ovarian carcinoma in a dose-dependent manner [52]. The same team showed that ACF efficiently targeted colorectal cancer

cells as well [52]. Moreover, ACF treatment of mice with prostate cancer xenografts prevented tumor growth and vascularization [53]. This was also observed by Shay *et al.*, who demonstrated that ACF limits tumor progression of established colitis-associated colon cancer (CAC) in immune-competent mice in a HIF-1 dependent manner [54]. Furthermore, a preclinical study introduced ACF in brain tumor rat models via biodegradable wafers, which showed improved survival, resulting in nearly 100% long term survival [38]. Recent studies show that ACF inhibits hypoxia-induced pathways in glioma-implanted rodents, which participate in creating a tumor-immunosuppressive microenvironment [55]. These reports have also shown that the systematic administration of ACF decreased tumor size during the first two weeks, showing the promising use of ACF in glioma therapy [55]. Interestingly, ACF increased the cytotoxic effect of 5-fluorouracil (5-FU) in colorectal cancer cell lines, suggesting the development of new chemotherapy regimens that administer ACF in 5-FU-resistant patients with advanced colorectal cancer [56]. The pretreatment of human perihilar cholangiocarcinoma cells (SK-ChA-1) with ACF enhanced photodynamic therapy (PDT) efficacy through the inhibition of HIF-1 and topoisomerases I and II [57]. This was also noticed in murine breast carcinoma 4T1-bearing mice, where ACF efficiently enhanced the antitumor effect of sunitinib, a VEGF receptor tyrosine kinase inhibitor [58]. This combination significantly decreased VEGF and TGF-β expression along with reduced tumor vasculature leading to intra-tumor necrosis and apoptosis, thus proposing the use of HIF-1 inhibitors with antiangiogenic therapy for cancer patients [58]. Moreover, Wong *et al.,* demonstrated that ACF inhibited HIF-1-mediated invasion and metastasis in human breast carcinoma xenografted mice [59]. The anti-tumor effects of ACF have also been assessed in human hepatocellular carcinoma (HCC). Lee *et al.,* revealed that ACF treated mice experienced a 4-fold decrease tumor growth compared to vehicle-treated mice, which could provide a new therapeutical approach to extend the survival rate of patients in advanced stages (Table **2**) [60].

6. HUMAN USES, DOSES, AND SAFETY

ACF has repeatedly shown to be an efficient HIF-1α inhibitor with anti-tumor effects along with its safe use *in vivo*, thus allowing its integration in clinical trials along with other conventional therapy for diseases other than cancer (Table **3**). The first-ever published administration of ACF in patients, with promising results, was for the treatment of gonorrhea by daily irrigations of the bladder [61]. ACF was also used later in 1930 to treat severe undulant fever and its complications. It was intravenously administered to 7 patients, and results showed that the course of undulant fever was shortened along with the prevention of the development of arthritis compared to untreated patients [47]. In addition, ACF was tested in Phase I-II clinical trials, which involved 10 volunteers with HIV infections (Patent ref. FR2711527A1). It was administered orally at increasing doses of 5 to 200 mg per day for 4 weeks to 8 months. Clinical trials revealed that the treatment allowed a stabilization of the disease in 6 patients. Interestingly, ACF was highly effective in HIV patients who are resistant to conventional treatment by dropping the levels of P24 antigen, showing a decrease in viral proteins in the blood.

Dosages of ACF were 25 to 250 mg per day of active ingredient orally administered. For parenteral administration, 5 to 50 mg per day of the active ingredient is directed. Moreover, the oral administration of ACF in HIV-1 patients, in combination with AZT-ellipticin, as an antiviral agent for 5 months did not evolve major side effects, thus suggesting that it is suitable for use in clinical trials 59. This observation was also noted by the same group after the treatment of HIV patients with ACF for 18 months to 6 years 60. The median lethal dose (LD50) of ACF in humans is unclear, but the LD50 of ACF in mice is 30 mg/kg. ACF is also introduced in an ongoing Phase IV clinical trial engaging 284 patients with recurrent cystitis, a bacterial infection in the urinary tract, having ≥2 episodes within the past 6 months (Clinical trials ref. NCT03379389). The study aims to assess the efficacy of different treatments, which include or not ACF.

7. CML STILL NEEDS A NEW THERAPY

CML is a clonal disease that affects hematopoietic stem cells (HSCs), resulting in the accumulation of malignant hematopoietic cells in the blood and bone marrow (BM) of patients. CML affects 0.6-1 person per 100 000 each year, with an average age at diagnosis around 57-60 years [63, 64].

This neoplasia is characterized by the presence of a chromosomal abnormality, caused by reciprocal translocation t(9;22)(q34;q11), which results in the expression of the constitutively active tyrosine kinase BCR-ABL [65]. This chimeric protein is expressed in 95% of CML cases, and it is responsible for the activation of several cellular pathways that are implicated in the malignancy of CML. Therefore, CML therapy is currently based on tyrosine kinase inhibitors (TKIs); 3 genera-

Table 2. Effect of ACF in cancers.

Cancer	Material	Dose of ACF	Outcomes	References
Breast Cancer	- Human MDA-MB-231 and MDA-MB-435 cells - Implantation of breast cancer cells into SCID mice	*in vivo*: daily intraperitoneal (i.p.) injections at 4 mg/kg	- ACF blocked hypoxia-induced expression of LOX and LOXL proteins, collagen cross-linking, $CD11b^{+}$ bone marrow-derived cell recruitment - ACF blocks breast cancer metastasis in an orthotopic breast cancer model	Wong *et al.*, (2012) [59]
Breast Cancer	- Murine breast cancer cells - BALB/c mice	*in vivo*: daily i.p. injections at 2 mg/kg	- ACF, in combination with sunitinib, decreased VEGF and TGF-β expression and reduced tumor vasculature, increased intratumor necrosis and apoptosis, and decreased accumulation of myeloid-derived suppressor cells in the spleen	Yin *et al.*, (2014) [58]
Hepatocellular Carcinoma (HCC)	- Mahlavu, SK-Hep1, Hep3B, Huh-7, and PLC/PRF/5 cell lines - BALB/cAnN. CgFoxn-1^{nu}/CrlNarl mice	-*in vitro*: 1, 2, 5 & 10 μM -*in vivo* : daily i.p. injections at 2 mg/kg	- *in vitro*: ACF suppresses HCC cell growth through the caspase-3 activation pathway - *in vivo*: ACF decreased tumor growth to approximately 500 mm3 compared to vehicle-treated mice	Lee *et al.*, (2014) [60]
Osteosarcoma	MG63 cell line	*in vitro*: 0.1, 1, 5, &10 μM	- ACF inhibited cell proliferation of MG63 cells in a dose-dependent manner, which was attributed to apoptosis and autophagy	Fan *et al.*, (2014) [46]
Ovarian carcinoma	Patient-derived tumor cells (PDTC)	*in vitro*: 0.2, 1 and 5 μM	- ACF inhibited cell proliferation in a dose-dependent manner	Hassan *et al.,* (2011) [52]
Colorectal cancer (CRC)	- PDTC - HCT116 colon cancer spheroids	*in vitro*: 0.2, 1 and 5 μM	- ACF inhibited cell proliferation in a dose-dependent manner in PDTC - Treatment with 5 μM of ACF reduced the spheroid volume compared to unexposed control spheroids	Hassan *et al.,* (2011) [52]
Colorectal cancer (CRC)	SW480, HCT116 and LS174T cell lines	*in vitro*: 0.07, 015, 0.31, 0.62, 1.25, 2.5 & 5 μM	-Pretreatment with ACF sensitized CRC cells 5-FU -Simultaneous treatment with ACF and 5-FU did not alter the resistance of CRC cells to 5-FU - ACF is a more potent agent than irinotecan for enhancing the antitumor activity of 5-FU - ACF did not alter the mRNA levels of either HIF-1α or topoisomerase 2	Zargar *et al.,* (2018) [56]
Colitis-associated colon cancer (CAC)	Balb/C mice	*in vivo*: daily i.p. injections at 2 mg/kg	- ACF inhibits the progression of an autochthonous model of established CAC in immunocompetent mice. - ACF decreased tumor number, size and advancement of CAC - ACF decreased macrophage infiltration and vascularity in colorectal tumors - ACF inhibited the hypoxic induction of M-CSFR and the expression of VEGF	Shay *et al.*, (2014) [54]
Perihilar Cholangiocarcinomas	SK-ChA-1 cells	*in vitro*: 1, 3 & 5 μM	- ACF increases expression of VEGF, CD105, CD31/Ki-67, and GLUT-1 - ACF was translocated to the nucleus under hypoxic conditions in SK-ChA-1 cells - Pretreatment with ACF enhanced PDT efficacy via inhibition of HIF-1 and topoisomerases I and II	Weijer *et al.,* (2016) [57]
Brain cancer	- Cell lines: Human U87 cell line, mouse GL261 cell line, rodent F98 cell line, 9L gliosarcoma - Human primary brain tumor stem cell neurosphere lines, GB1A, and primary brain tumor JHH 1113 stem cell line - F344 rats	*in vivo*: daily i.p. injections at 5 mg/kg	- ACF induces cytotoxicity and promotes apoptosis in brain cancer cell lines - ACF reduces the expression PGK1, VEGF and HIF-1α *in vitro* and *in vivo* - ACF promotes 100% long term survival	Mangraviti *et al.,* (2017) [38]

Table 3. Administration of ACF in clinical trials.

Disease	Trial	Study Title	Patient Population	Outcomes	References
Undulant Fever	---	The Results of The Use of Acriflavine Hydrochloride in The Treatment of Undulant Fever	-N = 14 patients -Sex: All - 20 ≥ Age ≤ 49	- Untreated patients suffered from fever from 9 months until 2 years, 2 patients died -Fever was arrested in 5 treated patients within 1 month -2 treated patients still had fever but at a lower level compared to untreated	Thurber D.S., (1930) [47]
HIV	Phase I	AIDS therapy with two, three or four agent combinations, applied in short sequences, differing from each other by drug rotation. i. first of two parts: a phase i trial equivalent, concerning five virostatics: azt, ddi, ddc, acriflavine and an ellipticine analogue	-N = 10 patients -Duration of treatment: 5 months	-Decrease of viral load to undetectable levels after four months -No side effects	Mathé *et al.,* (1996) [45]
HIV	Phase II	Data of pre-clinical and early clinical trials of acriflavine and hydroxy-methyl-ellipticine reviewed, enriched by the experience of their use for 18 months to 6 years in combinations with other HIV1 virostatics	-N = 15 patients -26 ≥ Age ≤ 45 -Duration of treatment: 1-8 months	- A slight increase of transaminases in 2 patients -Decreases of the p 24 antigens in 3 patients - Stabilization of p24 antigen in 2 patients	Mathé *et al.,* (1998) [62]
HIV	Phase I-II	Use of acriflavine as an anti-HIV agent	-N = 10 patients -Ratio male:female 9:1 -Av. age: 35.8 years -Duration of treatment: 4 weeks – 8 months	-ACF stabilized the expression of P24 antigen in 6 patients -ACF was effective in resistant patients - ACF and its derivatives, can be used in the treatment of AIDS	FR2711527A1
Cystitis	Phase IV	Clinical Assessment of Urinary Antiseptics Methenamine and Methylthioninium in Recurrent Cystitis	-N = 284 participants -Sex: All -18>Age (years)< 80 -Healthy donors: No -Duration of treatment: 6 days	-Recruiting	NCT03379389

tions of which were released. Imatinib Mesylate (IM), the first, was introduced in 2001 as a selective BCR-ABL inhibitor, which improved the estimated overall survival of CML patients to 89%, among which 60% of IM-treated patients completed a cytogenetic response [66]. However, despite the success of IM, 15% of patients were refractory to the treatment from the beginning, while 50% of patients relapsed after IM discontinuation, demonstrating that IM is not totally curative [67]. IM resistance is explained by *de novo* mutations deforming the IM binding region of BCR-ABL, or by alternative activation of signaling pathways implicated in CML malignancy [68]. In addition, the hypoxic BM microenvironment maintains residual leukemia stem cells (LSCs) survival and stem cell potential, favoring self-renewal of LSCs in CML. LSC potential and self-renewal capacities are highly associated with CML-patients relapse after TKIs treatment [69]. This encouraged the development of secondgeneration TKIs, in the hope of overcoming IM resistance. Indeed, Dasatinib and Nilotinib were introduced and FDA approved in 2006 and 2007, respectively [70, 71]. Later, a third generation TKI (Ponatinib) was released and approved in 2013 [72]. These last two generations are highly associated with several side effects, including cardiovascular diseases and heart failure [73]. Therefore, devel-

oping a new therapeutic strategy for CML treatment appears of extreme importance, and targeted CML therapy could be supported by drug repurposing of a previously existing drug.

8. ACF AS AN ANTI-LEUKEMIC AGENT

The first-ever reported anti-leukemic effect of ACF dates back to 1997 by Kim *et al.,* [74] who tested the cytotoxic effects of ACF in Yac-1 leukemic mouse cells. The authors showed that ACF caused morphological changes in these cells, which could represent energy depletion. In 2011, a study showed that ACF inhibited chronic lymphoblastic leukemia (CLL) cell survival and proliferation [52]. Our team recently demonstrated a cytotoxic effect of ACF on MV-4-11 cell line, a current model for acute myeloid leukemia (AML) cells [75]. This is also in agreement with a study by Sabolova *et al.,* who showed that ACF induces a dose-dependent inhibition of proliferation of HL-60 AML cell line and L1210 mice leukemia cells [76]. Furthermore, ACF was described for its potential anti-leukemic activity in K-562 and KCL-22 CML cell lines, which is related to its inhibitory potential against HIF [77].

HIFs are transcription factors that play crucial roles in the progression of several cancers, including leukemia [78]. Indeed, several studies confirmed that HIF-1 is a critical factor in CML progression and plays an important role in chemoresistance [79]. Its oncogenic activity in CML could be explained by the hypoxic nature of the BM microenvironment, since low oxygen levels (<7%) stabilize HIF-1α, which binds to HIF-1β and activates the transcription of a large number of genes regulating cell survival, proliferation, and angiogenesis [80]. In addition, resident LSCs in the hypoxic BM are associated with chemoresistant CML through the activation of HIFs [81]. The genetic knockout of HIF-1α in CML murine models prevents CML development, by targeting LSC cell cycle progression and apoptosis [82]. In addition, chemical inhibitors of HIFs showed promising results in leukemia treatment. For instance, the HIF-1α-binding activity blocker "echinomycin" induced apoptosis in AML LSCs, resulting in a decreased leukemia burden, and prolonged mice survival [83]. On the other hand, high concentrations of L-ascorbic acid (AA) have been shown to inhibit the expression of HIF-1α and reduce tumor growth in CML [84]. Moreover, the treatment of APL cell lines with the HIF-1α inhibitor EZN-2208 conferred anti-leukemic activity by affecting cell migration and clonogenicity. Furthermore, *In vivo* combination treatment of EZN-2208 with ATRA (all-trans retinoic acid) eradicated leukemia cells, and prolonged mice survival [85]. In U973 cell lines, treatment with YC-1 inhibited HIF-1α expression and triggered leukemia cells via mitochondrial-dependent apoptosis [86]. On the other hand, *in vivo* administration of YC-1 in murine models of leukemia reduced leukemia burden and enhanced the survival rate [87]. Finally, ACF was reported as a selective inhibitor of HIF-1 activity, by binding directly to the PAS-B subdomain of both HIF-1α and HIF-2α subunits, preventing their hetero-dimerization with HIF-1β, thereby inhibiting HIF-1-DNA-binding and transcriptional activity [53]. Indeed, the inhibitory activity of ACF against HIF-1 has been reported to induce blockage of tumor growth and tumor vascularization [53, 88].

Based on this information, ACF has become an interesting potential therapeutic approach for leukemia treatment, specifically targeting CML therapy. In 2017, Cheloni *et al.,* showed that ACF targets LSCs of CML *in vitro*, where it was able to suppress the stem cell potential in CML cell lines and primary cells at the hypoxic condition. This capacity of suppressing stem cell potential of CML LSCs was reported even after long incubation of CML cells in hypoxia, revealing the importance of ACF anti-leukemic activity. Indeed, LSCs in CML patients are already incubated and adapted to low oxygen levels at the time of drug administration, which is usually associated with chemoresistance [89, 90]. The authors further confirmed the anti-leukemic effects of ACF *in vivo* using BCR-ABL-induced CML mice models, where ACF was capable of blocking leukemia development and reducing CML LSCs maintenance. In addition, ACF was also able to reduce the overall CML burden by reducing the severe splenomegaly and the number of CML cells in the BM and blood circulation of CML mice models treated with ACF [77].

In a recent study, our team demonstrated that ACF affected the growth and survival of CML and AML cell lines by targeting the STAT5 and STAT3 signaling pathway, without affecting BCR-ABL expression (Fig. **2**) [75]. In fact, STAT3 and STAT5 are two transcription factors that are aberrantly activated in CML models by the constitutive activation of BCR-ABL that phosphorylates STAT factors at their tyrosine residues, causing their excessive activation [91]. In addition, STAT3 and STAT5 aberrant activation via JAK phosphorylation has been reported to contribute to the TKI-resistance in CML [92]. Furthermore, STAT5 activation has been associated with CML LSCs maintenance

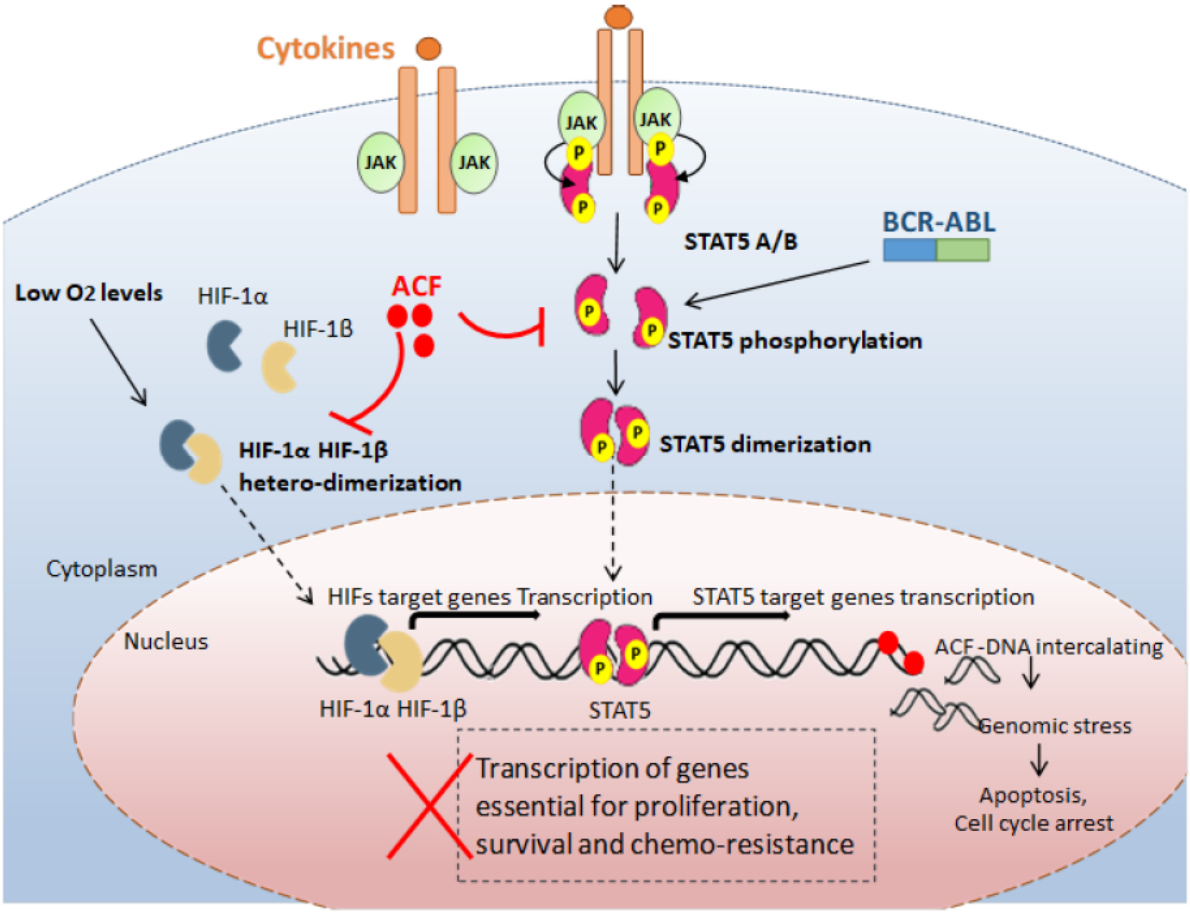

Fig. (2). Mechanism of action of ACF in CML. Canonical activation of STAT5 starts after binding of cytokines to their specific membrane receptors, inducing receptor dimerization, followed by tyrosine phosphorylation and activation of the receptor-associated tyrosine kinase JAK. In its turn, JAK mediates the tyrosine phosphorylation of STAT5 that dimerizes. The active STAT5 dimers are translocated to the nucleus, to bind with their target DNA sequences. In CML, the expression of the proto-oncogene BCR-ABL, leads to the constitutive activation of STAT5, directly by the tyrosine kinase activity of BCR-ABL. At the same time, the low oxygen levels in the bone marrow microenvironment stabilize the two hypoxia inducible factors HIF-1α and HIF-1β leading to their dimerization. The active dimer HIF-1α/HIF-1β is translocated to the nucleus to activate the transcription of its target genes. Both activation of STAT5 and HIF-1α/HIF-1β are associated with transcription of genes essential for proliferation, survival and chemo-resistance of CML cells. ACF inhibits both HIF-1α/HIF-1β dimerization by binding directly to HIF-1α, and STAT5 expression and phosphorylation, resulting in inhibition of transcription of oncogenes, therefore inducing apoptosis and cell cycle arrest. In addition, ACF intercalates directly in the DNA sequence causing genomic stress. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

[93]; thus STAT3/5 were considered as critical targets and the development of drugs inhibiting these transcription factors holds promise as a treatment for CML. The results of extensive research on STAT3/5 and their inhibitors are presented in an important monograph on STATs [94]. Several natural and synthetic molecules were identified as STAT inhibitors and tested in different cancers, including leukemia. In addition to that, several drugs were repurposed to be used as STAT inhibitors, Pimozide is one of them. This neuroleptic drug works by decreasing the activity of dopamine in the brain and it is approved to be used in Tourette syndrome [95]. Pimozide has later been identified in a cell screening assay as a STAT5 inhibitor that blocks CML cell growth [96, 97]. It also showed a synergistic effect with IM and Nilotinib in eradicating CML cells, thus overcoming TKI resistance [97]. Thus, targeting both HIFs and STAT signaling by ACF might potentially eradicate CML LSCs, and hence, prevent CML patients relapse after TKI treatment. Therefore, ACF- induced downregulation of STAT5 expression/activity in CML, along with the upregulation of STAT3 phosphorylation, could potentially lead to the inhibition of CML cells' survival and proliferation[75]. For instance, the anticancer chemotherapeutic agent doxorubicin showed STAT3 activation through JAK kinase phosphoryla-

tion, and was associated with growth inhibition in response to stress generation [98]. Moreover, we demonstrated that increased ACF doses induced DNA damage, associated with its DNA intercalating property, thus triggering apoptosis of CML cells. In line with these data, ACF was also reported as an inhibitor of topoisomerase I/II, which plays a crucial role in controlling DNA structure and DNA repair. Indeed, inhibitors of such proteins were reported as anti-cancer agents in several solid cancers and leukemia [52], thus, the ACF DNA-intercalating property is suggested to trigger apoptosis. Furthermore, activating the $P21^{Waf1}$ protein, an important marker of cellular senescence, was reported at a low concentration of ACF, suggesting that ACF might also induce a senescent phenotype in CML cells. Finally, ACF showed positive effects in IM-resistant CML cells sensitization, suggesting that ACF might be a useful drug that could be used alone or in combination with IM in order to alleviate resistant cells [75]. In summary, ACF presents a promising therapeutic strategy against CML, by inhibiting both HIF-1 and STAT5 transcription factors, and by eradicating TKI-resistant leukemia cells. Different studies of ACF in leukemia are summarized in Table **4**. Since ACF has proven to be safely used in humans, thus repurposing ACF in CML therapy presents an interesting strategy that has to be further investigated.

9. CHALLENGES AND OPPORTUNITIES OF THE THERAPEUTIC IMPLICATION OF ACF

Unlike other drugs that inhibit HIF-1 activity indirectly, ACF has shown to bind directly to both HIF-1α and HIF-2β [99, 100]. ACF has been administered to patients for targeting different malignancies without any major side effects [38, 47, 62]. In addition, the incorporation of ACF, along with conventional therapy of various diseases showed that ACF increases the antitumor effects of the latter without affecting normal homeostasis [77]. Thus, these observations propose ACF as the lead compound for the development of drugs that are efficient in treating patients with diseases characterized by increased HIF-1α or HIF-2α levels that are essential for malignancy progression. However, the challenge lies in the validation of the specificity of ACF towards its targets, which should be further investigated in order to assure the absence of any off-target effects. Moreover, the implication of ACF in clinical trials has not been extensively applied. Thus, further studies should be involved in order to determine the exact doses of ACF that should be administered. An-

Table 4. The effect of ACF in leukemia.

Leukemia	Material	Dose of ACF	Outcome	References
T Cell Lymphoma	-Yac-1 mouse leukemia cells	*In vitro*: various concentrations of ACF	-IC_{50} value is 2.11 μM; -ACF leads to morphological changes in Yac-1 cells, including bleb formations, swelling and ballooning	Kim *et al.,* (1997) [74]
Chronic Lymphocytic Leukemia (CLL)	-Patient-derived tumor cells (PDTC)	*In vitro*: 0.2, 1 and 5 μM	-ACF inhibits cell proliferation in a dose-dependent manner in PDTC	Hassan *et al.,* (2011) [52]
Acute Myeloid Leukemia (AML)	-MV-4-11 cell line	*In vitro*: 0.5, 1, 2, 4, 8 μM	-ACF downregulates STAT5 expression -ACF exerts a cytotoxic effect on AML cells	Hallal *et al.,* (2020) [75]
	-HL-60 cell line -L1210 cells	*In vitro*: various concentrations of ACF	-ACF has cytotoxic effect against HL-60 cells with IC_{50} value = 4.94 μM -IC_{50} value = 5.99 μM for L1210 cells	Sabolova *et al.,* (2020) [76]
Chronic Myeloid Leukemia (CML)	-CML cell lines -CML primary patient cells -C57BL/6J-CD45.1 mice	-*In vitro*: 0.5, 1, 5 μM -*In vivo*: 8 mg/kg, daily i.p. injection	-*In vitro*: ACF induced apoptosis and stem cell potential in CML cell lines and primary CML cells; ACF did not affect normal hematopoietic stem/progenitor cells -*In vivo*: ACF decreased leukemia development and reduced LSC maintenance	Cheloni *et al.,* (2017) [77]
	-K-562 cell line -KCL-22 cell line	*In vitro* : 0.5, 1, 2, 4, 8 μM	-ACF downregulates STAT5 expression -ACF induces apoptosis in a dose-dependent manner -ACF suppresses the resistance of CML cells to Imatinib	Hallal *et al.,* (2020) [75]

other challenge includes the investigation of ADMET (absorption, distribution, metabolism, excretion, and toxicity) of ACF in order to better incorporate it in clinical trials. Regardless of the extensive work required for the study of ACF, its anti-tumor effects have shown to be efficient in different cancers previously mentioned, thus showing promising results in not only eradicating CML, but also in other malignancies and cancer types [52, 54, 75]

CONCLUSION

CML patients are still facing a lack of efficacy with available TKIs in around 15% of cases. Besides, resistance mechanisms are not yet fully understood. Thus, developing a new treatment for these refractory cases remains considerably challenging. Drug repurposing has recently emerged as a promising tool for novel drug discovery in a wide range of cancers. It is relatively rapid and cost-effective, with minimal risk on patients. We have proved a cytotoxic effect of ACF on CML cells, thus suggesting it as a promising candidate for drug repurposing in CML patients. Indeed, ACF has already demonstrated to be safely administered to patients, without showing major side effects. However, chemical modifications should be considered in order to have a better bioavailability of the drug. Overall, we suggest the use of ACF in CML therapy alone, or in combination with other conventional drugs for the treatment of CML resistant to TKIs.

LIST OF ABBREVIATIONS

5-FU	=	5-fluorouracil
ACF	=	Acriflavine
ADMET	=	Absorption, distribution, metabolism, excretion, and toxicity
AIDS	=	Acquired immunodeficiency syndrome
AML	=	Acute myeloid leukemia
APL	=	Acute promyeloctic leukemia
ATRA	=	All-trans retinoic acid
AZT	=	Azidothymidine
BAX	=	Bcl-2-associated
Bcl-2	=	B-cell lymphoma 2 protein
BCR-ABL	=	Breakpoint cluster region – Abelson
BM	=	Bone marrow
CAC	=	Associated colon cancer
CLL	=	Chronic lymphoblastic leukemia
CML	=	Chronic myeloid leukemia
CRC	=	Colorectal cancer
EHR	=	Electronic health records
FDA	=	Food and drug administration
GWAS	=	Genome wide association studies
HCC	=	Human hepatocellular carcinoma
HIF	=	Hypoxia inducible factor
HIV	=	Human immunodeficiency virus
HSC	=	Hematopoietic stem cells
IM	=	Imatinib
INN	=	International nonproprietary name
IP	=	Intraperitoneal
JAK	=	Janus kinase
LSC	=	Leukemia stem cells
PAS-B	=	Per-Arnt-Sim B subdomain
PDT	=	Photodynamic therapy
PDTC	=	Patient derived tumor cells
PGK1	=	Phosphoglycerate Kinase 1
PPI	=	Protein-protein interactions
SCID mice	=	Severe combined immunodeficient mice
STAT	=	Signal transducer and activator of transcription
STI	=	Sexually transmitted infection
TGF-β	=	Transforming growth factor beta
TKI	=	Tyrosine kinase inhibitor
VEGF	=	Vascular endothelial growth factor

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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