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New uses for an old remedy: Digoxin as a potential treatment for 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, antineoplastic, metabolic, and antimicrobial 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 infections, among other conditions. In this review, we provide a summary of the clinical uses of digoxin to date and discuss recent research on its emerging applications.

Key Words: Digoxin; Cardiac glycosides; 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. Preclinical work supports the repurposing of digoxin as therapy for 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 on the differential action of digoxin at high vs low concentrations and identify areas of further research necessary to harness its promising multifunctional use.

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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, Withering[2], 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, Withering [2] 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[2]. 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].

The molecular formula of digoxin is $C_{41}H_{64}O_{14}$, and its molecular weight is 780.9 g/mol. Similar to other cardiac glycosides (CG), 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[3-5]. 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 medication regimen in patients with left ventricular systolic dysfunction when symptoms persist despite optimization of treatment with an angiotensin-converting enzyme inhibitor, a β -blocker, and/or a diuretic[7-9]. The digoxin effect in treatment of supraventricular arrhythmia occurs through its parasympathomimetic stimulation *via* the vagus nerve, reducing 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, 0.1875 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 2.0 ng/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 of digoxin necessitates monitoring of serum digoxin levels, particularly in patients with chronic renal dysfunction or varying 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, the use of digoxin in cardiac disease has been waning. During this period, however, there have been several

advances in basic and preclinical work toward the potential repurposing of digoxin and other CGs for non-cardiac conditions. These studies indicate that the biological effects of CG are not limited to the inhibition of Na, K-ATPase but include various signal transduction pathways including nuclear receptors (NRs) involved in hormonal signaling, immune response, and 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 nonalcoholic steatohepatitis (NASH). The amplitude of sterile inflammation triggered by metabolic stress in the liver has major clinical consequences. Sterile inflammation is responsible for increasing amounts of liver damage and cell death in NASH[24]. NASH, as well as other diseases associated with sterile inflammation of the liver, lacks effective treatments. This is due to 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- α (HIF-1 α) pathway activation in macrophages for sustained inflammatory responses provided HIF-1 α 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-1 α pathway in sterile inflammation may have great clinical relevance due to the ability of digoxin to inhibit HIF-1 α activation[26]. Digoxin (1.0-0.05 mg/kg) effectively prevents acute and chronic hepatic damage, steatosis, and inflammation in both lipopolysaccharide- and high-fat diet-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-1 α transcriptional activity, thus disrupting HIF-1 α -mediated antioxidant pathways. Digoxin induced significant changes in gene transcripts related to HIF-1 α in metabolic processes and nucleic acid binding[27]. To understand the direct molecular mechanisms responsible for the digoxin effect on HIF-1 α 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 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 and adenosine diphosphate to pyruvate and ATP[29].

In addition to its pyruvate kinase function, PKM2 interacts with HIF-1 α in the nucleus and functions as a transcriptional coactivator for HIF-1 α , resulting in the stimulation of HIF-1 α responsive genes[30]. Interestingly, the interaction of digoxin with 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-1 α and its downstream genes, such as inflammatory genes and genes involved in oxidative stress (Figure 1). Further, digoxin reduced the binding of PKM2 to histones, suggesting that digoxin suppressed PKM2-mediated transactivation of HIF-1 α through chromatin modifications[27].

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 the HIF-1 α pathway-sustained inflammasome activity in macrophages. Despite the importance of PKM2-HIF1 α 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 they were significantly elevated in NAFLD and NASH. Pyruvate kinase L/R, 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 (Table 1).

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 the regulation of a variety of metabolic and physiological processes makes them interesting pharmacological targets.

The NR gene retinoic acid-related orphan receptor C gene encodes two protein products, the retinoid-related orphan receptor-gamma (ROR γ) and ROR γ T isoforms, which differ by 21 amino acids in their N-terminal A/B domains. The ROR γ isoform is broadly expressed[38] and is involved in the regulation of genes in the circadian cycle and metabolism[37,39,40]. The ROR γ T isoform is expressed exclusively in T helper 17 (Th17) cells and regulates expression of interleukins (IL)-17A and IL-17F[41,42] involved in autoimmune disease[43-45]. Pivotal evidence for digoxin involvement in the regulation of ROR γ T

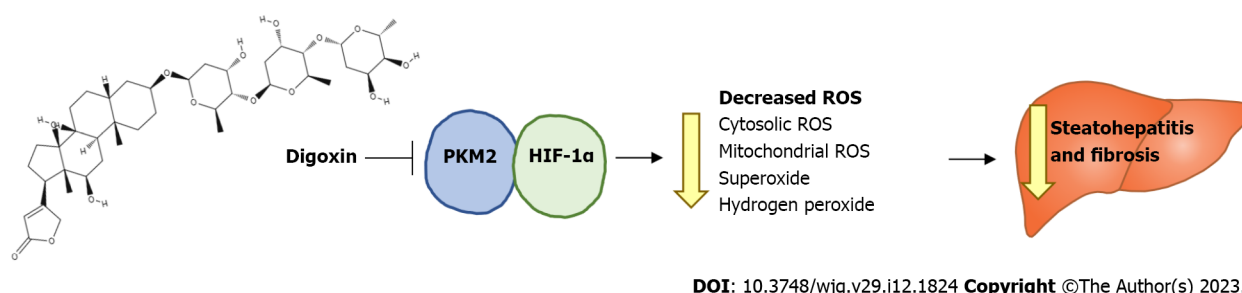
Table 1 Summary of the main findings from key original articles investigating non-cardiac applications of digoxin and other cardiac glycosides

Manuscript title	Publication year	Main biomedical/molecular findings	Main histological findings	Ref.
Cardiac glycosides inhibit p53 synthesis by a mechanism relieved by Src or MAPK inhibition	2009	Activation of Src/MAPK signaling pathways, resulting in reduction of p53 protein synthesis	NA	[72]
Human cytomegalovirus inhibition by cardiac glycosides: Evidence for involvement of the <i>HERG</i> gene	2012	CG reduced expression of the potassium channel gene, <i>hERG</i> , and reduced NF- κ B levels	NA	[85]
Digoxin Suppresses HIV-1 Replication by Altering Viral RNA Processing	2013	Reduction in HIV-1 viral mRNAs encoding structural proteins, with reduced synthesis of HIV-1 structural protein; altered viral RNA splice site use leading to loss of essential viral factor Rev; changed activity of CLK family of SR protein kinases and modification of SR proteins	NA	[91]
A novel cell-based high-throughput screen for inhibitors of <i>HIV-1</i> gene expression and budding identifies the cardiac glycosides	2014	Na-K ATPase- dependent but intracellular Ca ²⁺ -independent inhibition of <i>HIV-1</i> gene expression at the post-integration stage of the viral life cycle	NA	[90]
Digoxin Suppresses Tumor Malignancy through Inhibiting Multiple Src-Related Signaling Pathways in Non-Small Cell Lung Cancer	2015	Inhibition of proliferation, invasion, migration, and colony formation of A549 lung cancer cells; suppression of Src and related protein activity; reduced EGFR and STAT3 activity	NA	[75]
Synergistic effects of ion transporter and MAP kinase pathway inhibitors in melanoma	2016	Inhibition of the ATP1A1 Na ⁺ /K ⁺ pump, which is highly expressed in melanoma, resulting in selective toxicity to melanoma cells. Digoxin was also additive or synergistic with MEK inhibitor and/or BRAF inhibitor to induce cell death in melanoma cells; increased intracellular acidification, mitochondrial calcium dysregulation, and ATP depletion in melanoma cells	NA	[79]
Small-molecule TFEB pathway agonists that ameliorate metabolic syndrome in mice and extend <i>C. elegans</i> lifespan	2017	Activated TFEB, conferred hepatoprotection against diet-induced steatosis in mice, and extended lifespan of <i>Caenorhabditis elegans</i>	Amelioration of high-fat diet-induced steatosis, reversal of hepatocyte p62/SQSTM1 accumulation, suggesting enhanced autophagic flux	[62]
Targeting Intracellular Ion Homeostasis for the Control of Respiratory Syncytial Virus	2018	Findings suggested digoxin-mediated inhibition of RSV transcription and/or replication, likely dependent on changes in intracellular Na ⁺ and K ⁺	NA	[82]
Digoxin Suppresses PKM2 Promoted HIF-1 α Transactivation in Steatohepatitis	2018	Binding of PKM2 by digoxin downregulated HIF-1 α transactivation to decrease sterile inflammation in the liver. Digoxin suppressed ROS production both <i>in vivo</i> and <i>in vitro</i> from hepatocytes and immune cells	Reduction in hepatic damage, steatosis, and inflammation induced by endotoxin, high fat diet, or alcohol	[27]
Digoxin improves steatohepatitis with differential involvement of liver cell subsets in mice through inhibition of PKM2 transactivation	2019	Digoxin downregulated PKM2-PKM2-HIF-1 α axis and attenuated inflammasome activity in macrophages and hepatic oxidative stress response	Reduction of high fat diet-induced hepatic damage, steatosis, and liver inflammation	[28]
Antiviral activity of digoxin and ouabain against SARS-CoV-2 infection and its implication for COVID-19	2020	Inhibition of viral mRNA expression, copy number, and viral protein expression at the post entry stage of the viral life cycle	NA	[81]
Classical Drug Digitoxin Inhibits Influenza Cytokine Storm, With Implications for COVID-19 therapy	2020	Suppression of levels of the cytokines TNF- α , GRO/KC, MIP2, MCP1, and IFN- γ during cytokine storm	No difference in density of immune cells in rat lung sections, comparing digitoxin-treated and control lungs	[84]
Inhibition of the IL-17A axis in adipocytes suppresses diet-induced obesity and metabolic	2021	Digoxin inhibition of ROR γ T activity suppressed the IL-17A axis, thus preventing diet-induced obesity, metabolic alterations, and liver injury	Prevention of high fat diet-induced hepatic lipid accumulation, reduced	[59]

disorders in mice

fibrosis, increased browning
of adipose tissue

TFEB: Transcription factor EB; PKM2: Pyruvate kinase M2; IL-17A: Interleukin-17A; MAPK: Mitogen-activated protein kinase; CG: Cardiac glycoside; NF- κ B: Nuclear factor-kappaB; CLK: Cdc2-like kinases; SR: Serine-arginine; EGFR: Epidermal growth factor receptor; MEK: MAP kinase kinase; RSV: Respiratory syncytial virus; HIF-1 α : Hypoxia-inducible factor 1-alpha; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; TNF- α : Tumor necrosis factor-alpha; GRO/KC: Growth-regulated oncogene/keratinocyte chemoattractant; MIP2: Macrophage inflammatory protein 2; MCP1: Monocyte chemoattractant protein-1; IFN γ : Interferon-gamma; ROR γ T: Retinoid-related orphan receptor-gamma; NA: No application; ROS: Reactive oxygen species; STAT3: Signal transducer and activator of transcription 3.



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Figure 1 Digoxin reduces steatohepatitis by suppressing pyruvate kinase M2 dependent hypoxia-inducible factor 1-alpha activity and inhibiting reactive oxygen species production[27,28]. Digoxin structure derived from MolView. HIF-1 α : Hypoxia-inducible factor 1-alpha; PKM2: Pyruvate kinase M2; ROS: Reactive oxygen species.

activity was provided by Huh *et al*[46] in 2011 when they showed that digoxin inhibits the transcriptional activity of ROR γ T[46]. Inhibition of ROR γ T 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, Karaś *et al*[47,48] reported opposing findings with CGs activating ROR γ in HepG2 cells and ROR γ T in Th17 lymphocytes[47,48] when these compounds were used at much lower doses than originally used by Huh *et al*[46]. Thus, it appears that digoxin-mediated inhibition *vs* activation of ROR γ T may be dependent of the dose utilized[49].

ROR γ directly regulates glucose-6 phosphatase (G6Pase) and a number of genes involved in glucose regulation and insulin sensitivity. G6Pase facilitates glucose-6 phosphate hydrolysis into inorganic phosphate and free glucose[50-52], with suppression of hepatic G6Pase resulting in accumulation of glucose-6 phosphate and metabolic reprogramming involving increased carbohydrate response element binding protein activity and gene expression that lead to hepatic steatosis[53-56]. Digoxin-mediated activation of ROR γ 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 chronic feeding [58]. Digoxin (0.2-1.0 mg/kg) dose-dependently improved hepatic steatosis, neutrophil infiltration, and hepatocellular damage in ASH. The effect of digoxin was confirmed in human liver tissues, which showed a greater degree of upregulation of HIF-1 α and HIF-1 α -dependent genes in severe ASH compared to mild disease. 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-1 α 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 2).

DIGOXIN IN OBESITY AND METABOLIC DISORDERS

Overnutrition, inadequate physical activity, genetic and epigenetic factors, and other risk factors can

Table 2 Ongoing clinical trials of digoxin in non-cardiac diseases

Study title	Medication doses	Current status	Estimated study completion date	Clinical Trials.gov number	Number of participants	Country of trial
Phase 1						
Inhibition of Sterile Inflammation by Digoxin	Digoxin 3.00 mcg/Kg/day <i>vs</i> Digoxin 0.15 mcg/Kg/day <i>vs</i> placebo	Recruiting	July 2023	NCT03559868	45	United States
Phase IB Trial of Metformin, Digoxin, Simvastatin in Subjects With Advanced Pancreatic Cancer and Other Advanced Solid Tumors	Metformin 850 mg po/day, Simvastatin 5 mg po/day, Digoxin 0.0625 mg po/day <i>vs</i> Metformin 850 mg po/day then 1700 mg po/day, Simvastatin 20 mg po/day, Digoxin 0.25 mg po/day <i>vs</i> Metformin 850 mg po/day then 1700 mg po/day Simvastatin 40 mg po/day, Digoxin 0.25 mg po/day then 0.375 mg po/day	Recruiting	December 2023	NCT03889795	15	United States
Effect of Digoxin on Clusters of Circulating Tumor Cells in Breast Cancer Patients	Digoxin 0.125 mg or 0.250 mg digoxin based on renal function and target serum digoxin concentration	Recruiting	June 2022	NCT03928210	9	Switzerland
Phase 2						
Digoxin In Treatment of Alcohol Associated Hepatitis	Digoxin titration to goal 0.5 and 1.1 ng/mL <i>vs</i> no digoxin	Recruiting	August 2024	NCT05014087	60	United States
Evaluating the Effect of Digoxin and Ursodeoxycholic Acid in Patients With Rheumatoid Arthritis	Digoxin 0.25 mg + DMARDS <i>vs</i> UCDA 500 mg + DMARDS <i>vs</i> placebo + DMARDS	Recruiting	July 2022	NCT04834557	90	Egypt
FOLFIRINOX With Digoxin in Patients With Resectable Pancreatic Cancer	FOLFIRINOX + digoxin 0.125 or 0.250 mg for target digoxin level 0.8 to 1.2 ng/mL	Recruiting	February 2025	NCT04141995	20	United States
Topical Ionic Contra-Viral Therapy in Actinic Keratosis	Digoxin topical gel 0.125% <i>vs</i> furosemide topical gel 0.125% <i>vs</i> digoxin and furosemide gel 0.125% <i>vs</i> vehicle gel	Unknown	September 2019	NCT03684772	32	Netherlands
Phase II Multicentric Study of Digoxin Per os in Classic or Endemic Kaposi's Sarcoma (KADIG 01)	Digoxin goal 0.6 to 1.2 ng/mL for age < 75 yr; Digoxin goal 0.5-0.8 ng/mL for age > 75 yr	Unknown	September 2019	NCT02212639	17	France

DMARDS: Disease-modifying antirheumatic drug; FOLFIRINOX: FOLinic acid, 5-Fluorouracil, IRINotecan and Oxaliplatin; UCDA: Ursodeoxycholic acid.

predispose individuals to metabolic syndrome[59] with associated comorbidities[60]. Inhibition of ROR γ T-mediated IL-17A production by digoxin abolishes the IL-17A axis[46], suppressing diet-induced obesity and leading to increased brown adipose tissue[61]. Brown adipose tissue is an essential site for thermogenesis and critical for maintaining body temperature regulated by mitochondria uncoupling protein-1[61]. The metabolic effects observed with digoxin can also be achieved by the ubiquitous deletion of IL-17 receptor A. 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-dependent mechanisms and by promoting autophagolysosomal activity, an adaptive catabolic process that generates nutrients and energy during starvation[62]. Calcium is stored in cells in three different compartments, including lysosomes, mitochondria, and the endoplasmic reticulum[63], and TFEB activators can differentially affect calcium stores in these compartments. Digoxin induces lysosomal calcium release through mucolipin 1, leading to activation of TFEB with resultant anti-obesity effects[59].

CGs also appear to hold promise for heritable metabolic disorders. Familial hypercholesterolemia, characterized by elevated serum low-density lipoprotein-cholesterol, is a genetic disorder caused primarily by mutations in the low-density lipoprotein receptor. Patients with compound heterozygous

or homozygous mutations in the low-density lipoprotein receptor have low-density lipoprotein-cholesterol levels > 500 mg/dL, leading to the formation of xanthomas, severe cardiovascular disease, and early death[64]. Hepatocyte-like cells derived from induced pluripotent stem cells from patients with homozygous familial hypercholesterolemia have been used to screen for potential pharmacological therapies[65]. CGs reduced apoB, the crucial protein component of very-low-density lipoprotein and low-density lipoprotein 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 the *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

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

Th17 and T1 play a crucial role in rheumatoid arthritis, 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 rheumatoid arthritis patients and 10 healthy controls, there was a significant reduction in the population of Th17 cells through suppression of the transcription factor ROR γ T and a decrease in the levels of IL-1 β , IL-6, IL-17, and IL-23 cytokines[67]. Digoxin treatment did not modify the expression of transforming growth factor-beta 1 and interferon-gamma (IFN- γ) at the level of 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 the ear or back skin of mice 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 enzyme, which breaks down endogenous lipid mediators like epoxyeicosatrienoic acids 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 10 years, was found to be associated with a 25% lower risk of prostate cancer[71]. Although the methods through which digoxin reduced prostate cancer risk are unclear, one potential mechanism involves the increased influx of intracellular calcium into prostate cancer cells triggering apoptosis through the cyclin-dependent kinase 5/p25 pathway. Activated Src/mitogen-activated protein kinase (MAPK) signaling results in inhibition of p53 synthesis, suggesting that CGs may have utility in the treatment of cancers with 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 calcium signaling [74], and inhibition of HIF-1 α synthesis[26]. The DIG-HIF-1 pharmacodynamic trial, which sought to test whether digoxin can reduce the expression of HIF-1 α protein in surgically resected breast cancer tissue, was terminated early due to difficulty with accrual (NCT01763931). We 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 and single-strand break repair and reducing the cardiotoxicity of adriamycin[72]. Cotreatment 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, suppressing lung cancer cell proliferation, migration, and invasion through inhibition of phosphatidylinositol 3-kinase, focal adhesion kinase, stress-activated protein kinases/Jun amino-terminal kinases, paxillin, and

p130Cas activities. Digoxin also reduces mRNA expression 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 200000 small molecules for inhibitory effect against primary human melanoma cells showed that several CGs, including digoxin, demonstrated toxicity against melanoma cells *vs* normal human melanocytes[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 melanoma regression in patient-derived xenografts, they showed synergistic effects with inhibitors of MAPK pathway to mediate regression in both *BRAF* wildtype and *BRAF* mutant melanomas[77]. Polarization of CD4⁺ T cells into the Th17 subtype in a transgenic mouse model resulted in destruction of advanced B16 murine melanoma through IFN- γ dependent mechanisms[78]. A recent phase 1B clinical trial of digoxin and trametinib, a MAP kinase kinase inhibitor, in patients with *BRAF* wildtype metastatic melanoma who were refractory or intolerant to immune checkpoint blockade showed that 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 2). 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 coronaviruses and other viruses[80]. It inhibits the cytokine storm generated by severe acute respiratory syndrome coronavirus 2 (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, with > 99% viral inhibition compared to controls or patients on chloroquine at 48 h post-infection[81]. In other cases, digoxin suppressed viral mRNA expression (99%) more effectively than remdesivir (> 60%) or chloroquine (> 30%)[82]. Host cell entry by Middle East respiratory syndrome and SARS-CoV is inhibited through the silencing or inhibition of the Na, K-ATPase α 1-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 viral mRNA expression, copy number, and viral protein expression at half-maximal inhibitory concentration of 0.043 nM[81].

In rat models infected with influenza virus, administration of digoxin analog digitoxin suppressed cytokine levels, including tumor necrosis factor- α , growth-regulated oncogene/keratinocyte chemoattractant, macrophage inflammatory protein 2, monocyte chemoattractant protein-1, and IFN- γ in the rat lung[84]. The inhibition of Na-K-ATPase by CGs decreased intracellular potassium, inhibiting the host cell translational machinery and decreasing influenza virus replication[80].

Digoxin and other CGs also inhibit replication of cytomegalovirus, a herpesvirus pathologic agent of important human diseases, at nanomolar concentrations, with an additive effect when combined with antiviral drugs for cytomegalovirus such as ganciclovir[80]. CGs reduced the levels of viral proteins and cellular nuclear factor-kappaB, 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 HPV 1, 2, 27, and 57[87,88], while genital warts are typically caused by HPV 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 contra-viral 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 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 HIV-1 inhibitor with the potential for rapid development into antiretroviral therapy. The dose-limiting toxicities observed with CGs in humans are typically related to toxic increases in cardiac contractility driven by increases in intracellular calcium. As the mechanism of CG inhibition of HIV-1 appears to be independent of such calcium 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 *et al*[94] 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

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 nonalcohol 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 CGs 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 the Src/MAPK pathway, which regulates a number of downstream signaling pathways. Also at high doses, digoxin binding to the ligand-binding domain of the NR ROR γ T inhibits its transcriptional activity, leading to inhibition of Th17 activity and IL-17 release[59] and suppressing nuclear factor-kappaB activity[85], altogether reducing the inflammatory response. At lower doses, digoxin activates ROR γ T 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 determines 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, the decline of digoxin 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.

FOOTNOTES

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