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MINIREVIEW

Raloxifene as a treatment option for viral infections

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused corona virus disease 2019 (COVID-19) pandemic and led to mass casualty. Even though much effort has been put into development of vaccine and treatment methods to combat COVID-19, no safe and efficient cure has been discovered. Drug repurposing or drug repositioning which is a process of investigating pre-existing drug candidates for novel applications outside their original medical indication can speed up the drug development process. Raloxifene is a selective estrogen receptor modulator (SERM) that has been approved by FDA in 1997 for treatment and prevention of postmenopausal osteoporosis and cancer. Recently, raloxifene demonstrates efficacy in treating viral infections by Ebola, influenza A, and hepatitis C viruses and shows potential for drug repurposing for the treatment of SARS-CoV-2 infection. This review will provide an overview of raloxifene's mechanism of action as a SERM and present proposed mechanisms of action in treatment of viral infections.

Keywords: raloxifene, drug repositioning, COVID-19, SARS-CoV-2, SERM, estrogen receptor

Introduction

Coronavirus disease 2019 (COVID-19) pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has resulted in mass casualty (Coccolini *et al.*, 2020; Myers *et al.*, 2020). As of November 17, 2020, World Health Organization (WHO) reports 1.3 million SARS-CoV-2-related deaths and 53.7 million confirmed cases worldwide (WHO, 2020). Although the SARS-CoV-2 is mainly known to cause respiratory syndrome and is primarily detected in lungs, recent studies indicate that the virus can also infect and affect multiple other organs including brain, liver, heart, intestine, and kidneys (Gavriatopoulou *et al.*, 2020; Prasad and Prasad, 2020; Zhang *et al.*, 2020). Furthermore, many studies report significantly higher risk of morbidity and mortality in patients with one or more other clinical conditions such as diabetes, cardiovascular disease, and chronic kidney disease and a large variation in clinical manifestations in patients infected by SARS-CoV-2 (Cappuccio and Siani, 2020; Dariya and Nagaraju, 2020; Jordan and Adab, 2020; Ozma *et al.*, 2020). Thus, even though global scientific communities and health organizations have been putting major efforts into the development of therapeutic means and vaccines to treat COVID-19, safe and effective drugs have not been yet developed possibly owing to this highly contagious and complex nature of SARS-CoV-2.

As a strategy to accelerate the discovery of new cures for the COVID-19, drug repurposing or repositioning can be effectively utilized. Drug repurposing or repositioning is a strategy for investigating pre-existing drug candidates for novel applications outside their original medical indications and have also been used in the development of treatment options for COVID-19. For example, remdesivir, a nucleoside analogue inhibitor targeting viral RNA-dependent RNA polymerase, has been first discovered as an antiviral agent against Ebola virus. Recently, it has been tested and found to be effective in the early stage COVID-19 patients and was authorized for a compassionate use in late-stage COVID-19 patients by the U.S. Food and Drug Administration (FDA) (Lo *et al.*, 2020; Pardo *et al.*, 2020). More recently, the use of remdesivir has been expanded and is no longer limited to the treatment of late-stage patients. Moreover, remdesivir can now be given to pediatric patients less than 12 years of age in addition to the previously-approved patients who are 12 years and older and weigh at least 40 kg (Mahase, 2020). Not only drugs that directly target viral replication, but possibly owing to the complicated clinical manifestations of COVID-19 other pre-existing drugs that are effective on other medical indications hold promise as a possible cure for the pandemic. Dexamethasone, which is an FDA-approved glucocorticoid receptor agonist used to treat rheumatoid arthritis and other inflammatory diseases, also demonstrates efficacy in treatment of COVID-19 (Kesharwani *et al.*, 2019; Ledford, 2020). The last application has garnered significant attention because glucocorticoid receptor agonists improve the symptoms of COVID-19 mainly by antagonizing the increase in pro-inflammatory and decrease in anti-inflammatory cytokines, a biological response that leads to the development of severe inflammatory symptoms upon viral infections (Rhen

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and Cidlowski, 2005; Matthay and Wick, 2020; Patel *et al.*, 2020).

Another drug that was not initially discovered as an antiviral agent but demonstrates antiviral effects is raloxifene. Raloxifene is a selective estrogen receptor modulator (SERM) that was first approved by the FDA in 1997 for prevention and treatment of postmenopausal osteoporosis and cancer (Levenson *et al.*, 1998; Lippman and Brown, 1999; Rey *et al.*, 2009). Recently, raloxifene demonstrates a strong efficacy in treating infectious diseases caused by RNA viruses such as Ebola, influenza A, and hepatitis C viruses (Peretz *et al.*, 2016). Understanding and reviewing possible mechanisms of its efficacy in treatment of viral infections can open up a possibility of drug repurposing of raloxifene for the treatment of SARS-CoV-2. This review will first provide a general overview of the mechanism of action of raloxifene as a safe and effective SERM in treatment and prevention of postmenopausal osteoporosis and cancer, and later summarize possible mechanisms of its efficacy in viral infections by Ebola, influenza A and hepatitis C viruses.

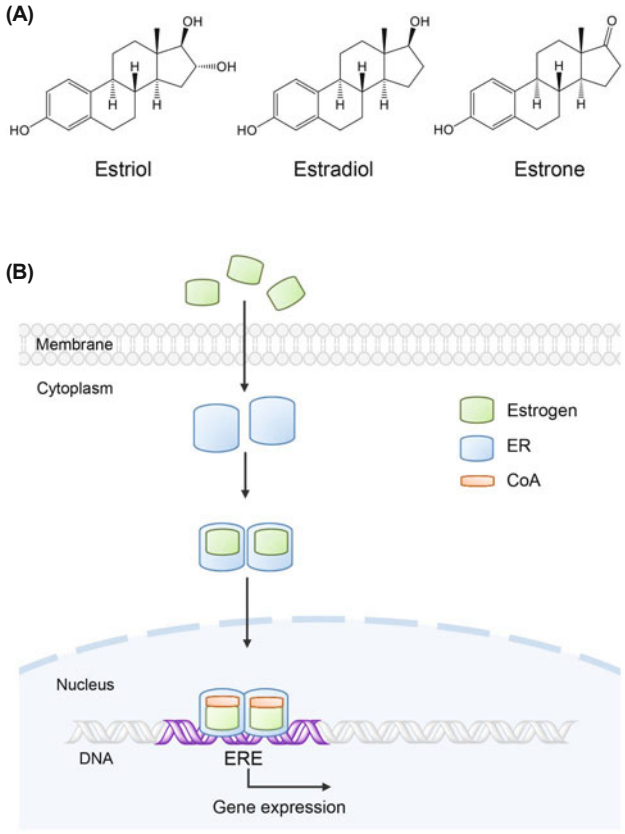

Fig. 1. Schematic of estrogen-estrogen receptor-estrogen response element complex resulting in gene transcription. (A) Estrogen is found in three forms: estriol, estradiol, and estrone. Estradiol, also known as 17β-estradiol, is the most potent and majorly-used form. (B) Estrogen as a steroid hormone crosses the cell membrane and binds to the estrogen receptor (ER) in the cytoplasm. Upon estrogen's binding to ER, ER forms a dimer. ER dimer enters the nucleus and binds to a specific region of DNA known as estrogen response element (ERE) through hinge region and DNA binding domain (DBD), respectively. The interaction between DBD, hinge region, and ERE opens up an activation factor-2 cleft of the activation factor domain (AFD) and prompts a binding of coactivator A (CoA) on the activation factor-2 cleft. The binding of CoA results in the transcription of genes encoded by ERE.

Mechanism of action of raloxifene

Role of estrogen and estrogen receptors in diseases

Estrogen is a steroid hormone that is mainly responsible for the maturation and proper functioning of female reproductive system but is also responsible for the maintenance of bone mass, healthy cardiovascular, and central nervous system (Levenson *et al.*, 1998; Pearce and Jordan, 2004; Heldring *et al.*, 2007; Patel and Bihani, 2018). Estrogen is found in three different forms estriol, estradiol, and estrone; and estradiol, also known as 17β-estradiol, is the most potent and majorly-used form (Clemett and Spencer, 2000). As shown in Fig. 1A, there are three structurally different forms of estrogen. To exert its activity, estrogen as a cellular steroid crosses cellular membrane and interacts with estrogen receptors (ERs) inside the cell. ER is found in two isoforms ERα and ERβ and the distribution of these two isoforms determines varying response of different cell types to the estrogen (Riggs and Hartmann, 2003; Patel and Bihani, 2018).

Both ERα and ERβ share four common domains: ligand binding domain (LBD), DNA binding domain (DBD), hinge region, and activation function domain (AFD) (Fig. 2A). Biochemical analysis demonstrates highly preserved homology in the DBD but significant difference in their AFD of ERα

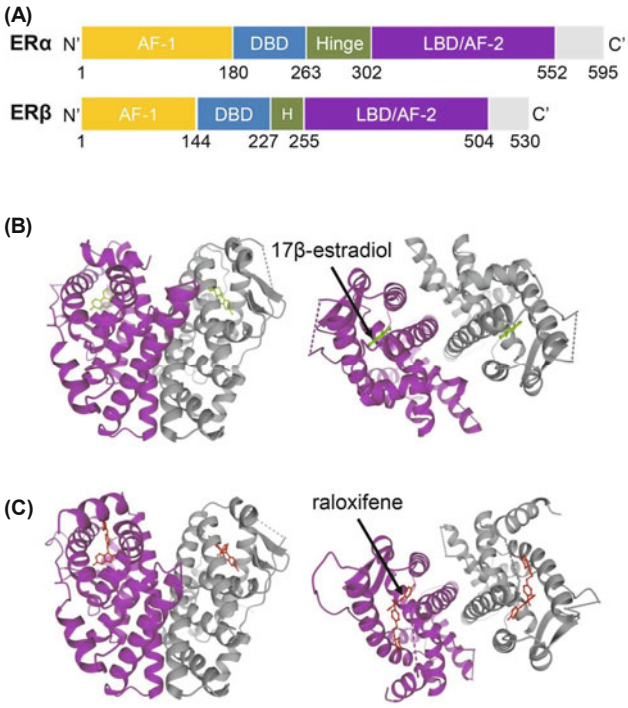

Fig. 2. Domains of estrogen receptor. (A) Estrogen receptor (ER) is found in two isoforms ERα and ERβ and share four common domains: ligand binding domain (LBD), DNA binding domain (DBD), hinge region, and activation function domain (AFD). (B) Estrogen can directly bind to ER through LBD. (C) Raloxifene also binds to LBD upon interaction with ER.

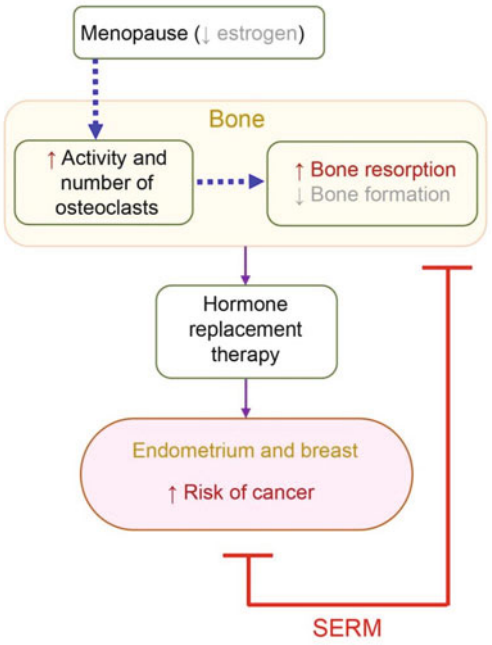

Fig. 3. Diseases related to imbalance of estrogen and estrogen receptor. The decreased estrogen level results in increased activity and number of osteoclasts increasing bone resorption leading to osteoporosis. Hormone replacement therapy (HRT) is able to prevent the loss of bone density. However, continuous exposure to estrogen during HRT causes imbalance in the levels of ER in the breast and endometrium thereby increasing the risk of cancer development. SERMs can act as agonists on bone cells and prevent/treat osteoporosis while acting as antagonists on breast and endometrium thereby preventing the development of cancer in these organs.

and ERβ isoforms (Heldring *et al.*, 2007; Kumar *et al.*, 2011). Upon crossing the cell membrane, estrogen binds to the LBD of ER in the cytoplasm, and the binding of estrogen triggers dimerization of ER (Fig. 1B). The molecular detail for the interaction between the LBD and estrogen has been established as shown in Fig. 2B. The resulting ER dimer can directly enter the nucleus and bind to a specific region, known as estrogen response element (ERE), in the chromosomal DNA through hinge region and DBD, respectively (Kumar and Chambon, 1988; Levenson *et al.*, 1998; Patel and Bihani, 2018). The binding of estrogen to its receptor prompts helix-12, one of the helices of LBD, to open up the activation factor-2 cleft of AFD to which coactivator A (CoA) can bind. Through the binding of CoA to AFD, a specific set of target genes encoded by ERE undergo transcriptional activation (Fig. 1B) (Tzukerman *et al.*, 1994; Brzozowski *et al.*, 1997; Levenson *et al.*, 1998; Pawlak *et al.*, 2012; Patel and Bihani, 2018). Through the direct estrogen-ER-ERE interaction, a wide range of genes are regulated, but this regulation can be limited or even abrogated in various physiological conditions, which can result in related diseases.

A major physiological condition that affects the estrogen and ER levels in women is menopause. Menopause is mainly characterized by cessation of menstrual cycles and other physical conditions caused by a significant decrease in estrogen production in women at their mid-to-late 40s (Nelson, 2008). Since estrogen is responsible for proper functioning of various organs, including reproductive organs, breast, and bone, the decrease in estrogen production can result in dysfunctions of these organs. Specifically, bone cells contain both isoforms of ER and respond to estrogen for maintenance of a suitable bone density. Post-menopause, estrogen deficiency increases the number and activity of osteoclasts thereby increasing bone turnover and resorption rather than its formation resulting in reduction of bone mass (Riggs, 2000; Riggs and Hartmann, 2003). A common therapy used to prevent the loss in bone mass and many other postmenopausal symptoms is hormone replacement therapy (HRT), supplementation of women with synthetic hormones to make up for insufficient levels of body-produced hormones. However, prolonged exposure to estrogen by HRT can result in dysregulation of ER levels in endometrium and breast eventually causing cancer (Fig. 3) (Beral and Million Women Study, 2003; Haldosen *et al.*, 2014; Omoto and Iwase, 2015; Collaborative Group on Hormonal Factors in Breast Cancer, 2019). Especially, breast cancer is the most frequently occurring carcinoma in women, and about 75% of all breast cancers is classified as ER-positive (ER+) carcinoma (Perou *et al.*, 2000; Patel and Bihani, 2018). In order to provide safe and efficient treatment for postmenopausal osteoporosis and many other ER-related diseases, several SERMs that selectively target and modulate ERs have been developed.

Mechanism of action of raloxifene in treatment of postmenopausal osteoporosis

SERMs are mainly divided into several classes, triphenylethylenes, benzothiophenes, phenylindoles, and tetrahydro-

Table 1. Chemical structure of selective estrogen receptor modulators

Triphenylethylene			Benzothiophene	2-Phenylindole	Tetrahydro-naphthalene
Tamoxifen	Afimoxifene	Endoxifen	Raloxifene	Bazedoxifene	Lasofoxifene
Toremifene	Droloxifene	Idoxifene	Arzoxifene	Pipendoxifene	

naphthalenes, according to their structures (Table 1). SERMs are chemically diverse and can exert their activity as agonists or antagonists in a tissue-specific manner possibly due to a differential distribution of ER isoforms in different tissues (Patel and Bihani, 2018). Tamoxifen is a SERM that was approved in the early 1970s for treatment of ER+ breast cancer of all stages (Gottardis and Jordan, 1987; Levenson *et al.*, 1998). It belongs to a triphenylethylene class of SERM and acts as an antagonist (i.e. exerts an anti-estrogenic action) in the breast tissue while acting as an agonist or a partial agonist (i.e. exerts an estrogenic action) in uterus, bone and heart (Jordan, 2008). Therefore, tamoxifen is an effective treatment for ER+ breast cancer and can prevent postmenopausal osteoporosis but results in increased risk of endometrial cancer and cardiovascular diseases (Vogel *et al.*, 2006; DeMichele *et al.*, 2008). To meet the need for a more selective and effective SERM that does not affect the endometrium and cardiovascular organs, raloxifene has been introduced to the clinic.

Raloxifene belongs to the benzothiophene class of SERMs in which its aromatic scaffold benzothiophene is used as a main site of drug action and contributes to its tissue specificity (Gottardis and Jordan, 1987; Patel and Bihani, 2018). Same as estrogen, raloxifene binds to the LBD of ER. The interaction between LBD and raloxifene is structurally represented in Fig. 2C. Raloxifene serves as an agonist in the bone, cardiovascular system and the liver, while it shows antagonistic properties in human breast and uterus (Levenson *et al.*, 1998; MacGregor and Jordan, 1998; Clemett and Spencer, 2000). In uterine and breast tissues, raloxifene's benzothiophene ring binds to ER, but its basic side-chain, which is large and inflexible, protrudes. This protrusion blocks the interaction between raloxifene and ERE preventing the associated genes from being activated thereby acting as an estrogen antagonist in these tissues. In bones, on the contrary, raloxifene binds to ER and activates genes that estrogen would normally activate thereby acting as an agonist (Fig. 3) (Bryant *et al.*, 1996). For example, raloxifene increases the production of transforming growth factor-β_3 (TGF-β_3) which plays an important role in bone remodeling and decreases production of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) which constitute important mediators of bone resorption (Yang *et al.*, 1996; Gianni *et al.*, 2004).

The tissue specificity of raloxifene makes it an effective treatment for postmenopausal osteoporosis and a preventive therapy for breast cancer without increasing the risk of endometrial cancer. Its efficacy is demonstrated by several preclinical and clinical studies (Table 2). A study with ovariectomized rats demonstrated that raloxifene improved bone remodeling but did not promote uterine growth, while HRT similarly improved bone remodeling but exerted its activity on uterine tissue (Turner *et al.*, 1994; Evans *et al.*, 1996). In clinical trials, raloxifene increased bone mineral density and decreased incidences of vertebral and non-vertebral fractures without affecting endometrial thickness (Delmas *et al.*, 1997, 2003; Ettinger *et al.*, 1999; Fugére *et al.*, 2000; Goldstein *et al.*, 2000). All these beneficial effects were observed without increased risk for breast cancer (Cummings *et al.*, 1999).

Proposed mechanisms of antiviral activity of raloxifene

Recently, raloxifene has demonstrated its efficacy in treating viral infections and has shown potential for drug repurposing. In fact, the efficacy of raloxifene in viral infections may be directly related to its activity through modulation of ER and the related pathways. A meta-analysis suggests that severity and clinical outcome of patients with viral infections vary by sex and age. For example, young adult women demonstrate more severe clinical outcome by influenza virus infection compared to age-matched male participants (Peretz *et al.*, 2015). Similarly, the severity of influenza virus infection changes during pregnancy in women (Straub, 2007). Furthermore, the infection by hepatitis C virus (HCV) progresses more rapidly in men than in women, and early menopause in women is associated with reduced treatment efficacy and accelerated progression of HCV-associated liver fibrosis (Shimizu, 2003; Di Martino *et al.*, 2004; Furusyo *et al.*, 2012). These clinical observations suggest that estrogen and ER might be involved in progression and prognosis of viral infections. Indeed, treatment with estradiol and raloxifene has decreased viral titer in nasal epithelial cells isolated from female patients infected with influenza A virus, while no effect is observed in the cells isolated from male patients infected with the same virus (Peretz *et al.*, 2016). Moreover, raloxifene has demonstrated its efficacy as an adjuvant to the standard treatment strategy in postmenopausal women infected with HCV, possibly due to a protective role of raloxifene as an ER agonist in hepatocytes (Furusyo *et al.*, 2012).

Another possible mechanism of action of raloxifene in viral infections is through direct targeting of viral life cycle. Virus proliferates by exploiting and scavenging the host cellular machinery and resources. Although there are variations to

Table 2. Pre-clinical and clinical studies demonstrating efficacy of raloxifene in postmenopausal osteoporosis

Drug action	Main findings	Reference
Increase in bone remodeling	Raloxifene increased the production of transforming growth factor-β_3 (TGF-β_3) which plays an important role in bone remodeling	Yang *et al.* (1996)
Increase in bone remodeling	A study with ovariectomized rats demonstrated that raloxifene improved bone remodeling but did not promote uterine growth, while HRT similarly improved bone remodeling with effects on uterine	Turner *et al.* (1994) Evans *et al.* (1996)
Decrease in bone resorption	Raloxifene decreased production of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) which constitute important mediators of bone resorption	Gianni *et al.* (2004)
Increase in bone mineral density	Clinical trials demonstrated that raloxifene increased bone mineral density measured with X-ray absorptiometry without affecting endometrial thickness	Delmas *et al.* (1997) Fugére *et al.* (2000) Goldstein *et al.* (2000)
Decrease in incidences of bone fractures	Raloxifene decreased incidences of vertebral and non-vertebral fractures in postmenopausal women	Ettinger *et al.* (1999) Delmas *et al.* (2003)

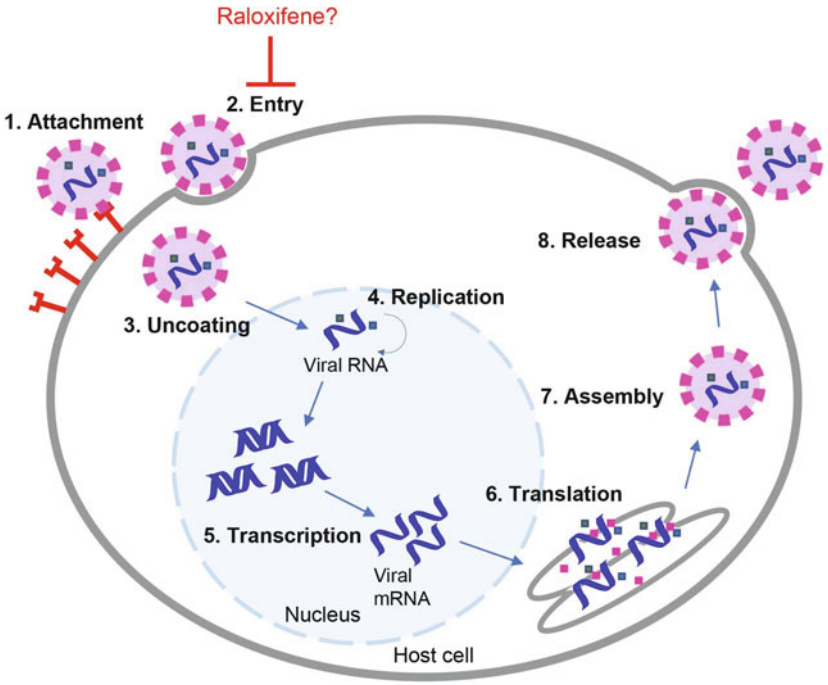

Fig. 4. Raloxifene's action on the viral life cycle. The virus enters the host cells through its specific receptors on the host cell, then the reproduction of viral genome and proteins initiate. The resulting viral materials are packaged in the host cell to generate progeny viral particles that exit the host cell through exocytosis.

their life cycle, commonly viruses replicate in the host cell as described in Fig. 4. Virus enters the host cell through the attachment to their specific receptors presented on the surface of the host cell. After attachment to the receptors, virus can enter through either membrane fusion or endosomal uptake. In the later route, inside the endolysosomes virus undergoes uncoating. Upon release from the endolysosomes, viral RNA enters the nucleus for replication of viral genome and proteins. The resulting genome and proteins are packaged in the cytoplasm to generate progeny viral particles that exit the host cell through exocytosis and infect the host body (Fig. 4) (Hoenen *et al.*, 2019; Jones *et al.*, 2020). Several studies suggest that raloxifene can directly target the viral life cycle. Takeda *et al.* (2012) demonstrate that raloxifene inhibited RNA replication of HCV in a cell line. In addition, Yoon *et al.* (2020) demonstrated that raloxifene repress expressions of VP40 matrix protein and the envelope glycoprotein, which inhibits the replication of Ebola virus.

As noted, the viral entry is one of the most promising antiviral targets, and thus the entry of SARS-CoV-2 has been extensively studied since the COVID-19 outbreak (Ragia and Manolopoulos, 2020). Importantly, Fan *et al.* (2017) provide a specific mechanism of action of raloxifene in prohibiting entry of Ebola virus. Ebola virus enters the cell through lysosomal uptake route, and the virus exit the endolysosomes by an elaborate level of sphingosine in the host cell. The high level of sphingosine induces an outflow of calcium ions from the endolysosomes, which leads to a conformational change of the outer membrane of the virus. This change allows the virus to fuse with the endolysosomal membrane and exit. Treatment with raloxifene results in significant decrease in the level of sphingosine in the membrane of the host cell, which leads to an accumulation of calcium ions inside the endolysosomes and prevention of viral escape from the endolysosomes thereby preventing its replication inside the host cells (Fig. 4) (Fan *et al.*, 2017). Furthermore, recently, raloxifene and bazedoxifene were found to be effective on acute respiratory distress syndrome (ARDS) by antagonizing IL-6 signaling in severe COVID-19 patient (Smetana *et al.*, 2020). The mechanisms of action of raloxifene in treatment of viral infections are summarized in Table 3. All these mechanisms strongly suggest raloxifene as a potential treatment option for SARS-CoV-2 infection and therefore its antiviral efficacy deserves further investigation.

Table 3. Mechanism of action of raloxifene in viral infections

Proposed mechanisms		Antiviral activity	Reference
General antiviral activities	Influenza A	Raloxifene has decreased viral titer in nasal epithelial cells isolated from female patients infected with influenza A virus, while no effect is observed in the cells isolated from male patients	Furusyo *et al.* (2012)
	HCV	Raloxifene has demonstrated its efficacy as an adjuvant to the standard treatment method in postmenopausal women infected with HCV	Peretz *et al.* (2016)
Specific antiviral activates	HCV	Raloxifene inhibited RNA replication of HCV	Takeda *et al.* (2012)
	Ebola virus	Raloxifene reduced the expression of sphingosine which led to an accumulation of calcium and prevention of viral entry into the cells	Fan *et al.* (2017)
	Ebola virus	Raloxifene also downregulated the expression of different types of G protein-coupled receptors that are responsible for replication of Ebola virus	Yoon *et al.* (2020)

Summary

Imbalance in the levels of a steroid hormone estrogen and its receptor ER are involved in several disease conditions. Decrease in estrogen production in women post-menopause results in an imbalance of ER in the bone cells, and this imbalance can result in postmenopausal osteoporosis. Furthermore, continuous exposure of breast cells to a high level of estrogen leads to an imbalance of ER in breast cells thereby causing cancer. Therefore, supplying postmenopausal women with synthetic estrogen in HRT might result in breast cancer. To treat these ER-related diseases, SERMs have been used as a safe and efficient treatment method. SERMS, through their specific interactions with ER, act as agonists in some cells but as antagonists in other cell types. Raloxifene is a SERM that can act as an agonist in bone cells thereby serving as a treatment method for osteoporosis but acts as an antagonist in breast cells thereby preventing the development of breast cancer.

Recently, in addition to ER-related osteoporosis and cancer, meta-analysis demonstrates a possible connection between ER levels and progression of viral infections. In support, raloxifene demonstrates efficacy in treatment for infection by Ebola, influenza A, and hepatitis C viruses. In addition, several studies demonstrate that raloxifene can directly target the viral life cycle. All these results suggest raloxifene as a possible treatment option for treatment of recently-emerging COVID-19 pandemic that is caused by SARS-CoV-2. Although COVID-19 is predominantly known as a respiratory disease, recent observations indicate that SARS-CoV-2 can infect many other organs other than the lung. Repurposing raloxifene that can selectively and differentially target different cell types can yield two beneficial effects. First, drug repurposing in which previously approved drug candidates are re-examined for a novel indication outside their original medical indication can save time in drug development. Recently emerging COVID-19 is affecting the global community and is leading to a mass casualty incident. Safe and effective treatment is urgently needed. Second, examining efficacy of raloxifene, drug for which the action is tissue-specific, in treatment of SARS-CoV-2 can help further reveal and understand how viral infections affect various organs in human bodies. We note in closing that recently major research institutes in both the European Union and South Korea have started a collaborative research for further development of raloxifene as a treatment for COVID-19, and they found a significant efficacy of raloxifene in cell-based SARS-CoV-2 infection model and *in silico* molecular docking simulation. In view of its potency against COVID-19, clinical trials are underway to progress the use of raloxifene as a promising curing option for SARS-CoV-2 infection.

Acknowledgments

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Conflict of Interest

We have no conflicts of interest to report.

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