Toward a repositioning of the antibacterial drug nifuroxazide for cancer treatment

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Nifuroxazide (NFX) is a broad-spectrum antibacterial drug that has been used for the treatment of infectious diarrhoea since 1966. In 2008, the discovery of potent inhibition of the transcription factor signal transducer and activator of transcription STAT3 by NFX prompted studies as a potential anticancer agent. Subsequently, it was shown that NFX induces cancer cell apoptosis and inhibits tumour growth. Recently, NFX was identified as a potent inhibitor of aldehyde dehydrogenase ALDH1 that selectively kills ALDH^{high} cancer-initiating cells. These two landmark discoveries – STAT3 and ALDH1 inhibition – strongly support the potential repositioning of NFX as a targeted anticancer agent. The related antiparasitic drug nifurtimox is undergoing clinical development for the treatment of paediatric tumours. The anticancer potential of NFX is highlighted here.

Nitrofurans as antibacterial drugs

The discovery of the therapeutic potential of nitrofuran derivatives can be traced back to 1944 when their antibacterial activities were discovered [1]. Their mode of action is unclear. It is assumed that these drugs interfere with the activity of dehydrogenases and inhibit protein synthesis in pathogenic bacteria. The 5-nitro group on the furan ring is reduced under aerobic conditions leading to the formation of toxic free radicals, thus increasing the antibacterial activity. This initial discovery led to the development of a complete family of synthetic 5-nitro furan hydrazones, acting as prodrugs – they require bioactivation of the 5-nitrofuran for activity – including nifuroxazide (NFX), nifurtimox, nitrofurantoin, furazolidone and others (Fig. 1). Some of these drugs have been withdrawn from the market owing to an unfavourable benefit/risk ratio. However, most of them remain used today in human or veterinary medicine. Here, we shall principally focus on NFX, the leading compound in the series, which has been in clinical use for many decades as an oral intestinal disinfectant for the treatment of diarrhoea of bacterial origin or colitis, in adults.

NFX as an anti-diarrhoea drug

NFX is a gastrointestinal antibiotic first patented in 1961 (France) and 1966 (USA) by Laboratoires Robert & Carrière SA (France) [2]. It was extensively used in the 1970s and largely promoted, as illustrated in Fig. 2. After oral administration, the drug can be absorbed and metabolised in the liver [3]. Very low concentrations can be detected in the blood and the urine. The drug shows an almost exclusive enteral aseptic action, without systemic antibacterial activity. NFX is well tolerated but there are occasional side effects in the forms of a disturbance in the digestive process and eventually allergic reactions such as cutaneous rash, urticaria, angioedema (and rare cases of serious immune-allergic reactions and anaphylactic shock) [4]. Depending on blood concentration, NFX exerts bacteriostatic or bactericidal effects and is primarily used to treat acute, infectious diarrhoea. It is active against the majority of causative agents of intestinal infections: Gram-positive (*Staphylococcus* family) and Gram-negative (*Enterobacteriaceae* family: *Escherichia*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Salmonella*, *Shigella*, *Yersinia*), and also *Vibria cholerae*, but inactive against the *Pseudomonas* and *Proteus*. NFX is mainly used for the treatment of infectious colitis and diarrhoea but it can be associated with other drugs, for example with *Plantago ovata* mucilage for the treatment of constipation (Fig. 2).

NFX was suspected to be mutagenic in mammalian cells [5]. In a transgenic mouse model to evaluate the mutagenic effects of nitrofurantoin and NFX, a weak mutagenic response was seen with nitrofurantoin, not with NFX [6]. The carcinogenicity of

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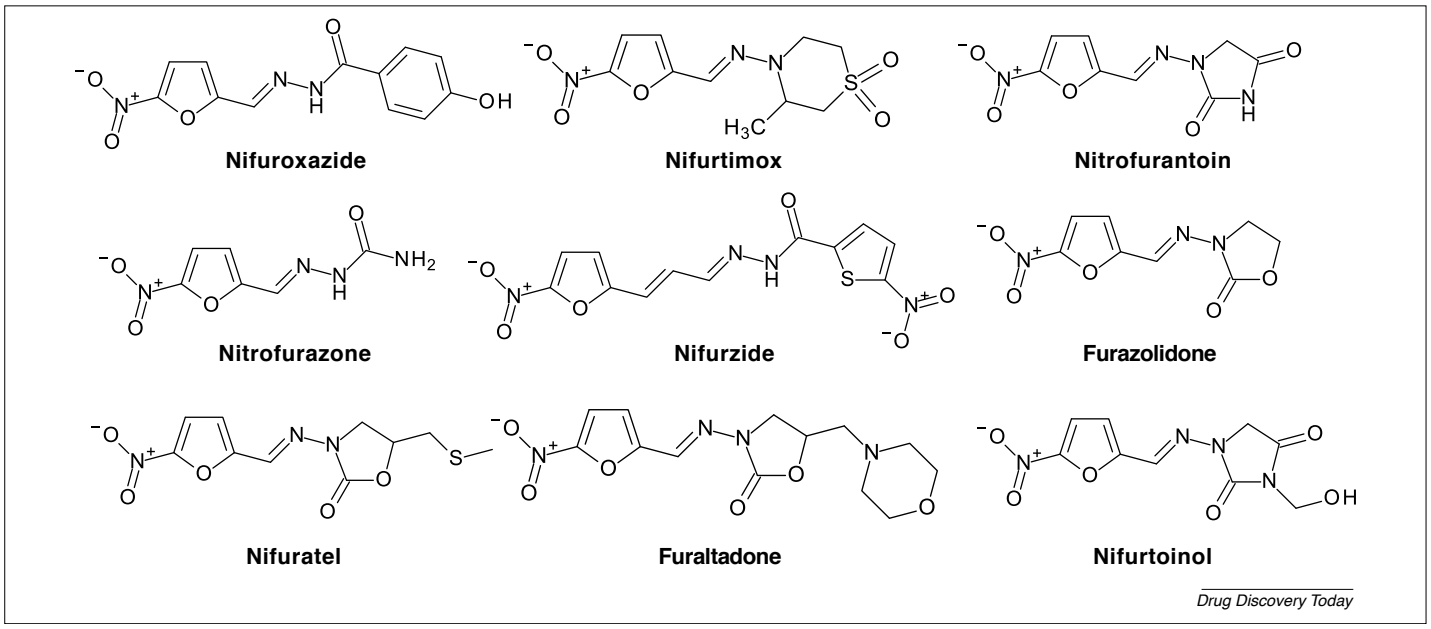

FIGURE 1

Chemical structures of nifuroxazide (NFX) and related nitrofuran drugs.

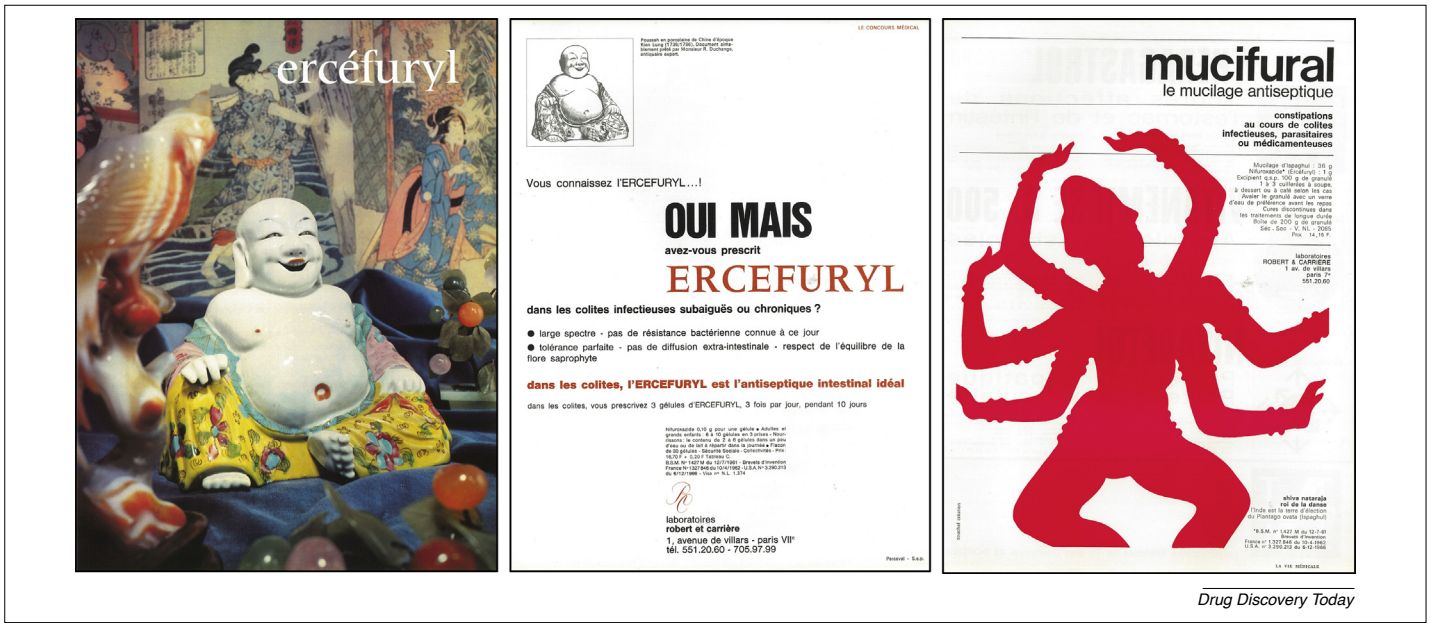

FIGURE 2

The antibacterial activity of nifuroxazide (NFX) promoted in the early 1970s. NFX is the active pharmaceutical ingredient of Ercefuryl® (treatment of chronic or subacute infectious colitis) and found also in Muciferal® (treatment of constipation: combination of NFX and *Plantago ovata* mucilage).

certain nitrofurans has led to the prohibition of their use in food-producing animal species in many regions, including the EU, USA, Canada and Australia. Despite a diminution of its use over the past two decades, NFX remains available in several countries for the treatment of acute diarrhoea. In a recent prospective observational study in Bosnia & Herzegovina, NFX (Enterofuryl®) demonstrated a better efficiency than treatment with a probiotic preparation containing lactic acid bacteria. The drug proved to be well tolerated and safe [7].

NFX and the design of novel antimicrobial drugs

New anti-infective agents are needed to fight microbial resistance, in particular to combat methicillin-resistant *Staphylococcus aureus* (MRSA) strains, responsible for complicated and difficult to treat pathologies. NFX derivatives have been designed and tested as potential anti-MRSA agents. Interesting antibacterial NFX-based compounds have been obtained [3,8,9] but none of them proved sufficiently efficient to warrant full drug development. NFX itself is not very potent against MRSA. However, the drug could be useful

to combat other infectious diseases, such as infection by the opportunistic pathogen *Pseudomonas aeruginosa*. NFX was shown to alter *Pseudomonas aeruginosa* biofilm through a blockade of bacterial intracellular communications (quorum sensing) [10]. Novel antimicrobial agents based on the NFX archetype, in particular preserving the key nitrofuran and the central azomethine unit (-NH-N = CH-), are regularly explored as novel antiseptic drug candidates [9,11].

Antiprotozoal activity of NFX

Nifurtimox is a well-known antitrypanosomal nitrofuran, used since the 1970s for the treatment of human African trypanosomiasis and for Chagas disease – a parasitic infection caused by *Trypanosoma cruzi*. The drug acts through production of reduced oxygen metabolites, superoxide and hydrogen peroxide, for which the parasites have a lower detoxification capacity. NFX was also explored as a potential antiparasitic agent. The drug was tested against *Gardia lamblia* infection but it was found to exhibit only a modest antigardial activity [12]. NFX was evaluated for the treatment of Chagas disease; it revealed a noticeable but not major activity against *T. cruzi*. Nitrofuran derivatives were designed, some of them appeared promising in the first selection tests but none was apparently developed [13,14]. NFX proved to be much more potent than nifurtimox and nitrofurantoin against *Trypanosoma brucei rhodiense in vitro* but it is also more cytotoxic [15]. NFX is active against *Leishmania donovani* and *T. cruzi in vitro* but it is not more potent than nifurtimox, while being more cytotoxic [15]. Therefore, it is unlikely that NFX could be repositioned as an antiparasitic agent for the treatment of neglected tropical diseases. However, the use of NFX has been recommended in *T.-cruzi*-infected patients with an immune-compromised condition (typically AIDS patients) to decrease the risk of reactivation and to minimise clinical complications [16].

NFX is a potent STAT3 inhibitor

The capacity of NFX to inhibit signal transducer and activator of transcription STAT3 was discovered in 2008 during the course of a functional screen to identify STAT3 inhibitors (Fig. 3). The screening of the Prestwick Library of 1200 bioactive compounds (off-patent marketed drugs and natural compounds) led to the characterisation of NFX as a potent inhibitor of STAT3-dependent gene expression. Complementary biochemical studies revealed that NFX inhibits STAT3 phosphorylation through inhibition of the JAK family kinases JAK2 and Tyk2. This kinase inhibitory activity accounts for the antiproliferative activity of NFX in myeloma cells with a constitutive activation of STAT3, with minimal effects on normal cells [17]. This study also showed that NFX can promote the antiproliferative activity of a histone deacetylase inhibitor or a MEK inhibitor, thus providing a rationale to design combinatory treatments [17]. This landmark discovery study was not immediately exploited and no other proof of the anticancer activity of NFX was published for the next 7 years. Then, from 2015, several studies highlighted the anticancer potential of NFX and its capacity to trigger apoptosis in cancer cells.

Apoptosis and tumour growth inhibition by NFX

STAT3 is a point of convergence of multiple oncogenic signalling pathways and is constitutively active in a variety of human

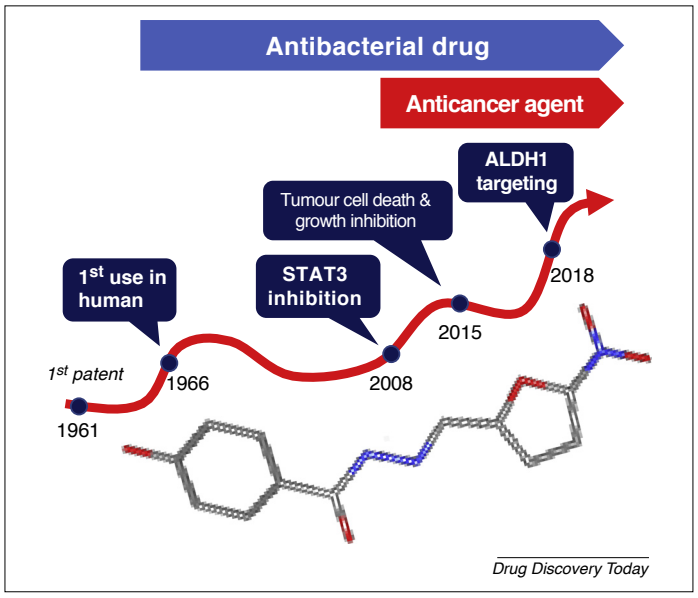

FIGURE 3

Schematic of nifuroxazide (NFX) discovery and development as an antibacterial drug and then as a targeted anticancer therapeutic. Fifty-eight years of evolution of NFX, to illustrate its main clinical applications and biochemical properties.

cancers. In particular, STAT3 is activated in 70% of breast tumours, notably triple-negative tumours. On this basis, Yang *et al*. demonstrated that NFX dose-dependently inhibits proliferation, induces apoptosis and restricts migration and invasion of breast cancer cells [18]. Most importantly, the drug was shown to decrease mammary tumour growth and lung metastasis *in vivo*. NFX also reduced the infiltration of myeloid-derived suppressor cells in the lung, without significant toxicity [18].

The study in breast cancer was followed by complementary works in other indications (Table 1) where STAT3 plays an important part as a tumour driver, such as melanoma. NFX was shown to inhibit the proliferation and cell migration and invasion of several melanoma cell lines [19]. NFX inhibited the expression levels of different mediators of cell migration and invasion, such as matrix metalloproteinase MMP-2, MMP-9, vimentin, P-FAK and P-Src. Moreover, as for the initial study in breast cancer, the mechanism of lung metastasis suppression was shown to implicate a drug-induced inhibition of the infiltration of myeloid-derived suppressor cells [19]. Significant, although not outstanding, *in vivo* activity was observed upon treatment with NFX on mice bearing A375 melanoma tumours, with a reduction of p-STAT3 *in vivo* [19]. The same investigators reported the activity of NFX in colon cancer. Here again, NFX was shown to inhibit cell growth, to induce apoptosis and to impair migration and invasion of different types of colorectal cancer cells [20]. An *in vivo* antitumour activity was evidenced, as well as an antimetastatic effect, accompanied with a reduction of the number of myeloid-derived suppressor cells in the spleen, tumour and blood. Interestingly, an increased infiltration of $CD8^+$ T cells and a reduction of the number of M2-type macrophages in the tumour were noticed, suggesting a drug-induced stimulation of immune response [20].

Structural analogues of NFX were shown to display antitumour activity in a melanoma model. In particular, two bromo- and

TABLE 1

Nifuroxazide (NFX) anticancer mechanisms of action in various models

Targets and activities	Tumour types	Refs
Inhibition of STAT3 via kinases JAK2 and Tyk2. Cell growth inhibition	Multiple myeloma	[17]
Drug-induced apoptosis and tumour growth inhibition *in vivo*. Inhibition of cell migration and invasion to limit lung metastasis	Breast carcinoma	[18]
NFX nanoparticles: apoptosis. Inhibition of STAT3 phosphorylation and tumour growth		[27]
Inhibition of the infiltration of myeloid-derived suppressor cells. ↘ MMP expression	Melanoma	[19]
Binding to ALDH1. Selective killing of ALDH1high cancer-initiating cells		[29]
NFX derivatives: ↘ Akt; ↗ Bim expression		[21]
Apoptosis. Tumour growth inhibition. Antimetastatic effects.	Colon carcinoma	[20]
Stimulation of immune response: ↗ infiltration of $CD8^+$ T cells and ↘ M2-macrophages		
Combination with loaded dendritic cells: immune response to promote tumour growth inhibition	Hepatocellular carcinoma	[23]
Combination of NFX and sorafenib: suppression of CD133 leading to tumour growth inhibition		
Drug-induced apoptosis. Inhibition of cell migration and invasion	Osteosarcoma	[25]
Inhibition of cell proliferation	Thyroid carcinoma	[26]
Furazolidone: drug-induced myeloid differentiation and apoptosis	Leukaemia	[30]
Nitrofurantoin: apoptosis, ↗ Bax, ↘ Bcl-xl		[31]
Clinical efficacy of nifurtimox. ↘ N-Myc expression. Drug-induced apoptosis and impact on glucose oxidative metabolism	Paediatric tumours: neuroblastoma, medulloblastoma	[32,33]

iodo-derivatives of NFX (the nitrofuran system was replaced by a benzofuran unit) showed the best activity in a series of 23 derivatives in a murine melanoma model *in vitro* and *in vivo*. The antitumour effect implicated downregulation of p-AKT and upregulation of BIM [21].

STAT3 is connected upstream with Janus kinase family proteins (notably JAK2) and is capable of integrating inputs from different signalling pathways [22]. STAT3 is involved in cancer progression and its persistent activation can promote chronic inflammation. The modulation of the JAK2/STAT3 pathway by NFX opens many possibilities for drug combinations. For example, NFX was shown to promote an antitumour response when it was combined with tumour-cell-lysate-loaded dendritic cells, in a hepatocellular carcinoma (HCC) model *in vivo* [23]. In HCC, STAT3 binds nuclear factor NFκB p65 and the formed dimer interacts with hypoxia-inducible factor HIF-1α promoter in the nucleus of hypoxia-stimulated Huh7 cells. NFX combined with the multi-kinase inhibitor sorafenib remarkably suppressed tumour growth *in vivo*. The antitumour effect is associated with a suppression of CD133 expression, consequently decreasing the levels of downstream targets such as cyclin A/D1, Bcl-2 and Mcl-1 [24]. Recently, NFX was shown to inhibit proliferation and induce apoptosis of osteosarcoma and thyroid carcinoma cells [25,26]. Self-assembling nanoparticles formed with a lipase-labile phospholipid NFX prodrug revealed remarkable growth inhibition of breast cancer cells *in vivo*, associated with an inhibition of STAT3 phosphorylation [27].

NFX targets ALDH1

The 5-nitrofurans require bioactivation to exert their anticancer activity. NFX can be bioactivated by aldehyde dehydrogenase (ALDH) enzymes which are highly expressed in certain cancer-initiating cells ($ALDH1^{high}$ stem cells). Although ALDH2 is a direct target of nifurtimox [28], NFX is selective for bioactivation by ALDH1 isoform over ALDH2 [29]. The drug would fit into the substrate pocket of ALDH1A1/A3 isoforms, interacting with two cysteine residues in the active site of the enzyme. This binding leads to an oxidation and inactivation of the enzyme. The interaction occurs *in vivo*. NFX was shown to selectively kill the $ALDH1^{high}$ cancer-initiating cells, which correspond to a highly tumorigenic subpopulation. In sharp contrast, $ALDH^{low}$ cells were found to be resistant to NFX. *In vivo* experiments, using mice grafted with A375-L2T melanoma cells, revealed that the subpopulation of $ALDH1^{high}$ cancer cells are extremely sensitive to NFX, which totally eradicated this population *in vivo* [29]. This key discovery opens the door to the design of new melanoma treatment protocols with patient stratification based on the unique $ALDH1^{high}$-dependent activity of NFX.

NFX-related anticancer agents

Other nitrofuran derivatives that are direct analogues of NFX have revealed anticancer properties. The antibiotic furazolidone, used for decades as an antibacterial drug, was shown to display a potent antileukaemic activity, inhibiting proliferation and inducing apoptosis in different acute myeloid leukaemia cell lines. It can also induce myeloid cell differentiation [30]. Nitrofurantoin was also shown to induce apoptosis in leukaemia cells, via an upregulation of BAX and downregulation of BCL-xl [31]. But the most advanced repositioning strategy in this group of nitrofuran derivatives concerns the antiprotozoan drug nifurtimox, which was shown to inhibit cancer cell growth *in vitro* and *in vivo*, and was subsequently tested in humans, in particular for the treatment of paediatric tumours. Nifurtimox was shown to exert an antitumour activity and to induce apoptosis of neuroblastoma and medulloblastoma tumours [32,33]. The drug induces a shift of glucose metabolism from production of lactate to oxidative phosphorylation and a reduced expression of N-Myc (which is frequently amplified in certain high-risk tumours) in neuroblastoma cells [34]. It showed a significant activity against a panel of neural tumour cells, in particular when it was combined with the γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine [35]. Nifurtimox can also inhibit the clonogenic growth of tumour cells under hypoxic conditions [36]. In early-phase clinical trials, nifurtimox was found to be well tolerated by children with relapsed neuroblastoma and tumour responses were observed as a single agent and in combination with chemotherapy [37,38]. A Phase II study of nifurtimox combined with cyclophosphamide and topotecan for the treatment of young patients with refractory or relapsed

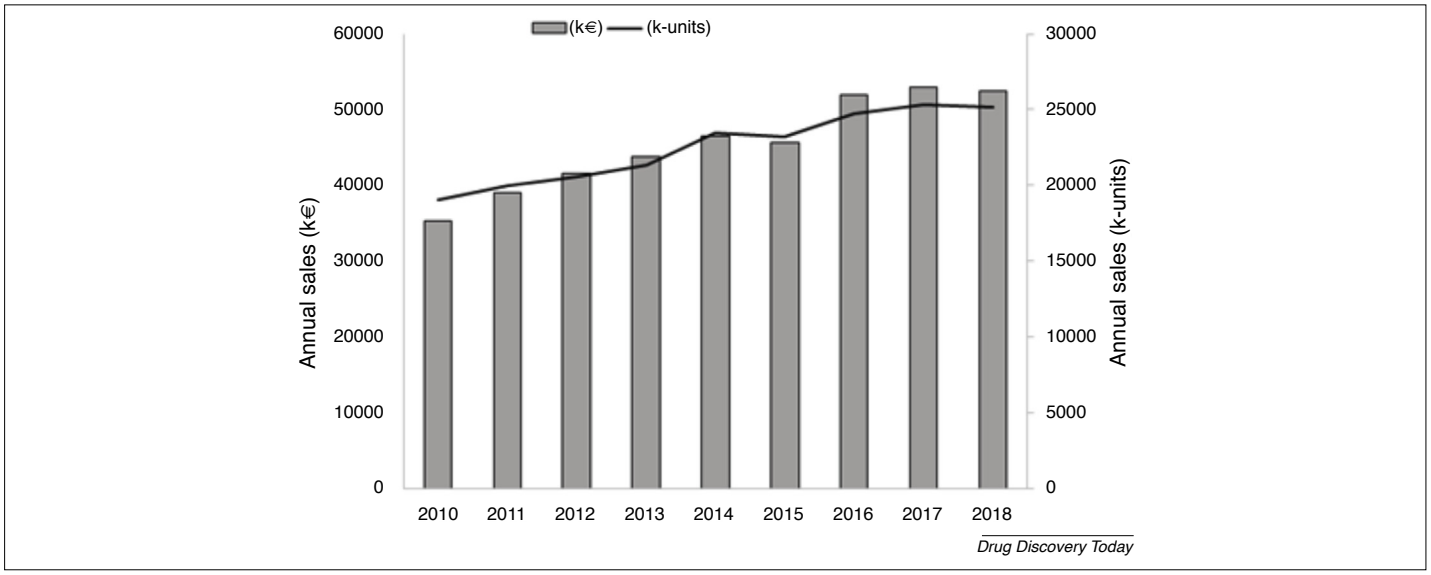

FIGURE 4

Pharmaceutical sales of nifuroxazide (NFX) for years 2010–2018 (Market Analysis & Estimates). The graph shows the annual sales in k€ (left axis) and in k-units (right axis). Numbers for year 2018 are estimates based on the June 2018 moving annual total (MTA) data.

neuroblastoma and medulloblastoma was initiated in 2008 [39]. The results are awaited.

We can also refer to other 5-nitrofuran derivatives that display antitumour activities. In particular, three drugs warrant mention: (i) the 5-nitrofuran EMBL-153441 (designated Thanatop) characterised as a topoisomerase II inhibitor [40]; (ii) Eeyarestatin 1 (ES1) which inhibits protein dislocation from the endoplasmic reticulum in the cytosol [41]; and (iii) the anticancer small molecule NSC59984 which targets the p53 signalling pathway [42]. The case of ES1 is particularly interesting because its 5-nitrofuran-2-acrylaldehyde hydrazine moiety functions as an electrophilic warhead that supports endoplasmic-reticulum-associated protein degradation (ERAD) inhibitory and cytotoxic activities, via an interaction with the p97 ATPase (also known as valosin-containing protein) [43]. Indirectly, these three examples reinforce the conclusion that the nitrofuran-hydrazide moiety of NFX plays a key part in the anticancer activity.

NFX and inflammation

The capacity of NFX to inhibit STAT3 signalling opened new perspectives in oncology, but also in other therapeutic domains, notably for the treatment of certain inflammatory diseases. Inflammation is a major player in the development and progression of diabetic nephropathy, which is a serious complication of diabetes mellitus. NFX was shown to mitigate the inflammatory burden and to protect against diabetes-induced nephropathy in a rat model. The drug reduced macrophage infiltration and fibrosis, thus suggesting the potential use of NFX as an alternative anti-inflammatory therapy in this indication [41,44].

Another well-thought-out hypothesis for the use of NFX as a STAT3 inhibitor is the treatment of the acute graft-vs-host disease (aGvHD) which is a major and potentially lethal complication of allogenic bone-marrow transplantation. Two interesting studies have been published recently [42,43,45,46]. It was shown that blocking STAT3 activation by NFX reduced $CD4^+$ T lymphocytes, decreased secretion of interferon IFN-γ and tumour necrosis factor TNF-α cytokines. NFX suppressed the development of aGvHD and significantly delayed aGvHD-induced lethality [43,46]. The use of NFX as a prophylactic or as a second-line therapy for aGvHD was proposed.

Concluding remarks

NFX has been used to treat bacterial infections and associated diarrhoea for >50 years (Fig. 3). Although its human use decreased during 2000–2010, it remains largely prescribed in some countries. The drug is sold in at least 22 countries, in Europe, Africa, Latin America and Asia. A market analysis for the past 9 years indicated that the pharmaceutical sales of NFX-containing products is progressing (+25% in volume, +40% in value), as illustrated in Fig. 4. NFX is not an expensive drug. The global market remains modest but significant (€50 m/year). It is likely that the drug will remain used as an enteral antibacterial agent for many years to come.

The discovery in 2008 of the inhibition of STAT3 by NFX was a turning point in the history of the drug, providing a new perspective for its repurposing for the treatment of cancers or certain inflammatory diseases. Over the past 10 years, NFX has revealed interesting anticancer properties, inducing apoptosis in different cancer cell lines and models (Table 1). The drug displays antiproliferative and antimetastatic effects. The antitumour activity observed in the clinic with the related drug nifurtimox bodes well for the potential repurposing of a nitrofuran derivative in oncology. The key to successful development would be the selection of a well-defined patient subpopulation with molecular characteristics adapted to the drug mechanism of action in oncology. In this context, the recent discovery of the targeting of ALDH1 and extreme sensitivity of $ALDH1^{high}$ cancer cells to NFX is remarkable. This is the exact type of mechanism-based precision therapy that could be efficiently developed. NFX is on track to be reprofiled, from a broad-spectrum antibacterial drug with a multifaceted mechanism of action to a molecularly targeted anticancer agent

susceptible to address a well-defined tumour type. It is of course too early to predict the success of this approach and the future of NFX in oncology. But the path is open and well traced.

Repurposing is a drug development strategy that seeks to use existing medications for new indications. In oncology, there is an increased level of activity looking at the use of noncancer drugs as possible cancer treatments. The Repurposing Drugs in Oncology (ReDO) project [44,47] is emblematic of this strategy. An interesting parallel can be established between 5-nitrofurans such as NFX and fluoroquinolones such as ciprofloxacin. In both cases, it is about a family of antibacterial drugs with a multifaceted mechanism of action that have been characterised later as anticancer agents inducing cell cycle perturbations, apoptosis, enhancing anticancer activity in drug combinations and promoting antimetastatic effects. The repositioning of a fluoroquinolone into an anticancer molecule is a plausible option, owing to their marked immunomodulatory and/or antiproliferative or antimotility activities [45,48]. I believe that the possibility to repurpose a 5-nitrofuran derivative such as NFX is even stronger, considering the recent subcategory of cancer (ALDH1^{high} tumours) exquisitely sensitive to NFX [46]. Perhaps it will be necessary to modify the structure of NFX to mask a potential adverse effect (a questioned mutagenic potential for example). It is an interesting and timely challenge, to reinforce the discovery of effective and economically affordable medications for specific cancers. Drug repositioning is an efficient and economic strategy, which has met with some success in oncology [46–51]. Let's hope that NFX, or a 5-nitrofuran analogue, will confirm the benefit of such a strategy.

Conflicts of interest

The author declares no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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