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New uses for an old remedy: digoxin as a potential treatment for steatohepatitis and other disorders

Digoxin in steatohepatitis and other disorders

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Abstract

Repurposing of the widely available and relatively cheap generic cardiac glycoside digoxin for non-cardiac indications could have a wide-ranging impact on the global burden of several diseases. Over the past several years, there have been significant advances in the study of digoxin pharmacology and its potential non-cardiac clinical applications, including anti-inflammatory, anti-neoplastic, metabolic, and anti-microbial use. Digoxin holds promise in the treatment of gastrointestinal disease, including nonalcoholic steatohepatitis and alcohol-associated steatohepatitis, as well as in obesity, cancer, and treatment of viral infection, among other conditions. In this review, we provide a brief summary of the clinical use of digoxin to date and discuss recent research on its emerging applications.

Key Words: Digoxin; cardiac glycosides; sodium potassium pump; oxidative stress; nonalcoholic steatohepatitis; alcohol associated steatohepatitis; sterile inflammation

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Core Tip: Digoxin has been used primarily as a cardiac drug for treatment of arrhythmias and heart failure. Recent preclinical work support the potential repurposing of digoxin as therapy for a number of non-cardiac conditions, including alcohol-associated steatohepatitis, nonalcoholic steatohepatitis, obesity and metabolic disorders, autoimmune and inflammatory conditions, malignancy, and viral infections, among others. Here, we provide an overview of findings to date on the potential clinical applications of digoxin and mechanisms of action in steatohepatitis and other non-cardiac disorders. We discuss evidence so far on the differential action of digoxin at high *vs* low concentration and identify areas of further research necessary to harness its promising multifunctional use.

INTRODUCTION

Digoxin in a Nutshell: an overview of 200 years

Digoxin (also known by the broader term digitalis) is derived from the purple foxglove, a medicinal plant that can be traced to Irish monks and Germans and was cultivated during the time of Charles the Great (700s – 800s). Its Latin scientific name *Digitalis purpurea,* was coined by Leonard Fuchs in 1542 based on the translation of the German word describing the shape of the flower as a Fingerhut or thimble. Digitalis was mentioned in herbal remedies in England in the 1500s and 1600s for several purposes, including epilepsy, vertigo, swelling/fluid accumulation, tuberculosis, and skin diseases [1]. Subsequently, digitalis fell out of favor due to reports of its toxicity. Animal experiments involving the administration of digoxin leaves to turkeys and roosters resulted in fits and death [1].

In the late 1700s, William Withering, an English botanist and physician, heard about a family recipe containing over twenty different herbs used in the cure of fluid overload, referred to as dropsy [2]. After realizing that the active ingredient in the herbal remedy was likely from the foxglove plant, Dr. Withering administered foxglove tea as a cure to a patient with dropsy. That patient did well, and over the ensuing decade, he performed a comprehensive case series of digitalis by administering a decoction prepared from dried foxglove leaves to 163 patients with fluid retention, of whom 101 experienced relief. He noted that digitalis was especially helpful for patients with dropsy after having scarlet fever or bad sore throats. Withering's work inspired other physicians to try digitalis as a therapy in dropsy. For further information on the evolution of digoxin as a medical therapy, the reader is referred to an excellent review by Wray *et al* [1].

Digoxin has a molecular formula $C_{41}H_{64}O_{14}$ and molecular weight of 780.9 g/mol. Similar to other cardiac glycoside, digoxin increases the force of contraction of the heart by reversibly inhibiting the activity of the myocardial Na-K ATPase pump, an enzyme

that controls the movement of potassium ions into the heart [3, 4]. The most common cardiac uses of digoxin include heart failure and supraventricular arrhythmia. Its role in heart failure is due to its inotropic properties, inhibiting the Na-K ATPase pump thus increasing the intercellular calcium concentration [6]. This lengthens the cardiac action potential, lowering the heart rate and increasing myocardial contractility. The American College of Cardiology/American Heart Association guidelines recommend that digoxin be added to the heart failure regimen in patients with left ventricular systolic dysfunction when symptoms persist despite optimization of treatment with an angiotensin-converting enzyme (**ACE**) inhibitor, a β-blocker, and/or a diuretic [7-9]. Digoxin effect in treatment of supraventricular arrhythmia occurs through its parasympathomimetic effect involving activation of vagus nerve activity, reducing the automaticity of the sinoatrial node and slowing atrioventricular conduction [10].

Current clinical use of digoxin is limited to the cardiac arena. Oral digoxin is available as a solution (0.05 mg/mL) or as tablets (0.0625 mg, 0.125 mg, and 0.25 mg). Dosing is typically maintained between 0.125 to 0.25 mg daily, with lower doses considered in patients 70 years of age or older [11]. The steady-state volume of distribution of digoxin is decreased in chronic renal failure; therefore, both loading and maintenance dosing should be decreased in such patients [12]. Digoxin has a narrow therapeutic window, with the rate of toxicity increasing as serum concentration reaches over 2.0 ng/mL. However, toxicity can also occur at levels below 2.0ng/mL in the setting of risk factors such as age, decreased renal function, hypokalemia or other electrolyte abnormalities, or interacting medications [13]. The narrow therapeutic window for digoxin necessitates monitoring of serum digoxin levels, particularly in patients with chronic renal dysfunction or changing renal function.

With the discovery of many effective cardiac drugs for heart failure and supraventricular arrhythmias over the past few decades, and difficulty maintaining the narrow digoxin therapeutic index while preventing toxic side effects, the use of digoxin in cardiac disease has been waning. During this period, however, there have been several advances in basic and clinical work toward the potential repurposing of digoxin

and other cardiac glycosides for non-cardiac conditions. These studies indicate that the biological effects of cardiac glycoside are not limited only to the inhibition of Na, K-ATPase, but modulate various signal transduction pathways including nuclear receptors involved in hormonal signaling, immune response, carcinogenesis, among others [14-19].

Digoxin in steatohepatitis

Overnutrition and obesity impair metabolic homeostasis and trigger sterile-type inflammation [20-23], contributing to the development of nonalcoholic fatty liver disease (**NAFLD**) and advanced nonalcoholic steatohepatitis (**NASH**). The amplitude of sterile inflammation triggered by metabolic stress in the liver has major clinical consequences. It is responsible for increasing amounts of liver damage and death in NASH [24]. NASH, as well as other diseases caused by sterile inflammation of the liver, lack effective treatments. This is limited by the relatively poor understanding of the initiating steps in sterile inflammation and the dysregulation of a wide range of pathways, making it difficult to know which ones to target.

Identification of Hypoxia-inducible factor 1-alpha (**HIF-1α**) pathway activation in macrophages for sustained inflammatory responses provided HIF-1α with a key role in the core regulatory machinery for the transition from acute self-limiting to sustained chronic inflammation [25]. These mechanistic insights into the role of the HIF-1α pathway in sterile inflammation may have great clinical relevance due to the ability of digoxin to inhibit HIF-1α activation [26]. Digoxin (1.0-0.05 mg/kg) effectively prevents acute and chronic hepatic damage, steatosis, and inflammation in both lipopolysaccharides (**LPS**)- and high-fat diets-driven animal models [27].

Digoxin reduces oxidative stress during liver injury by maintaining cellular redox homeostasis and protects the liver from a wide variety of insults [27, 28]. Digoxin reduces HIF-1α transcriptional activity, and the antioxidant effect of digoxin is dependent on HIF-1α. Digoxin induced significant changes in gene transcripts related to HIF-1α in metabolic processes and nucleic acid binding [27]. To understand the direct

molecular mechanisms responsible for the digoxin effect on HIF-1α transcription, pyruvate kinase M2 (**PKM2**) was identified as the major digoxin binding protein using a novel approach of digoxin-immunized agarose beads coupled with liquid chromatography with tandem mass spectrometry (**LC-MS/MS**) analysis [27]. The ability of digoxin to bind to PKM2 was an unexpected finding and provided novel insights into PKM2 biology and the role of PKM2 in sterile inflammatory liver diseases. PKM2 is best known as the rate-limiting glycolytic enzyme that catalyzes the conversion of phosphoenol pyruvate (**PEP**) and adenosine diphosphate (**ADP**) to pyruvate and adenosine triphosphate (**ATP**) [29].

In addition to its pyruvate kinase function, PKM2 interacts with HIF-1α in the nucleus and functions as a transcriptional co-activator for HIF-1α, resulting in the stimulation of HIF-1α responsive genes [30]. Interestingly, the interaction of digoxin to PKM2 did not alter its pyruvate kinase ability or reduce its nuclear translocation. Digoxin, however, reduced the ability of PKM2 to upregulate the transcription of HIF-1α and its downstream genes, such as inflammatory genes and genes involved in oxidative stress. Further, digoxin reduced the binding of PKM2 to histones, suggesting digoxin-suppressed PKM2 promoted HIF-1α transactivation through chromatin modifications.

Oral digoxin significantly reduced high-fat diet-induced hepatic damage, steatosis, and liver inflammation across a wide dosage range [27]. The lowest dose of digoxin (0.125 mg/kg) showed significant protective effects against liver injury and sterile inflammation. Interestingly, digoxin had direct effects on the inhibition of inflammasome activation. Digoxin had a small effect on typical inflammasome activity while strongly inhibiting HIF-1α pathway sustained inflammasome activity in macrophages. Despite the importance of PKM2-HIF1α pathway activation in immune cells during NASH development [27], its direct effect on hepatocytes was unclear. PKM2 Levels in healthy human liver cells were very low, but in NAFLD and NASH, it was significantly elevated, whereas pyruvate kinase L/R (**PKLR**), the major isoform of pyruvate kinase in the liver, was unchanged. Digoxin treatment directly inhibited

PKM2 transactivation leading to the improvement of hepatocyte mitochondrial dysfunction, steatosis, and hepatocellular injury in the obese mouse model (Figure 1).

Nuclear receptors (**NRs**) are ligand-activated transcription factors that are involved in a wide array of physiological processes. These transcription factors typically have different domains responsible for ligand-independent interactions with corepressors and coactivators, recognition and binding of response elements within target genes, interaction with other proteins or facilitation of protein translocation, as well as ligand-dependent functions [31-37]. The involvement of NRs in regulation of a variety of metabolic and physiological processes makes them interesting pharmacological targets. The retinoic acid-related orphan receptor C (**RORC**) gene is a nuclear receptor with two protein products, the RORγ and RORγT isoforms which differ by 21 amino acids in their N-terminal A/B domains. The RORγ isoform is broadly expressed [38] and is involved in regulation of genes in the circadian cycle and metabolism [37, 39, 40]. The RORγT isoform is expressed exclusively in Th17 cells and regulates expression of interleukins IL17A and IL17F [41, 42] involved in autoimmune disease [43-45]. Pivotal evidence for digoxin involvement in regulation of RORγT activity was provided by Huh *et al* in 2011 when they showed that digoxin inhibits the transcriptional activity of RORγT [46]. Inhibition of RORγT by digoxin or its non-toxic derivatives selectively inhibits Th17 differentiation, delaying the onset and severity of autoimmune reactions in murine models [46]. More recently, Karas *et al* reported opposing findings with cardiac glycosides activating RORγT in HepG2 cells [47, 48] when these compounds are used at much lower doses than originally used Huh *et al* Thus, it appears that digoxin-mediated inhibition *vs* activation of RORγT is dependent on the dose utilized [49].

RORγT directly regulates glucose-6 phosphatase (G6Pase) and a number of genes involved in glucose regulation and insulin sensitivity. G6Pase facilitates glucose-6 phosphate (G6P) hydrolysis into inorganic phosphate and free glucose [50-52], with suppression of hepatic G6Pase resulting in accumulation of G6P and metabolic reprogramming involving increased carbohydrate response element binding protein

(**ChREBP**) activity and gene expression that lead to hepatic steatosis [53-56]. Digoxin-mediated activation of RORγT upregulates G6Pase, resulting in improved glucose homeostasis and decreased NAFLD phenotype.

In many respects, the pathophysiological changes seen in alcohol-associated steatohepatitis (**ASH**) are similar to those seen in NASH, including increased oxidative stress and sterile inflammation manifested as steatohepatitis [57]. The ability of digoxin to improve ASH was tested in a well-accepted Lieber-Decarli ethanol liquid diet (5% ethanol) plus a single ethanol binge mouse model during a chronic feeding [58]. Digoxin (0.2-1.0 mg/kg) dose-dependently improved hepatic steatosis, neutrophil infiltration, and hepatocellular damage in ASH. The presence of the digoxin target pathway was confirmed in human liver tissues, which showed a greater degree of up-regulation of HIF-1α and HIF-1α dependent genes in severe, as compared to early ASH patients. It was concluded that long-term treatment with digoxin reduced chronic liver damage, inflammation, and steatosis in experimental models of NASH and ASH without affecting cardiac chronotropic and inotropy. Digoxin is notable for producing cardiotoxicity at concentrations that are close to its effective concentration. Remarkably, however, digoxin did not have any cardiac or other toxicity at lower doses. These studies identified an entirely novel application of this old drug at doses significantly below the dose required for the cardiac effect. Digoxin showed the potential to therapeutically inhibit liver injury in both ASH and NASH through the regulation of PKM2-HIF-1α pathway activation with the involvement of multiple cell types. Because of the large clinical experience with oral digoxin, this may have significant clinical applicability in human ASH and NASH. Digoxin is currently being investigated in a phase II pilot study in patients with ASH (NCT05014087) (Table 1).

Digoxin in obesity and metabolic disorders

Overnutrition, inadequate physical activity, genetic and epigenetic factors, and other risk factors can predispose individuals to metabolic syndrome [59] with associated comorbidities [60]. Inhibition of RORγT-mediated interleukin (IL)-17A production by

digoxin abolishes the IL-17A axis [46], suppressing diet-induced obesity and leading to increased brown adipose tissue (**BAT**) [61]. BAT is an essential site for thermogenesis and critical for maintaining body temperature regulated by mitochondria uncoupling protein-1 (**UCP-1**) [61]. The metabolic effects observed with digoxin can also be achieved by the ubiquitous deletion of IL-17 receptor A (Il17ra). Modulation of IL-17A signaling may thus serve as a strategy to inhibit obesity and related complications [59].

Metabolic disorders, including obesity, liver steatosis, and aging, may be improved by caloric restriction or starvation, which activates the transcription factor EB (**TFEB**) that regulates lipid metabolism and the biogenesis of lysosomes. Agents that activate TFEB can confer metabolic changes resembling starvation and thus have utility in the treatment of these metabolic disorders. Recently through a nanotechnology-enabled high-throughput screening of various small molecules, digoxin was one of three small molecules identified that activate TFEB [62]. This activation occurs through distinct Calcium (Ca2+)-dependent mechanisms and also by promoting autophagolysosomal activity, an adaptive catabolic process that generates nutrients and energy during starvation [62]. Ca+ is stored in cells in 3 different compartments, including Lysosomes, mitochondria, and endoplasmic reticulum (ER) [63], and TFEB activators can differentially affect calcium stores in these compartments. Digoxin induces lysosomal calcium release through mucolipin 1 (**MCOLN1**), leading to activation of TFEB with resultant anti-obesity effects [59].

Cardiac Glycosides (**CGs**) also appear to hold promise for heritable metabolic disorders. Familial hypercholesterolemia, characterized by elevated serum low-density lipoprotein-cholesterol (**LDL-C**), is a genetic disorder caused primarily by mutations in the low-density lipoprotein receptor (**LDLR**). Patients with compound heterozygous or homozygous mutations in LDLR have LDL-C levels >500 mg/dL, leading to the formation of xanthomas, severe cardiovascular disease, and early death [64]. In a recent study, hepatocyte-like cells derived from induced pluripotent stem cells (**iPSC**) from patients with homozygous familial hypercholesterolemia were used to screen for a drug in order to identify potential therapeutics [65]. CGs reduced apoB, the crucial protein

component of very-low-density lipoprotein (**VLDL**) and LDL particles, in human hepatocytes as well as in the serum of mice with humanized livers. The mechanism through which CG-mediated reduction of apoB and improvement of hypercholesterolemia occurred did not appear to involve the expression of APOB gene or the synthesis of apoB protein but rather the enhancement of proteolytic turnover of the apoB protein [65].

Digoxin in autoimmune and inflammatory conditions

T helper 17 (**Th17**) cells are an independent subset of T helper cells that produce IL-17 and are involved induction of inflammation and autoimmune disease. These cells have a unique transcription factor, RORγT [41], and are activated by IL-6 and transforming growth factor-beta 1 (**TGF-β1**). Because TH17 cells are inducers of inflammation and autoimmune disease, specific targeting of these cells can reduce inflammation. Digoxin down-regulates Th17 differentiation through suppression of ROR-γt transcriptional activity without effect on the differentiation of T cell lineages [66].

Th17 and T1 play a crucial role in rheumatoid arthritis (**RA**), a systemic autoimmune inflammatory disorder characterized by hyperplasia of the synovial membrane along with persistent inflammation of joints. In one study assessing the effect of digoxin on the peripheral blood mononuclear cells of 30 RA patients and ten healthy controls, there was a significant reduction in the population of Th17 cells through suppression of the transcription factor ROR-γt and a decrease in the levels of IL-1β, IL-6, IL-17, and IL-23 cytokines [67]. Digoxin treatment did not modify the expression of TGF-β1 and interferon-gamma (**IFN-γ**) at the level of messenger ribonucleic acid (**mRNA**) and protein. Psoriasis is another chronic inflammatory disease involving IL-17-producing Th17 cells [68]. The toll-like receptor 7 agonist imiquimod creates psoriasis-like lesions on mice's ear or back skin through an IL-17-dependent mechanism. Intraperitoneal digoxin differentially affects these skin lesions, reducing those on the ear and exacerbating those on the back [68]. This differential effect

of digoxin may relate to differences in target tissues, the imiquimod application dose, and digoxin bioavailability in different sites.

Digoxin might also be effective for managing pain [69]. Digoxin is a potent inhibitor of soluble epoxide hydrolase (**sEH**) enzyme, which breaks down endogenous lipid mediators like epoxyeicosatrienoic acids (**EETs**) that are known to have cardiovascular effects including vasodilation, anti-migratory actions on vascular smooth muscle cells, and anti-inflammatory actions [70]. Digoxin has antipyretic activity in rats and inhibits neutrophil infiltration and alveolar septal thickening in lung tissue [69]. Administration of digoxin at a low dose can reduce pain and allodynia and decrease edema and abdominal contraction [69].

Digoxin in cancer

In a study investigating potential new drugs for prostate cancer, digoxin was found to be highly potent in inhibiting prostate cancer cell growth *in vitro* [71]. Regular digoxin use, especially over ten years, was found to be associated with a 25% lower risk of prostate cancer [71]. Although the mechanism of action for reduced prostate cancer risk is unclear, one mechanism appears to be an increased influx of intracellular calcium into prostate cancer cells triggering apoptosis through the cyclin-dependent kinase 5 (**Cdk5**)/p25 pathway. Activated Src/mitogen-activated protein kinase (**MAPK**) signaling results in inhibition of p53 synthesis, suggesting that cardiac glycosides (**CGs**) may have utility in the treatment of cancers with a gain of function P53 mutations [72]. Other mechanisms proposed for the anticancer effects of digoxin include inhibition of Na^+/K^+-ATPase and topoisomerase [73], alterations of Ca^{2+} signaling [74], and inhibition of hypoxia-inducible factor 1 alpha (**HIF-1α**) synthesis [26]. The DIG-HIF-1 pharmacodynamic trial, which sought to test whether digoxin can reduce the expression of HIF-1α protein in surgically resected breast cancer tissue, was terminated early due to difficulty with accrual (NCT01763931). It is our hope that there will be subsequent studies that will shed light on this important question.

When given together with the anti-neoplastic drug Adriamycin, digoxin enhanced anti-cancer effects *in vitro* on non-small cell lung cancer by inhibiting both DNA double-strand break (**DSB**) and single-strand break (**SSB**) repair and reduced the cardiotoxicity of Adriamycin [72]. Co-treatment with digoxin blocked the Adriamycin-induced reduction in cardiomyocyte size, suggesting that digoxin can ameliorate the reduction of heart weight/body weight ratio by Adriamycin.

Digoxin suppresses lung cancer progression by inhibiting Src activation and related pathways [75]. In digoxin-treated cells, the phosphorylation of Src and its related proteins was inhibited, leading to the inhibition of lung cancer cell proliferation, migration, and invasion through inhibition of phosphatidylinositol 3-kinase (**PI3K**), focal adhesion kinase (**FAK**), stress-activated protein kinases (**SAPK**)/Jun amino-terminal kinases (**JNK**), paxillin, and p130Cas activities. Digoxin also reduces messenger Ribonucleic acid (**mRNA**) expression and the quantity of Src and related protein kinases [75]. Digoxin was also found to have effects on glioblastoma, a highly aggressive and lethal brain tumor, by enhancing apoptosis and reducing the levels of the anti-apoptotic protein through its proteasomal degradation [76].

A screen of 200,000 small molecules for inhibitory effect against primary human melanoma cells showed that several CGs, including digoxin, showed toxicity against melanoma cells *vs* normal human melanocytes and umbilical cord blood cells [77]. This effect involves inhibition of the ATP1A1 Na+/K+ pump that is crucial for the maintenance of ion gradients across the plasma membrane for substrate transport. Although CGs alone were insufficient to cause regression in patient-derived xenografts, they showed synergistic effects with inhibitors of MAPK pathway to mediate regression in both BRAF wild-type and BRAF mutant melanomas [reference 44 Eskiocak]. Polarization of CD4+ T cells into Th17 subtypes in a transgenic mouse model resulted in destruction of advanced B16 murine melanoma through interferon-gamma (IFN-gamma) dependent mechanisms [78]. In a recent phase 1B clinical trial of digoxin and trametinib, a MAP kinase kinase (**MEK**) inhibitor, in Stage IV BRAF wild-type metastatic melanoma patients who were refractory or intolerant to immune checkpoint

blockade, thirteen 13 out of 20 patients (65%) achieved disease control (NCT28278423)[79]. The results of this early study are encouraging and need to be expanded.

Digoxin is currently being studied in a phase 1B combination drug trial in pancreatic cancer and other advanced solid tumors (NCT03889795) (Table 1). It is also being studied for feasibility and safety when combined with Folinic acid, 5-Fluorouracil, Irinotecan, and Oxaliplatin in patients with resectable pancreatic cancer (NCT04141995).

Digoxin in viral infection

Digoxin inhibits coronavirus and other viruses [80]. It inhibits the cytokine storm generated by SARS-CoV-2 infection and blocks viral cell penetration and infectivity [81]. After single-dose digoxin treatment, SARS-CoV-2 titers were the same as achieved with treatment by remdesivir, resulting in >99% inhibition compared to controls or patients on chloroquine at 48 h post-infection [81]. In some cases, digoxin-mediated suppression of viral mRNA expression (99%) was more effective than remdesivir (>60%) and chloroquine (>30%) [82]. Host cell entry by Middle East respiratory syndrome (MERS-CoV) and SARS-CoV is inhibited through the silencing or inhibition of the Na, K-ATPase α1-subunit by low doses of CG. This disruption of cell entry occurs at an early stage by interfering with endocytosis through a non-elucidated pathway [80, 83]. In the post-entry stage, digoxin significantly inhibits replication at half maximal inhibitory concentration (IC_{50}) of 0.043 nM [33004837] and viral protein expression [81].

In rat models infected with influenza virus, administration of digoxin analog digitoxin suppressed cytokine levels, including tumor necrosis factor-alpha (TNFα), growth-regulated oncogene/ keratinocyte chemoattractant (**GRO/KC**), macrophage inflammatory protein 2 (**MIP2**), monocyte chemoattractant protein-1 (**MCP1**), and interferon-gamma (**IFNγ**) in the rat lung [84]. The inhibition of Na-K-ATPase by CGs decreases intracellular potassium, inhibiting the host cell translational machinery and decreasing influenza virus replication [80].

Digoxin and other CGs also inhibit replication of cytomegalovirus (**CMV**), a herpesvirus pathologic agent of important human diseases, at nanomolar concentrations, with an additive effect when combined with antiviral drugs for CMV such as ganciclovir [80]. CGs reduced the levels of viral proteins and cellular NF-KB, with the activity of CGs correlating with the expression of hERG, a potassium channel gene [85].

Human papillomaviruses (**HPVs**) rely on potassium ion influx for replication [86]. Cutaneous warts (including plantar warts or common warts) are typically caused by HPVs 1, 2, 27, and 57 [87, 88], while genital warts are typically caused by HPVs 6 and 11. CGs such as digoxin and the loop diuretic furosemide interact with the cell- membrane ion cotransporters Na^+/K^+- ATPase and Na- K- Cl and inhibit potassium flux thus inhibiting HPV replication [86]. The inhibitory effect on DNA replication appears most potent when digoxin and furosemide are combined; the term ionic contraviral therapy (**ICVT**) describes the topical application of these drugs in combination. A phase 1/2 open- label study of ICVT was safe and efficacious in 12 healthy patients with common warts [89]. A follow-up randomized, double- blind, placebo- controlled phase 2A proof- of- concept study assessed the efficacy, safety, and tolerability of ICVT in adults with cutaneous warts. Eighty adult patients were randomized to digoxin or furosemide alone, ICVT or placebo (NCT02333643) [87]. Reduction in HPV load and wart size was achieved in all active treatment groups but not in placebo, with a statistically significant reduction in wart diameter in those treated with ICVT *vs* placebo. On the contrary, a phase 2 study of ICVT for HPV-related genital lesions was terminated early due to a lack of effect on interim analysis (NCT03334240). Overall, digoxin appears promising for the treatment of HPV-induced lesions, especially the cutaneous subtype, and warrants further investigation in large multicenter studies.

A cell-based screen performed on cells transfected with proviral DNA constructs uncovered a number of compounds that inhibit HIV-1 virion production, including numerous CGs [90]. Digoxin selectively impaired HIV-1 replication at two levels: (1) through global alterations in the efficiency of HIV-1 RNA processing and (2) by

blocking the export of incompletely spliced viral RNAs to the cytoplasm [91]. The cardenolides and the bufadienolides, both subclasses of CGs, inhibited the late stages of the HIV-1 replication cycle. Although both are C(23) steroids, they differ in that cardenolides contain a butanolide five-membered lactone ring at C-17, whereas bufadienolides contain a six-membered lactone ring. Members of both classes of CGs inhibited late stages of HIV-1 production, and changes in structure resulted in changes in inhibition. Digoxin (and potentially the CG family of drugs) represents a novel class of HIV-1 inhibitors with the potential for rapid development into antiretroviral therapies. The dose-limiting toxicities observed with CGs in humans are related to toxic increases in cardiac contractility driven by increases in intracellular Ca^{2+}. As the mechanism of CGs inhibition of HIV-1 appears to be independent of such Ca^{2+} increases, it is possible that structural modification of the CGs could avoid cardiac toxicity while maintaining HIV-1 inhibition.

Digoxin in non-cardiac genetic disorders

CGs or their derivatives, including digoxin, also appear promising for treating certain genetic diseases, such as cystic fibrosis and Duchenne's muscular dystrophy, wherein truncated protein products encoded by the corresponding nonsense mRNAs are fully or partially functional [92, 93]. The nonsense-mediated mRNA decay (**NMD**) pathway selectively eliminates aberrant transcripts containing premature translation termination codons and regulates the levels of a number of physiological mRNAs. NMD modulates the clinical outcome of a variety of human diseases, including cancer and several genetic disorders. Using a dual-color bioluminescence-based NMD reporter system, Nickless A *et al* performed a high-throughput screen to identify drug candidates that can alter NMD activity in human cells [94]. The effects of seven of the inhibitor hits were found, and each validated compound inhibited NMD in a dose-dependent manner. Notably, the top five verified hits, including digitoxin, digoxin, lanatoside C, proscillaridin, and ouabain, are all CGs [95]. It should be noted that the concentrations of CGs used in this study to achieve more complete NMD inhibition without causing

significant cellular toxicity (for example, 500 nM for digoxin and 175 nM for ouabain) are much higher than standard clinical doses used for the treatment of cardiac failure. Thus, acute use of these drugs at the experimental working concentrations cannot directly translate to the clinic owing to *in vivo* toxic effects. However, the benefits of partial NMD inhibition with chronic treatment at clinically relevant doses may potentially be efficacious, but this will require further clinical pharmacology studies.

CONCLUSION

Conclusion and future directions

Until now, most of our knowledge and experience with digoxin pertains to its use in the cardiac field. However, in the past decade, digoxin has emerged as a potential pharmacologic agent in the management of several conditions, including steatohepatitis in the context of non-alcohol and alcohol-associated fatty liver disease, obesity and other metabolic disorders, autoimmune conditions, malignancy, and viral infection, among others. Clinical trials on the repurposing of digoxin for therapeutic use in a variety of non-cardiac conditions are still in their early stages but appear promising.

At relatively high concentrations (hundreds of nM), digoxin and other cardiac glycosides inhibit the Na-K ATPase pump, leading to accumulation of sodium ions in the cytosol that drives an influx of calcium into the heart, increasing contractility [96]. At lower doses (picomolar to low nanomolar), digoxin induces the Na-K ATPase to act as a receptor that can modulate a variety of pathways [5, 96], including Src/ MAPK pathway which regulates a number of downstream signaling pathways. Also at high doses, digoxin binding to the ligand-binding domain of the nuclear receptor RORγT inhibits its transcriptional activity, leading to inhibition of Th17 activity and IL-17 release [59] and suppressing NF-kB activity [85], altogether reducing inflammatory response. At lower doses, digoxin activates RORγT signaling, leading to induction of several Th17-specific genes, suggesting a potential role of digoxin in adoptive cell therapy [14, 47, 48].

Several questions remain to be clarified in the quest towards repurposing of digoxin, including the structure-activity relationships that direct its molecular targeting

in specific disease settings; whether dosing/concentration alone determine its activity as an inhibitor *vs* activator or whether other factors affect its action; and the ideal potency that can be utilized for pharmacologic intervention in a particular tissue while optimizing its safety profile. Indeed, digoxin's falling out of favor in the cardiac arena is largely attributable to its narrow therapeutic index and potential toxicity, thus it is very exciting that recent studies show potent biological activity of much smaller doses of digoxin than used historically in the clinical setting. Digoxin is commercially available as a relatively cheap generic drug, thus further elucidation of its biological effects and mechanisms of action especially at low non-toxic doses will facilitate its rapid therapeutic repurposing.

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