A drug repurposing study based on clinical big data for the treatment of interstitial lung disease

(間質性肺疾患の治療のための

臨床ビッグデータに基づくドラッグリパーパシング研究)

2020

Soni Siswanto

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令和２年９月２３日

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Title

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Summary

Interstitial lung disease (ILD) is a heterogeneous group of respiratory disorders characterized by inflammation and/or fibrosis in the pulmonary interstitial tissues. The etiology of ILD is still unknown. However, it is recognized that the drug treatment itself is one of the primary causes of ILD. Despite recent advancement in the understanding and classification of ILD, limited treatment options are associated with poor prognosis in ILD patients. In attempt to find a new treatment, fast and low cost drug development through drug repurposing approach has become a preferred alternative strategy. Several approaches have been made in drug repositioning studies. One of them is by utilizing clinical big data, which present a great resource for mining and analyzing real-world medication usage. This study conducted efforts aiming to discover a hypothetic drug interaction that is able to reduce the drug-induced ILD by analyzing clinical big data, and validate the hypothesis in pharmacological experiments through establishing an animal disease model.

Chapter 1. Data mining approach for an effective treatment of drug-induced ILD

For the large-scale analysis of clinical data, US Food and Drug Administration (FDA) adverse event reporting system (FAERS, 11 million cases) and Japanese Medical Data Center insurance claims (JMDC, 7.4 million cases) were utilized. In these clinical records, the incidence of ILD-related event was strongly associated with the prescription of an antiarrhythmic agent, amiodarone. Moreover, the incidence of amiodarone-induced ILD was significantly reduced in patients who received an anticoagulant, dabigatran. Since amiodarone and dabigatran are K^+ channel blocker and thrombin antagonist, respectively, their interaction are suggestive of new target molecule(s) in human body.

Chapter 2. Experimental validation for the effect of dabigatran on amiodarone-induced ILD

Firstly, I have established a mouse model of ILD. A repeated oral treatment of mice with amiodarone (300 mg/kg, 5 times per week) for 4 weeks caused decrease in body weight, which reflect the progression of respiratory dysfunction. In the lung of diseased mice, inflammatory and fibrotic changes were observed in histological staining using hematoxylin-eosin and Sirius Red respectively. In parallel, upregulation of inflammation and fibrosis marker *Pdgfra* and *Pdgfc* were observed in a time-dependent manner. In these mice, co-treatment with dabigatran reduced the decrease in body weight and pulmonary inflammation induced by amiodarone. Dabigatran co-treatment with amiodarone also found to reduce *Pdgfra* expression in mouse lung but did not reduce *Pdgfc* upregulation.

In conclusion, this study on clinical big data analysis found that amiodarone is associated with ILD. Meanwhile, dabigatran co-occurrence is found to suppress amiodarone-induced ILD. Furthermore, this study suggests that dabigatran co-treatment provides a beneficial result in mouse model of amiodarone-induced ILD associated with attenuation of *Pdgfra* expression.

Abbreviations

Acta2	actin alpha 2
ASR	adjusted sequence ratio
ATC	anatomical therapeutic chemicals
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CMC	carboxymethylcellulose
Col1a1	collagen type 1a1
Col3a1	collagen type 3a1
CSR	crude sequence ratio
FAERS	FDA adverse event reporting system database
FDA	Food and Drug Administration
HE	hematoxylin-eosin
HRCT	high resolution computed tomography
ICD	International classification of disease
ILD	interstitial lung disease
JMDC	Japanese medical data center insurance claims database
MedDRA	medical dictionary for regulatory activities
mRNA	messenger ribonucleic acid
Pdgfa	platelet-derived growth factor a
Pdgfc	platelet-derived growth factor c
Pdgfra	platelet-derived growth factor receptor a
Pum1	pumilio1
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RNA	ribonucleic acid
ROR	reporting odds ratio
SMQ	standardized MedDRA queries
SSA	sequence symmetry analysis
Tgfb1	transforming growth factor beta 1
Tnfa	tumor necrosis factor alpha

Chapter 1. Data mining approach for an effective treatment of drug-induced ILD

1.1. Introduction

The term “interstitial lung disease” or “diffuse parenchymal lung disease” is a heterogeneous group of respiratory disorders characterized by inflammation and/or fibrosis in the pulmonary alveoli as well as in the pulmonary interstitial tissues (1). Most patients with interstitial lung disease (ILD) have been reported to suffer from exertional dyspnea with variable duration and speed of onset (2). Cough, which can be productive, also has been reported to be common and may be debilitating (2). The incidence and prevalence of ILD varies according to data collection methods, classification terms, and geographic area (3). Generally, incidence of ILD varies between 0.48 – 93.7 cases per 100,000 persons (3). Prevalence of ILD is reported to increase in older ages (3). Survival rates at 5 years range from 20% to more than 90% (4,5). Poor prognosis of ILD leads to lung transplantation, and in fact, more than 40 % patients undergoing lung transplantation according to worldwide registry data have ILD (6). Lung transplantation offers survival benefits for some patients, with 50% survival at 7 years (6).

The causes of ILD can be classified into known etiology (approximately 35% of patients) and unknown etiology (approximately 65% of patients). Adverse drug reactions, environmental exposure, and infections are common known exogenous factors of patients with ILD (5,7). As for unknown etiology, no convincing evidence has been reported of the precise causative agent(s) but exogenous factors have been shown to be the risk factors for ILD (5).

As long as the exposure threshold of exogenous factor to induce ILD is still unknown, in order to reveal a potential cause of ILD, it is essential to understand patient’s detailed history of past and present employment, smoking, medication, and environmental exposures

(2,8). Patient's family history of ILD is also a beneficial information in evaluating patients suspected with ILD (8,9).

The clinical investigation of patients with ILD can be classified into initial and specialized steps. End-inspiratory crackle is a key symptom for the early diagnosis of most patients with ILD (9,10). Although it is neither sensitive nor specific, serial chest radiography is able to help determine clinical classification of ILD (2). Laboratory investigations of full blood counting or inflammatory marker can provide supporting evidence for a suspected clinical diagnosis of ILD (2,9,11). For specialized investigation, high-resolution computed tomography (HRCT) is currently regarded as the standard investigation for the definitive diagnosis of ILD (9,12). HRCT can be used to guide a probe to the site of lung biopsy, and the biopsy specimen provides invaluable information for the pathological analysis of ILD (12). Specialized investigation of pulmonary function tests provide information concerning the degree of functional impairment and disease severity (13,14).

There are several basic histologic patterns of ILD. The first pattern is named acute lung injury. Basic elements of this pattern include interstitial edema, intra-alveolar fibrin, and reactive type 2 cells. The second basic pattern is named fibrosis. Basic element of this pattern is dense collagen deposition in the lung parenchyma. The third pattern is chronic inflammation infiltrated with basic elements such as inflammatory cells within alveolar walls. The fourth pattern is alveolar filling, named by the condition where alveoli in the biopsy specimen is filled with plenty of cells and non-cellular materials. The fifth pattern is named nodules, which are found in the biopsy specimen. The last pattern is named minimal changes, in which biopsy specimen shows little evident pathology at scanning magnification (12).

Treatment objectives in managing ILD are determined by the clinical disease behavior (2,15). Key decisions in the treatment of ILD include when to initiate a drug treatment and how to monitor the responses (2,15). In ILD with poor prognosis, key decisions include

assessing exercise-induced hypoxemia or referring to lung transplantation, and in a serious case, palliative care may be the best approach (2,5). Anti-inflammatory and anti-fibrotic agents are used for the treatment of ILD, however, only two drugs, nintedanib (tyrosine kinase inhibitor with multiple target) and pirfenidone, are approved at present for the treatment of idiopathic pulmonary fibrosis, IPF (15-17). A number of drugs have been clinically tried to treat fibrotic ILD, including imatinib (a tyrosine kinase inhibitor), ambrisentran (selective endothelin receptor A antagonist), macitentan and bosentan (dual endothelin receptor antagonists) as well as sildenafil (phosphodiesterase inhibitor). Yet, all trials failed to provide therapeutic benefit in the treatment of fibrotic ILD (15,16).

Drug repurposing (drug repositioning) is a practical drug development strategy to identify unintended usefulness of approved drug or clinically-tested investigational drugs different from the originally approved indications. This strategy is relatively fast and efficient when compared to the classical development from entirely-new compound (18). Repurposed drug has already been found to be sufficiently safe for humans since the early-stage trials have been completed, so the risk of safety failure is low (19,20).

Generally, drug repurposing strategy includes the step to identify candidate molecule for a given indication, the step to assess the drug efficiency in preclinical animal models, and then the step to evaluate its efficacy in phase II clinical trials (19,20). Among them, identifying candidate molecule is the most critical step in drug repurposing. Approaches for identifying candidate molecule can be divided into computational approaches and experimental approaches (19-21).

Computational approaches are largely data-driven, involving systematic analysis of big data that leads to the formulation of repurposing hypotheses (21). There are a number of approaches in computational approaches. The initial approach utilizes chemical structural data of drugs (ligands) and targets (receptors) in order to find novel interaction *in silico*. Later

approach utilizes various experimental data of transcriptomic, proteomic or metabolomics from disease phenotype, drug treatment or genetic variants in order to generate hypothesis of promising drug candidate affecting on a diseased metabolic pathway. Experimental approaches involve phenotypic screening approach, aiming to identify compounds that show disease‐relevant effects in model systems (19,20).

In this research, two clinical data were mined and analyzed in order to find drug that can suppress ILD. The databases are the Food and Drug Administration (FDA) adverse event reporting system database (FAERS) and Japan Medical Data Center insurance claims database (JMDC). These databases provide valuable resources, since the data represent real world medication usage.

The FAERS is a database of spontaneous adverse event reports. It was designed to support the FDA's pharmacovigilance programme (22). Adverse events are coded based on Medical Dictionary for Regulatory Activities (MedDRA) terminology (23). This database has been statistically examined for confounding factor that unexpectedly increases or decreases the occurrence of drug-induced adverse event (24). Utilizing FAERS as a source of clinical database provide several advantages: anonymized clinical data with many cases, a wide variety of adverse events that are collected from all over the world, easily identified symptom regardless of disease name, and freely accessible data since 2004. On the other hand, FAERS database also has disadvantages. For instances, unknown population with a large number of duplicate cases are included, drug name are diverse, and there are biases that shall be considered (25).

The JMDC is a database that is designed to monitor insurance claims in Japan (26). JMDC have been collected medical records of patients from medical facilities throughout Japan since 2005. Compared to spontaneous report, JMDC has several advantages: knowable total population of prescription, no duplication records (since each person has a single

identifier), and easy identification of prescribed drugs (according to the Anatomical Therapeutic Chemical/ATC classification system codes, days of supply, dosage information and mode of prescription). Therefore, sequential association between drug prescription and disease onset can be analyzed in a timeline history of patients (since the date of service information is specified up to the month and year). On the other hand, JMDC data has some limitation in identifying adverse events from diagnosis documented in the receipt (27).

The objective of this chapter is to scientifically discover drug-induced ILD and to discover concomitant drug that suppress the drug-induced ILD.

1.2. Methods

1.2.1. Analysis of FAERS

Reports from the first quarter of 2004 throughout the fourth quarter of 2019 of the FAERS were filtered by adopting the most recent case number to remove duplicated reports, and then analyzing the remaining unduplicated reports. Drug names were mapped into unified generic names via text mining. The adverse event term used in this research was standardized MedDRA queries (SMQ) code 20000042 (interstitial lung disease) from MedDRA ver.23.0.

Drugs associated with ILD were examined by plotting individuals report in the FAERS and dividing them into the following four groups: (a) individuals who received the drug of interest and exhibited ILD; (b) individuals who received the drug of interest, but did not exhibit ILD; (c) individuals who did not receive the drug of interest and exhibited ILD; and (d) individuals who did not receive the drug of interest and did not exhibit ILD. Concomitant drugs (drug B) that affect the occurrence of drug associated with ILD (drug A) were examined by plotting individuals report receive drug A in the FAERS and divide them into the four groups as mentioned above. Adverse event risk was evaluated by calculating the

reporting ORs (ROR, formula 1) and Z-score (formula 2). Incidence rate of ILD in FAERS database was calculated according formula 3.

$$\text{ROR} = \frac{ad}{bc} \quad \text{..... 1}$$

$$\text{Z score} = \frac{ln(OR)}{\sqrt{\frac{1}{a}+\frac{1}{b}+\frac{1}{c}+\frac{1}{d}}} \quad \text{..... 2}$$

$$\text{Incidence rate} = \frac{a}{(a+b)} \quad \text{..... 3}$$

1.2.2. Analysis of JMDC

The total number of patients in JMDC from January 2005 to August 2019 is 7,438,470 patients. A total of 17 International Classification of Disease (ICD)-10 terms related to ILD were used in this research (Table 1). Amiodarone hydrochloride (ATC code: C01BD01) and dabigatran etexilate methanesulfonate (ATC code: B01AE07) were picked as drug of interest.

Table 1.1. ICD-10 terms used in the research

No	Code	Japanese term	English term
1	J702	急性薬物誘発性間質性肺障害	Acute drug-induced interstitial lung disease
2	J703	慢性薬物誘発性間質性肺障害	Chronic drug-induced interstitial lung disease
3	J704	薬剤性間質性肺炎	Drug-induced interstitial pneumonia
4	J841	肺線維症	Pulmonary Fibrosis
5	J841	特発性間質性肺炎	Idiopathic interstitial pneumonia
6	J841	特発性器質化肺炎	Cryptogenic organizing pneumonia
7	J841	特発性肺線維症	Idiopathic pulmonary fibrosis
8	J841	びまん性間質性肺炎	Diffuse interstitial pneumonia
9	J841	急性間質性肺炎	Acute interstitial pneumonia
10	J841	非特異性間質性肺炎	Nonspecific interstitial pneumonia

No	Code	Japanese term	English term
11	J841	通常型間質性肺炎	Usual interstitial pneumonia
12	J841	リンパ球性間質性肺炎	Lymphocytic interstitial pneumonia
13	J841	炎症後肺線維症	Post-inflammatory pulmonary fibrosis
14	J841	びまん性肺胞傷害	Diffuse alveolar damage
15	J841	呼吸細気管支炎関連性間質性肺疾患	Respiratory bronchiolitis-associated interstitial lung disease
16	J841	剥離性間質性肺炎	Desquamative interstitial pneumonia
17	J849	間質性肺炎	Interstitial lung disease

Cases were extracted from JMDC and analyzed for event sequence symmetry analysis (SSA) with the previously described method (28). Briefly, patients with ILD and patients who received amiodarone were visualized to find new ILD diagnose or new amiodarone use. After identifying new use of amiodarone or new ILD diagnosis, sequences were ordered according to which came first between ILD and amiodarone. Sequence ratio was calculated according formula 4. Since SSA may be affected by prescribing trends over the time, sequence ratio was adjusted according to formula 5 and sequence ratio expressed as adjusted sequence ratio. Confidence interval of the adjusted sequence ratio was calculated according to formula 8 for ASR lower and 10 for ASR upper.

$$\text{Sequence ratio} = \frac{Number\ of\ patients\ using\ amiodarone \rightarrow ILD}{Number\ of\ patients\ ILD \rightarrow\ using\ amiodarone} \quad \ldots\ldots\ldots\ldots\ldots \quad 4$$

$$\text{Adjusted Sequence Ratio} = \frac{Sequence\ ratio}{Null\text{-}effect\ sequence\ ratio}\text{, where} \quad \ldots\ldots\ldots\ldots\ldots \quad 5$$

$$\text{Null-effect sequence Ratio} = \frac{Pa}{(1-Pa)}\text{, while} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad 6$$

$$Pa = \frac{\sum_{m=1}^{u}\left[Im \times \left(\sum_{n=m+1}^{m+i} Mn\right)\right]}{\sum_{m=1}^{u}\left[Im \times \left(\sum_{n=m-1}^{m-1} Mn + \sum_{n=m+1}^{m+d} Mn\right)\right]} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad 7$$

Note:

- Pa indicates the overall average probability that ILD will occur after amiodarone,
- *n* indicates consecutive month of the study period,
- *u* indicates the last month of the study period,
- *Mn* indicates the number of persons had ILD on certain month,
- *Im* indicates the number of persons who prescribed with amiodarone on that month, and
- *d* is the specified number of months for observation time window

$$\text{ASR}_{\text{lower}} = \frac{CSR_{lower}}{Null-effect\ sequence\ Ratio}\text{, where} \quad (8)$$

$$\text{CSR}_{\text{lower}} = \frac{\frac{AILD}{AILD+ILDA} - 1.96x\sqrt{\frac{AILD\ x\ ILDA}{(AILD+ILDA)^3}}}{1-\left(\frac{AILD}{AILD+ILDA} - 1.96\ x\sqrt{\frac{AILD\ x\ ILDA}{(AILD+ILDA)^3}}\right)} \quad (9)$$

$$\text{ASR}_{\text{upper}} = \frac{CSR_{upper}}{Null-effect\ sequence\ Ratio}\text{, where} \quad (10)$$

$$\text{CSR}_{\text{lower}} = \frac{\frac{AILD}{AILD+ILDA} - 1.96x\sqrt{\frac{AILD\ x\ ILDA}{(AILD+ILDA)^3}}}{1-\left(\frac{AILD}{AILD+ILDA} - 1.96\ x\sqrt{\frac{AILD\ x\ ILDA}{(AILD+ILDA)^3}}\right)} \quad (11)$$

Note:

- AILD = number of patients using amiodarone → ILD
- ILDA = number of patients using ILD → amiodarone

Cumulative ILD incidence of amiodarone with dabigatran and amiodarone without dabigatran were calculated (formula 12) (29). Cases were also extracted from JMDC to obtain the number of ILD risk in amiodarone with dabigatran and amiodarone without dabigatran. Cumulative incidence and number of ILD risk were visualized through Kaplan-Meier graph. Hazard ratio and log-rank test were calculated using R with “survival” package.

$$\text{Cumulative incidence} = \frac{\text{Number of people who get a ILD during a specified period}}{\text{Number of people free of ILD in the population at risk at the beginning of the period}} \quad \ldots. \quad 12$$

1.3. Results

1.3.1. Amiodarone is highly associated with ILD

After deduplication, a total of 11,430,831 reports in FAERS database remained as unique reports. Drugs associated with ILD were investigated and their numbers of report were plotted in Figure 1.1. Drug with higher value of $\log_{10}$(ROR) has higher association with ILD and drug with higher value of absolute Z-score, has more statistically significance. The highest association of drug and ILD was observed in amiodarone with ROR > 10 and Z-score > 100. Bleomycin, a drug that famously induces pulmonary fibrosis and an agent that is recommended for inducing fibrosis in animal model, had lower association with ILD compared to amiodarone in FAERS database. Total reports of bleomycin in FAERS database were observed quite low compared to those of amiodarone.

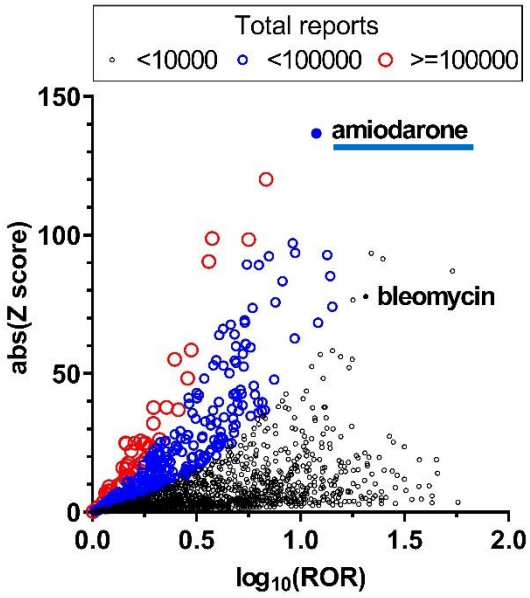


Figure 1.1. Drug association with ILD
Amiodarone was highly associated with ILD; meanwhile, bleomycin showed lower association with ILD

1.3.2. Concomitant dabigatran associated with lower occurrence of amiodarone-associated with ILD in FAERS

Within amiodarone user reports, every drug concomitantly prescribed with ILD was investigated and the statistical values were plotted with the number of reports (Figure 1.2a). Drugs plotted in positive x axis are drugs that are associated with higher occurrence of amiodarone-associated ILD. On the other hand, drugs plotted in negative x axis are drugs that are associated with lower occurrence of amiodarone-associated ILD. Several anticoagulant drugs including dabigatran, clopidogrel, rivaroxaban, apixaban, warfarin, and heparin were concomitantly prescribed and associated with lower occurrence of amiodarone-associated ILD. Among those anticoagulant drugs, a thrombin inhibitor, dabigatran, showed the highest significance with lowest occurrence of amiodarone-associated with ILD. Total report of dabigatran in amiodarone associated with ILD in FAERS database was sufficiently high, thus calculated ROR are not susceptible to sampling fluctuation.

Based on the FAERS database analysis, amiodarone was found to elevate ILD incidence rate to > 6%, while dabigatran did not increase ILD incidence rate by itself compared to control. Co-occurrence of dabigatran with amiodarone showed lower ILD incidence rate (< 2%) compared to amiodarone alone (Figure 1.2b).

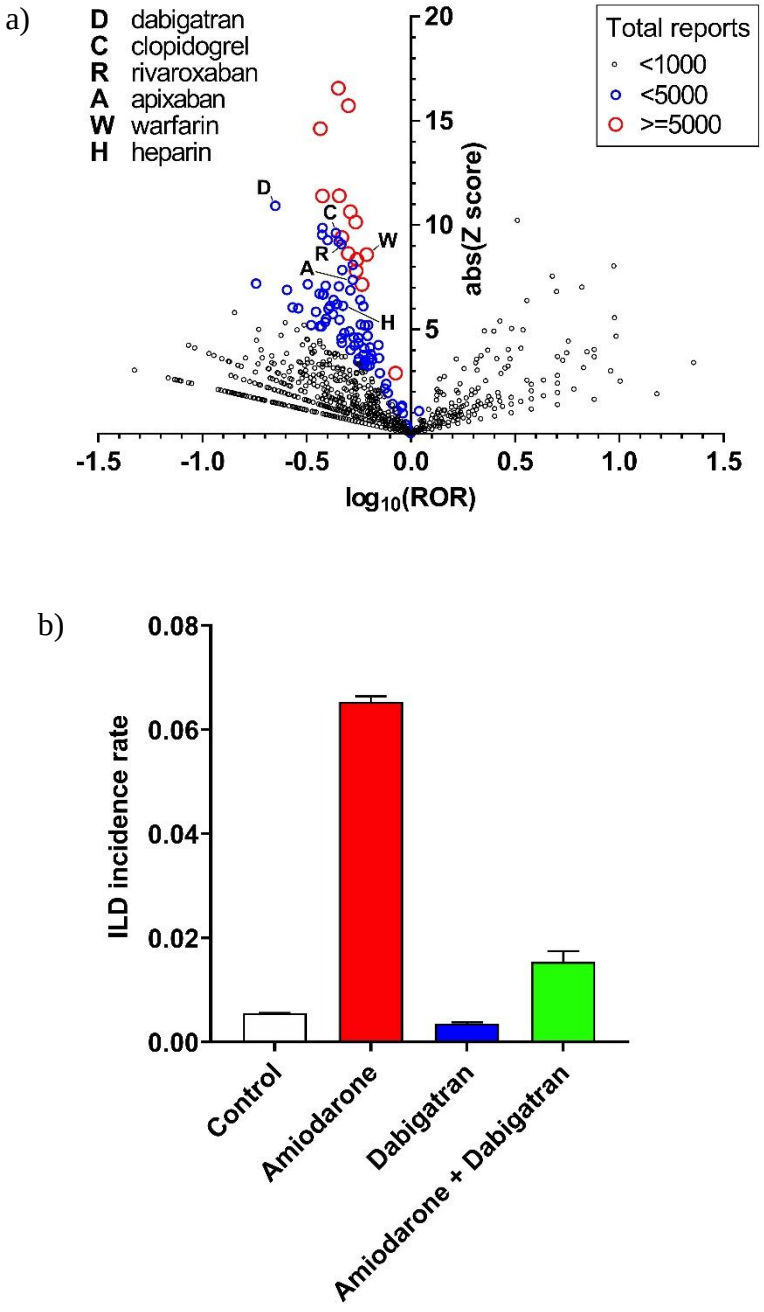


Figure 1.2. Concomitant dabigatran associated with lower occurrence of amiodarone-associated ILD.

a) Dabigatran had highest association with lower occurrence of amiodarone-associated ILD. b) Co-occurrence of amiodarone and dabigatran lowered ILD incidence rate compared amiodarone alone.

1.3.3. Dabigatran showed a beneficial effect in decreasing the risk of amiodarone-induced ILD in JMDC

Among the total patients extracted from JMDC, the number of predefined ILD patients were 22,855, and around 80% of them were diagnosed with interstitial lung disease (Table 1.2). After visualizing the first amiodarone prescription and ILD diagnosis timing distribution, it is clear that the number of both amiodarone prescription and ILD diagnosis were much higher in the first two months (Figure 1.3), suggesting that long-term patients received amiodarone with the diagnosis of ILD from the beginning of the insurance enrollment of them. This speculation is supported by the fact that the incidence of amiodarone prescription and ILD diagnosis became constant after 3 months from insurance enrollment. According to this result, three month will be sufficient for the run-in (washout) period to identify the first amiodarone prescription and the onset (or diagnosis) of ILD.

Table 1.2. Number of patients with ILD

No	Code	Japanese term	English term	Patients
1	J702	急性薬物誘発性間質性肺障害	Acute drug-induced interstitial lung disease	55
2	J703	慢性薬物誘発性間質性肺障害	Chronic drug-induced interstitial lung disease	3
3	J704	薬剤性間質性肺炎	Drug-induced interstitial pneumonia	565
4	J841	肺線維症	Pulmonary Fibrosis	1,704
5	J841	特発性間質性肺炎	Idiopathic interstitial pneumonia	1,068

No	Code	Japanese term	English term	Patients
6	J841	特発性器質化肺炎	Cryptogenic organizing pneumonia	1,048
7	J841	特発性肺線維症	Idiopathic pulmonary fibrosis	547
8	J841	びまん性間質性肺炎	Diffuse interstitial pneumonia	336
9	J841	急性間質性肺炎	Acute interstitial pneumonia	245
10	J841	非特異性間質性肺炎	Nonspecific interstitial pneumonia	142
11	J841	通常型間質性肺炎	Usual interstitial pneumonia	51
12	J841	リンパ球性間質性肺炎	Lymphocytic interstitial pneumonia	23
13	J841	炎症後肺線維症	Post-inflammatory pulmonary fibrosis	16
14	J841	びまん性肺胞傷害	Diffuse alveolar damage	12
15	J841	呼吸細気管支炎関連性間質性肺疾患	Respiratory bronchiolitis-associated interstitial lung disease	11
16	J841	剥離性間質性肺炎	Desquamative interstitial pneumonia	5
17	J849	間質性肺炎	Interstitial lung disease	17,024
			Total	22,855

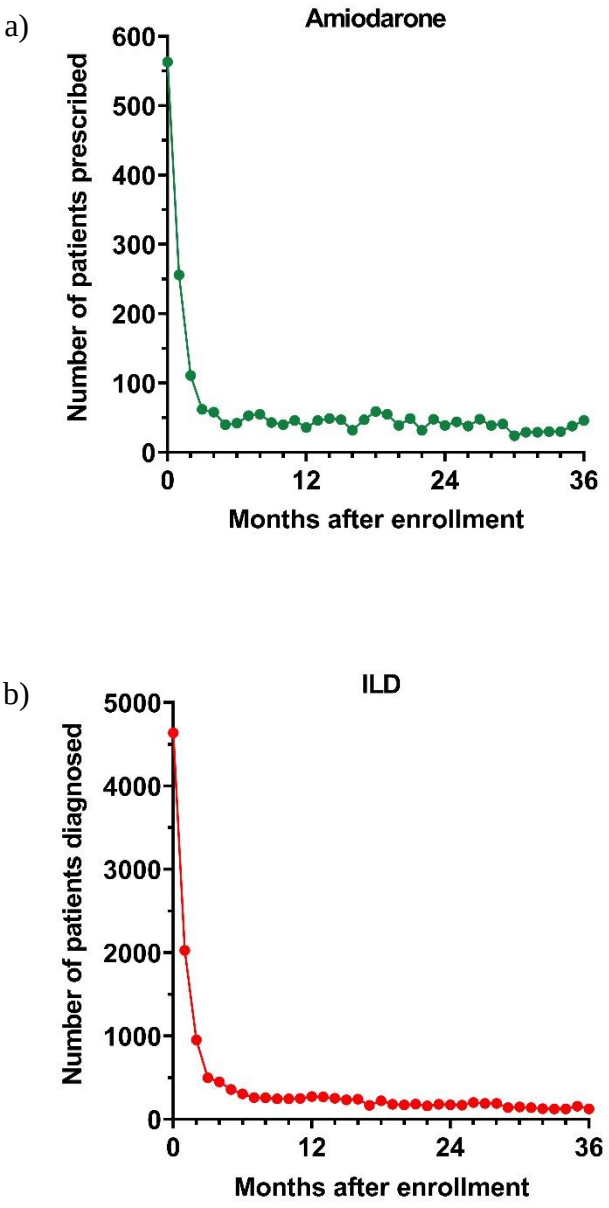
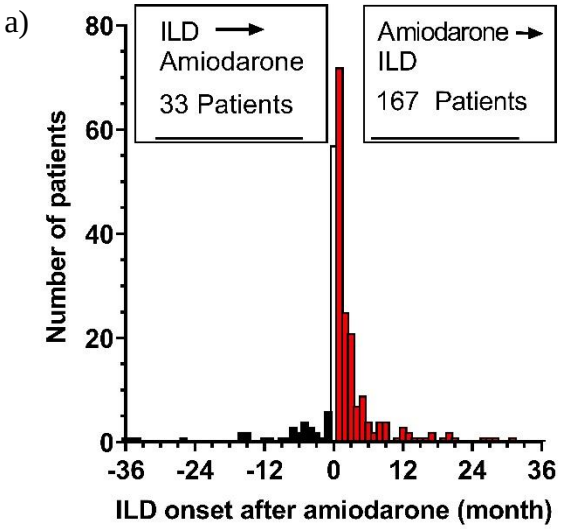


Figure 1.3. Amiodarone prescription and ILD diagnosis time distribution in JMDC claims database

a) Time distribution of patients prescribed with amiodarone. b) Time distribution of patients diagnosed with ILD

In the SSA of patients with ILD and patients receiving amiodarone, the number of patients with amiodarone then ILD sequence was observed higher (167 patients) than the number of patients with ILD then amiodarone sequence (33 patients) (Figure 1.4a). The adjusted sequence ratio of amiodarone associated with ILD was 7 (95% CI = 5.0 – 10.8).

Cumulative ILD incidence of amiodarone without dabigatran was observed > 10 %, while cumulative ILD incidence of amiodarone with dabigatran was < 6 % in 36 month observation period. At the 36th month, the ratio of number at risk of patients receiving amiodarone without dabigatran was 20 % and the ratio of number at risk of patients receiving amiodarone with dabigatran was 25 % of the number of patients at risk at the beginning of observation (Figure 1.4b). The hazard ratio of amiodarone with dabigatran was 0.55 (95% CI = 0.31 – 0.99) with log-rank test: p = 0.04



b)

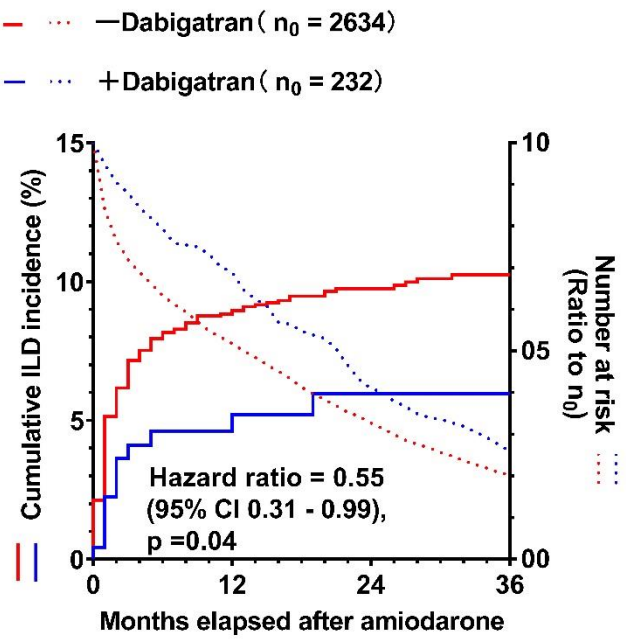


Figure 1.4. Dabigatran has a beneficial effect in decreasing the risk of amiodarone-induced ILD

a) Amiodarone was highly associated with increasing ILD by sequence symmetry analysis. b) Concomitant dabigatran reduced cumulative ILD incidence with the ratio of number at risk of patients receiving amiodarone without dabigatran was 20 % and the ratio of number at risk of patients receiving amiodarone with dabigatran was 25 % of the number of patients at risk at the beginning observation.

1.4. Discussion

The clinical database used in this research provides large data to be analyzed. Analysis of spontaneous adverse event report database such as FAERS is useful to identify drug associated with adverse event. In the FAERS database analysis, an anti-arrhythmia drug, amiodarone, was highly associated with ILD. This result is clinically relevant since amiodarone has been reported as a drug that induces ILD (7). This research finding on amiodarone and ILD association reconfirms previous reports on reproducible drug and clinical adverse event association on FAERS analysis, such as cisplatin-nephrotoxicity, carboplatin-myelosuppression, oxaliplatin-peripheral sensory neuropathy (30). In this analysis, bleomycin, a drug known to induce pulmonary fibrosis and a recommended agent as

inducer in animal model of idiopathic pulmonary fibrosis was less associated with ILD when compared to amiodarone. The nature of FAERS database that collect spontaneous report might potentially contain reporting bias.

In JMDC analysis, 3-month washout period was used to exclude patients who ever previously were prescribed with amiodarone or whoever had had ILD prior to insurance enrollment. Identification of initiation of amiodarone and of the new ILD diagnosis is required in sequence symmetry analysis methods because generally an event could occur soon after treatment initiation. By applying SSA method, it was revealed that amiodarone prescription increases ILD diagnosis in JMDC. This result reconfirms finding on FAERS analysis and clinical association of amiodarone with ILD. Low dose amiodarone also has been reported to increase incidence of pulmonary toxicity in Japanese patient (31).

A number of anti-coagulants were observed highly associated with suppression of amiodarone-associated ILD. Although pro-coagulant signaling could be a novel target for ILD (32,33), the result of warfarin clinical trial is considered not providing benefit and potentially harmful, which is unrelated to blood coagulation (34). Dabigatran, a direct thrombin inhibitor was observed to have the highest association with suppression of amiodarone-associated ILD. Thrombin has been reported to increase human lung proliferation (35). Dabigatran co-occurrence with amiodarone in JMDC analysis result also re-confirms analysis on FAERS database, where dabigatran co-occurrence reduces cumulative incidence of ILD.

As a conclusion, the current data mining on clinical database analysis provides finding that dabigatran can suppress amiodarone-induced ILD.

Chapter 2. Experimental validation for the effect of dabigatran on amiodarone-induced ILD

2.1. Introduction

Amiodarone, an iodinated benzofuran drug was originally developed more than 5 decades ago. To date, amiodarone remains to be one of the most effective antiarrhythmic drugs (36). However, its clinical use has been limited because of its numerous side effects that may involve the skin, eyes, lungs, liver, as well as central and peripheral nervous system (37). Patients prescribed with amiodarone had been reported to develop pulmonary toxicity ranging from 1% to 17% (37). Total cumulative dose is likely to correlate with amiodarone-induced interstitial lung disease rather than the daily dose or plasma concentration of amiodarone (38). Although the clinical sign and symptom of amiodarone-induced ILD are non-specific, including nonproductive cough, dyspnea, weakness, weight loss, and occasionally fever (39), amiodarone-induced ILD can be characterized by an increase of alveolar macrophages, widening of alveolar septum with a cellular inflammatory infiltrate, and varying degrees of interstitial fibrosis (40,41).

Key characteristics of amiodarone-induced ILD are increasing alveolar macrophages and varying degree of fibrosis (40,41). Macrophages are immune cells that reported to play a key role in the pathogenesis of fibrotic lung disease (42). Classically macrophages phenotypes are divided into M1 macrophages and M2 macrophages, where M2 macrophages produce TGFβ that drive fibrosis (43). Lung fibroblasts are a cell population of mesenchymal origin that play important role in fibrotic ILD (17). Fibroblast differentiation to myofibroblast with contractile phenotype is a major characteristic in fibrotic ILD (44).

Objective of this chapter is to establish amiodarone-induced ILD in mice and evaluate the effect of dabigatran co-treatment with amiodarone.

2.2. Methods

2.2.1. Drug and chemical

Amiodarone hydrochloride was purchased from Nacalai Tesque (Kyoto, Japan), dabigatran etexilate was purchase from Combi-Blocks (San Diego, USA). Amiodarone and dabigatran were suspended in CMC-NA (Nacalai Tesque, Kyoto, Japan) 0.5 % prior to use.

2.2.2. Animal and treatments

All animal experiments were approved by the Kyoto University Animal Research Committee in accordance with the ethical guidelines of the Committee. Male C57BL/6J mice (6–7 weeks of age) were purchased from Japan SLC (Shizuoka, Japan) and housed with a constant ambient temperature (22 ± 1 °C) and humidity (55% ± 10%) on a 12 h/12 h light/dark cycle. Mice had free access to food and water. All efforts were made to minimize the number of animals used and limit experimentation to only what was necessary to produce.

In order to evaluate amiodarone-induced lung disease, mice were randomized into 2 groups. The first group was treated with amiodarone 300 mg/kg/day, 5 times a week. The second group serves as control group, treated with CMC-Na 0.5% with equal volume to amiodarone group. Mice were monitored for body weight change. Histology specimens were prepared from mice lung collected at day 26. In order to evaluate gene expression, mice were sacrificed at day 1, 3, 5, 12, 19, 26 and their lungs were collected.

In order to evaluate dabigatran effect on amiodarone-induced ILD, mice were randomized into 4 groups. The first group was treated with amiodarone 300 mg/kg/day, 5 times a week. The second group was treated with dabigatran 60 mg/kg/day, 5 times a week. Third group was treated amiodarone 300 mg/kg/day + dabigatran 60 mg/kg/day, 5 times a week. The fourth group serves as control group, treated with CMC-Na 0.5% with equal volume. Mice were monitored for body weight change. Mice were sacrificed at day 26 and their lungs were collected for evaluating gene expression and histology specimens.

2.2.3. Quantitative RT-PCR

The levels of mRNA were analyzed using a quantitative RT-PCR approach. In brief, total RNA was isolated using the Isogen Reagent (Nippon Gene, Tokyo, Japan), and the isolated RNA (500 ng) was synthetized to cDNA using the ReverTra Ace (Toyobo, Osaka, Japan). Quantitative RT-PCR was performed using the StepOne Real-Time PCR System (Life Technologies, Carlsbad, USA) and the Thunderbird SYBR qPCR Mix (Toyobo). The following conditions were used for the amplification process: 10 min at 95 °C, followed by 35 cycles at 95 °C for 15 s and 60 °C for 1 min. The oligonucleotide primers used for RT-PCR were as follows: 5′-GCT TGT GCA GGA TCA GTA TGG-3′ and 5′-CAG CAC GCT CTG TAC GTG A -3′ for the *Pum1*; 5′-GAG CAC AGA AAG CAT GAT CCG-3′ and 5′ -GCC ATT TGG GAA CTT CTC ATC-3′ for *Tnfa*; 5′-GCA ACA ATT CCT GGC GTT ACC-3′ and 5′-TAT TCC GTC TCC TTG GTT CAG C-3′ for *Tgfb1*; 5′- AGA GGC GAA GGC AAC AGT CG-3′ and 5′- GCA GGG CCA ATG TCT AGT CC-3′ for *Cola1*; 5′- ACC AAA AGG TGA TGC TGG AC-3′ and 5′-GAC CTC GTG CTC CAG TTA GC-3′ for *Col3a1*; and 5′-TCA ACC CTA GTT CCT GCA TCC-3′ and 5′-GCT TGC AGA TCA TCC AGT CG-3′ for *Pdgfra*; 5′-CTT TCA TTG GGA TGG AGT CAG C-3and 5′-CAA TGC CTG GGT ACA TGG TG-3′ for *Acta2*; 5′-GGA AGA GAA GTA TTG AGG AAG CC-3 and 5′-AGA TCA GGA AGT TGG CCG ATG-3′ for *Pdgfa*; 5′-AGT ACC ATG AGG TCC TTC AGT TGA G-3and 5′-TCC TGC GTT TCC TCT ACA CAC-3′ for *Pdgfc*. The mRNA expression levels of each gene were normalized to that of *Pum1* mRNA.

2.2.4. Histological examination

Mice right lungs were fixed in 4% paraformaldehyde for 24 h at 4 ^{0}C and then transferred to ethanol 70%, dehydrated, and processed for paraffin embedding. Paraffin sections were cut into 5 μm thick, stained with hematoxylin-eosin (HE) and Sirius Red for evaluation of alveolitis and fibrosis under a microscope (Nikon, Tokyo, Japan).

2.2.5. Data analysis

Data analysis was performed using GraphPad Prism8. Mean differences of two groups were analyzed with unpaired t-test. Varian differences of groups were analyzed with one-way ANOVA with Dunnett's multiple comparison tests.

2.3. Results

2.3.1. Amiodarone decreases mouse body weight

In order to establish animal model of amiodarone-induced ILD, mice body weights were monitored. Amiodarone (300 mg/kg p.o., 5 days a week for 4 weeks) treatment was found to induce mice body weight decline. The decline started from the first week, and the body weight remained low from the end of second week to the end of fourth week (Figure 2.1). This result indicated amiodarone-induced ILD in mice that appeared correlated to clinical finding of amiodarone-induced ILD.

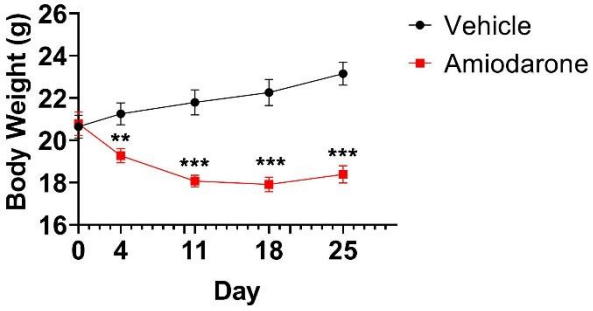


Figure 2.1. Amiodarone treatment reduced mice body weights
Mice were treated with amiodarone (300 mg/kg p.o., 5 days a week for 4 weeks) and body weights were monitored. n = 10 – 16, data expressed as mean ± SEM. ***P*<0.01, ****P*<0.001.

2.3.2. Amiodarone induces alveolitis

Lung histology was examined to confirm amiodarone-induced lung toxicity. In the lung of mice treated with amiodarone, increasing alveolar macrophages were observed (Figure 2.2a and 2.2b) but collagen was not increased in lungs of mice treated with

amiodarone (Figure 2.2c and 2.2d). This finding confirmed amiodarone-induced ILD with marked characteristic of alveolitis and interstitial thickening.

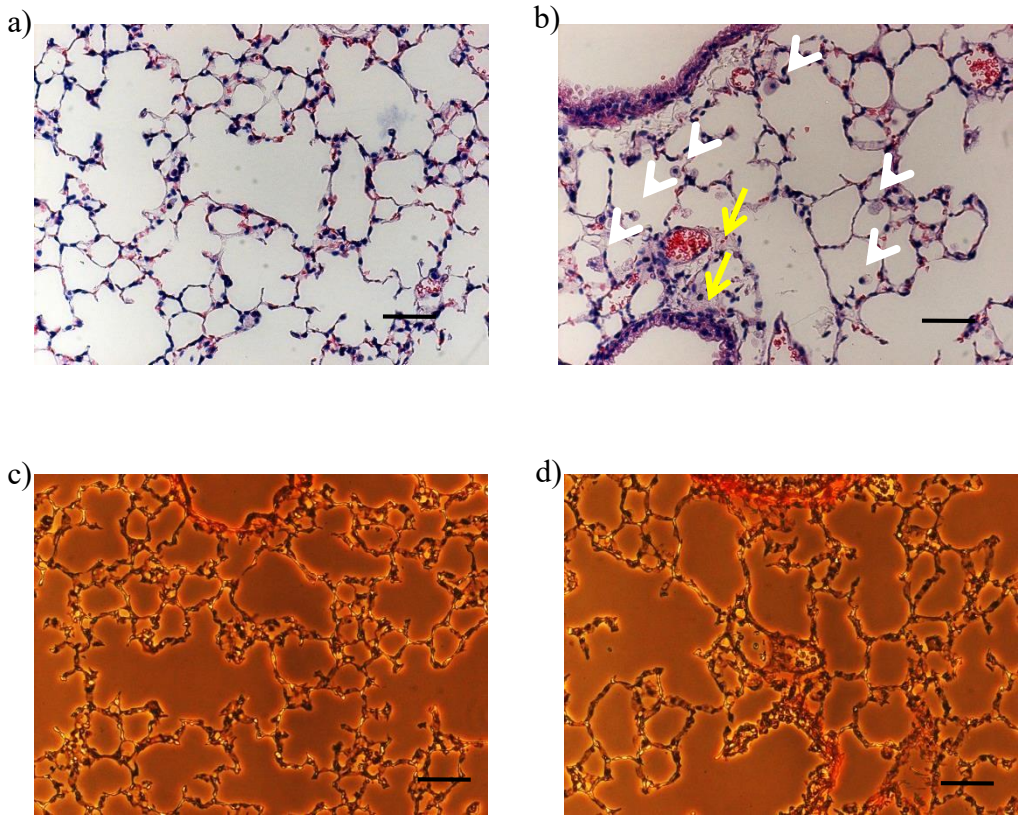


Figure 2.2. Amiodarone treatment induced alveolitis
Mice were treated with amiodarone (300 mg/kg p.o., 5 days a week for 4 weeks) and their lungs were collected at day 26. Representative HE staining picture of lung from mice treated with a) vehicle and b) amiodarone. Yellow arrow = interstitial thickening; white arrow head = alveolar macrophages, scale = 50 μm. Representative Sirius Red staining picture of lung from mice treated with c) vehicle and d) amiodarone. Amiodarone treatment did not induce collagen in mouse lung.

2.3.3. Amiodarone does not induce *Tnfa* nor *Tgfb1*

Since inflammation and fibrosis are characteristic in ILD, marker of inflammation *Tnfa* and marker of fibrosis *Tgfb1* were measured in the lung in time-dependent manner. Amiodarone treatment was found not to increase both *Tnfa* and *Tgfb1* (Figure 2.3).

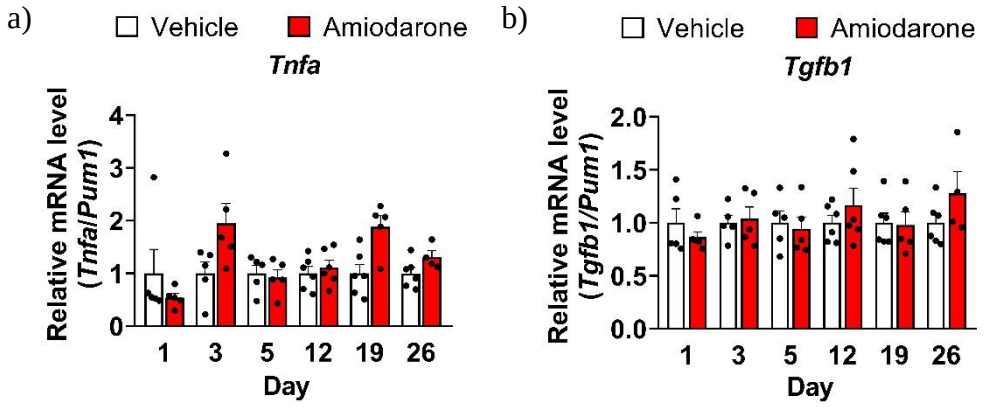


Figure 2.3. Amiodarone treatment did not induce *Tnfa* and *Tgfb1*
Mice were treated with amiodarone (300 mg/kg p.o., 5 days a week for 4 weeks) and their lungs were collected at day 1, 3, 5, 12, 19, and 26. The expression of a) *Tnfa* and b) *Tgfb1* were measured in the collected lungs. n = 4-6, data expressed as mean ± SEM.

2.3.4. Amiodarone downregulates *Col1a1* and *Col3a1*

Collagen accumulation is one of ILD characteristic. Marker for collagen accumulation, *Col1a1* and *Col3a1*, were measured in this research. Amiodarone treatment reduced *Col1a1* and *Col3a1* gene expression (figure 2.4).

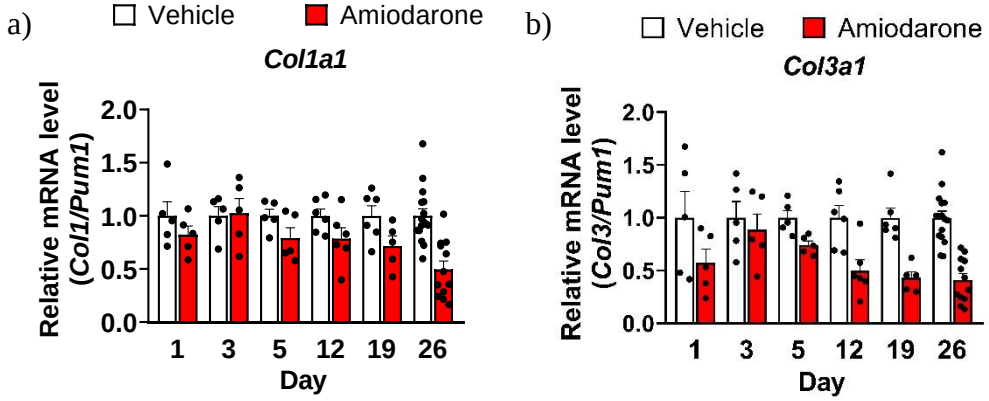


Figure 2.4. Amiodarone treatment reduced *Col1a1* and *Col3a1*
Mice were treated with amiodarone (300 mg/kg p.o., 5 days a week for 4 weeks) and their lungs were collected at day 1, 3, 5, 12, 19, and 26. The expression of a) *Col1a1* and b) *Col3a1* were measured in the collected lungs. n = 5-16, data expressed as mean ± SEM.

2.3.5. Amiodarone upregulates *Pdgfra* but not *Acta2*

Fibroblasts and myofibroblasts are key players in ILD. Marker for fibroblast proliferation, *Pdgfra*, was upregulated by amiodarone treatment but marker for myofibroblast accumulation, *Acta2*, was not upregulated by amiodarone treatment (Figure 2.5).

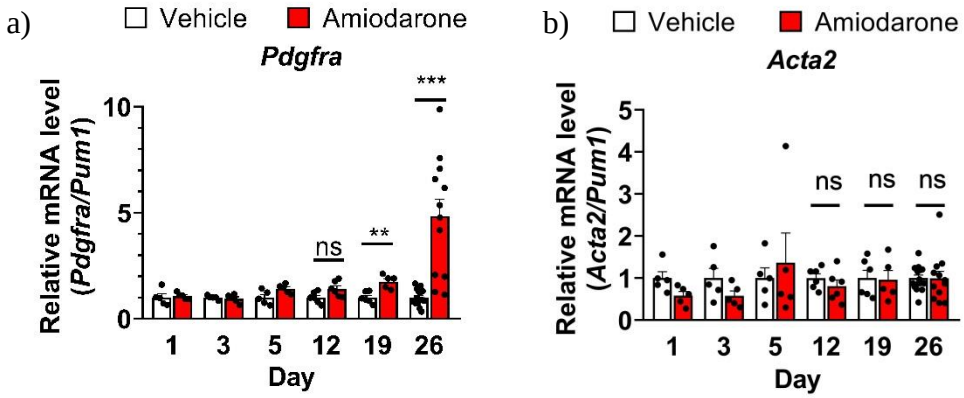


Figure 2.5. Amiodarone treatment induced *Pdgfra* but did not induce *Acta2*
Mice were treated with amiodarone (300 mg/kg p.o., 5 days a week for 4 weeks) and their lungs were collected at day 1, 3, 5, 12, 19, and 26. The expression of a) *Pdgfra* and b) *Acta2* were measured in the collected lungs. n = 5-16, data expressed as mean ± SEM. ns = not significant, ***P*<0.01, ****P*<0.001 vs control.

2.3.6. Amiodarone induced *Pdgfc* upregulation but not *Pdgfa*

Fibroblast surface protein expression (*Pdgfra*) was found to be upregulated by amiodarone. Its signaling was triggered by its platelet derived growth factor ligand. Amiodarone treatment induced *Pdgfc* upregulation but did not induce *Pdgfa* upregulation (Figure 2.6).

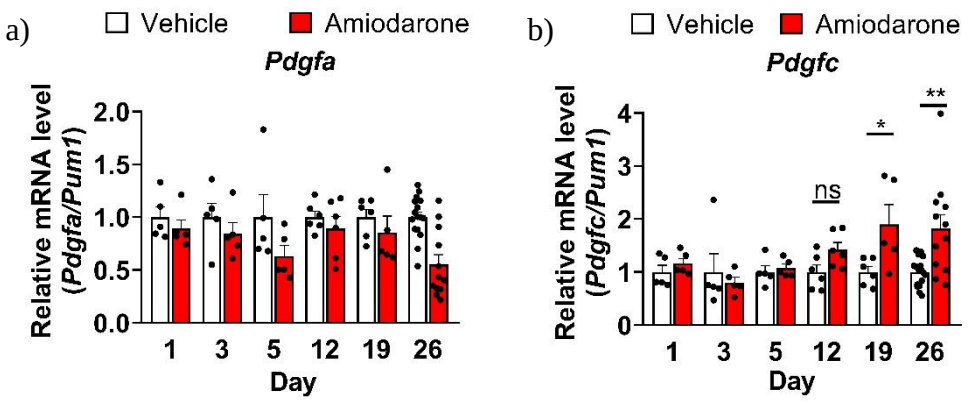


Figure 2.6. Amiodarone treatment induced *Pdgfc* but did not induce *Pdgfa*
Mice were treated with amiodarone (300 mg/kg p.o., 5 days a week for 4 weeks) and their lungs were collected at day 1, 3, 5, 12, 19, and 26. The expression of a) *Pdgfa* and b) *Pdgfc* were measured in the collected lungs. n = 5-16, data expressed as mean ± SEM. ns = not significant, **P*<0.05, ***P*<0.01 vs control.

2.3.7. Dabigatran co-treatment reduces amiodarone-induced body weight declining

After confirming amiodarone-induced ILD in mice, dabigatran co-treated with amiodarone was evaluated in this model. Dabigatran (60 mg/kg p.o., 5 days a week for 4 weeks) co-treatment with amiodarone reduced amiodarone-induced body weight declining (Figure 2.7).

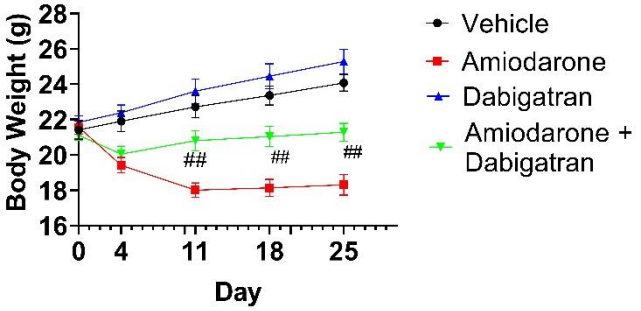


Figure 2.7. Dabigatran co-treatment reduced amiodarone-induced body weight declining
Mice were co-treated with dabigatran (60 mg/kg p.o., 5 days a week for 4 weeks) and their body weights were monitored. n= 8-11, data expressed as mean ± SEM. $^{\#\#}P$<0.01 vs amiodarone

2.3.8. Dabigatran co-treatment reduces amiodarone-induced alveolitis

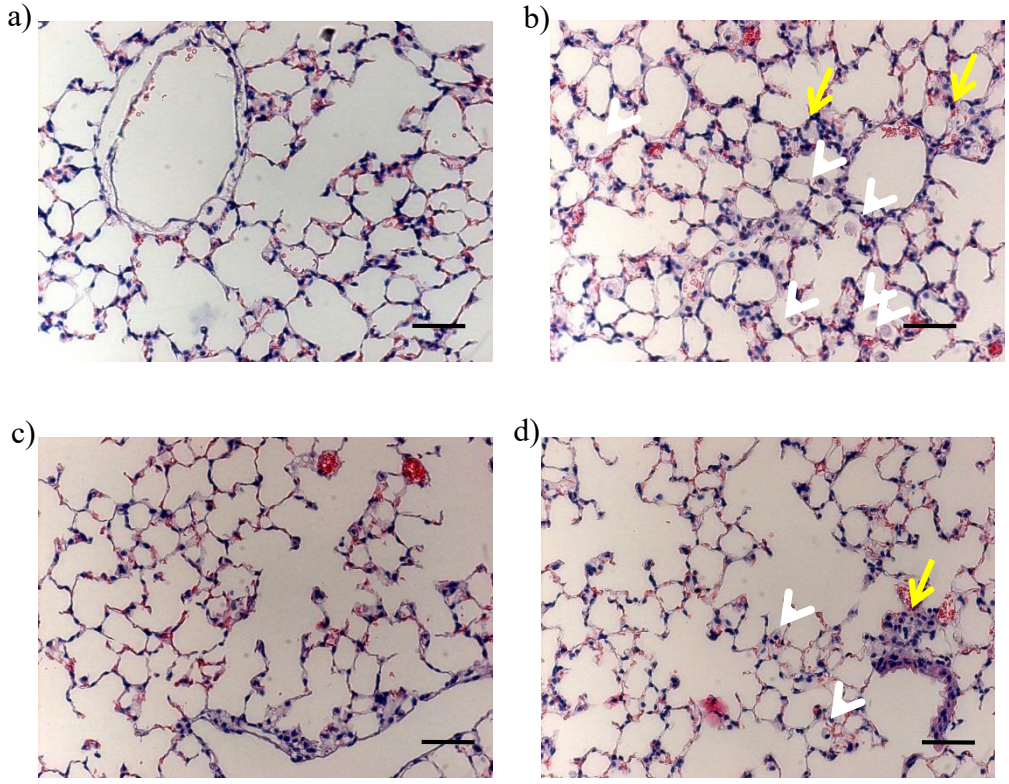


Figure 2.8. Dabigatran co-treatment reduced alveolitis and interstitial thickening
Mice were co-treated with dabigatran (60 mg/kg p.o., 5 days a week for 4 weeks) and their lungs were collected at day 26. Representative HE staining picture of lung from mice treated with a) vehicle, b) amiodarone (300 mg/kg p.o., 5 days a week for 4 weeks), c) dabigatran, and d) amiodarone and dabigatran. Yellow arrow = interstitial thickening; white arrow head = alveolar macrophages, scale = 50 μm.

Dabigatran co-treatment reduced intra alveolar macrophage and interstitial thickening in lung of mice treated with amiodarone (Figure 2.8).

2.3.9. Dabigatran co-treatment reduces amiodarone-induced *Pdgfra* upregulation but did not reduces amiodarone-induced *Pdgfc* upregulation

Dabigatran co-treatment with amiodarone reduced amiodarone-induced *Pdgfra* but not *Pdgfc* upregulation (Figure 2.9).

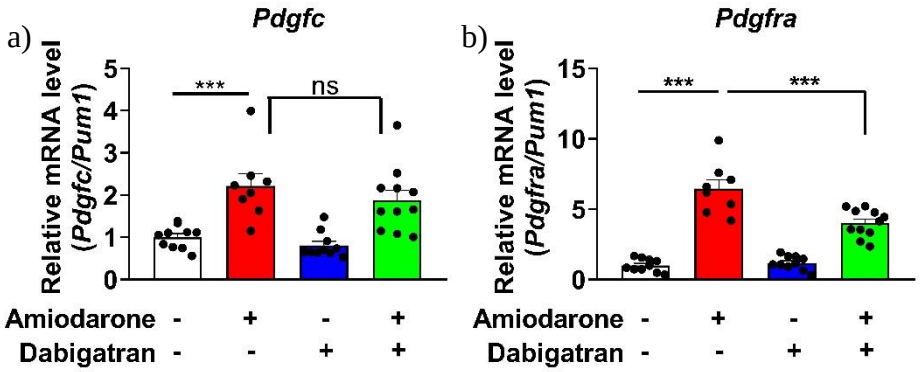


Figure 2.9 Dabigatran co-treatment reduced *Pdgfra* upregulation
Mice were co-treated with amiodarone (300 mg/kg p.o., 5 days a week for 4 weeks)-dabigatran (60 mg/kg p.o., 5 days a week for 4 weeks) and their lungs were collected at day 26. The expression of a) *Pdgfc* and b) *Pdgfra* were measured in the collected lungs.. n = 8 -11, data expressed as mean ± SEM. n.s = not significant, ***P<0.001 vs amiodarone treated mice.

2.4. Discussion

In this mouse model, alveolitis was observed in mice lungs after amiodarone treatment for 4 weeks. In lung histology, increased intra-alveolar macrophage and interstitial thickening were also observed but there was no increase in collagen deposition in lung mice. Amiodarone also has been described to induce body weight declining in a previously reported mouse model of amiodarone-induced lung toxicity (45).

Interstitial lung disease is characterized with inflammation and fibrosis. In this research, marker of inflammation *Tnfa* and marker of fibrosis *Tgfb1* were measured but neither of them was altered by amiodarone treatment. Since fibroblast is an important cell that has a role in interstitial lung disease, *Pdgfra* a marker for fibroblast surface protein and *Acta2* as marker of increasing myofibroblast were measured. In this research, *Pdgfra* was increased by amiodarone treatment, while *Acta2* was not increased. In this study, PDGFRα ligands were also examined. The expression of *Pdgfc* but not *Pdgfa* was increased by amiodarone treatment in time dependent manner.

Platelet-derived growth factors receptors (PDGFRs) and its ligand PDGFs represent a family of profibrotic growth factors (46). In a recently published report, PDGFRα^{+} cells were reported to have contribution to both injury healing and fibrosis (47). Upregulation of PDGFRα was associated with fibroblast proliferation (48), while its ligand PDGF-AA suppressed myofibroblast trans-differentiation (49). In another report, PDGF-CC showed more dominant in profibrotic role compared to PDGF-AA (50,51). Activation of PDGFRα by PDGF-CC stimulated receptor auto-phosphorylation and then activates PI3K and MAPK pathways (52). In bleomycin model, PDGF-CC was also upregulated in mouse lung (53). Cells that have reported expressing PDGF-CC were epithelial cell and macrophages (54).

In bleomycin model of lung fibrosis, thrombin and protease activated receptor 1 was upregulated. Direct thrombin inhibition reduced collagen, procollagen 1a1 and CTGF (55). In the same model, PAR1 knockout reduced CCL2, CTGF, TGFβ and total collagen (56). Alveolar epithelial type II was reported as a cell expressed PAR1 (57). In several animal model of fibrosis, dabigatran has been reported to have a benefit in attenuated fibrosis (58,59). In previous study, mechanism of dabigatran in fibrosis attenuation was reported to involve thrombin/protease-activated receptor 1/integrin $\alpha_v\beta_6$/TGFβ axis (60).

In this research, dabigatran co-treatment attenuated amiodarone-induced weight loss. Furthermore, dabigatran co-treatment has been found to improve outcome in histological findings in amiodarone-induced ILD. These results suggest that dabigatran co-treatment has beneficial effect in amiodarone-induced ILD. From the gene expression examinations, it was revealed that the effect of dabigatran co-treatment in amiodarone-induced ILD was associated with attenuation of *Pdgfra* upregulation.

Conclusion

In this research on drug repurposing strategy based on clinical data, it was identified that dabigatran, a thrombin inhibitor, has potential benefit in treatment for patient with ILD.

In the first chapter, analysis on FAERS, amiodarone was observed highly associated with ILD. This finding was reconfirmed by analysis on JMDC, where amiodarone prescription has increased ILD diagnosis. Amiodarone-ILD association observed by this analysis is clinically correlated since amiodarone has been reported as a causal of ILD. Dabigatran was associated with low amiodarone-associated ILD in FAERS analysis. In this database analysis, dabigatran co-occurrence was found to reduce ILD incidence rate. This finding was reconfirmed through analysis on JMDC database, where dabigatran co-occurrence with amiodarone reduced ILD cumulative incidence.

In the second chapter, amiodarone-induced ILD was confirmed in mice. Amiodarone treatment induced mice body weight decline. Alveolitis and interstitial thickening were observed in lungs of mice treated with amiodarone. Gene expression of fibroblast protein surface *Pdgfra*, was upregulated by amiodarone treatment. Gene expression of *Pdgfc* was also upregulated by amiodarone treatment. Dabigatran co-treatment has improved outcome in body weight declining and in histological findings in amiodarone-induced ILD. The beneficial effect of dabigatran co-treatment was associated with attenuation of *Pdgfra* upregulation.

This study demonstrated systematical analysis on clinical databases and suggested dabigatran as a new candidate drug to suppress amiodarone-induced ILD. Although the mechanisms or the exact target is still unclear, this study suggested that dabigatran has beneficial effect in mouse model of amiodarone-induced ILD. For further study it is essential to investigate the exact target or mechanism of dabigatran in amiodarone-induced ILD and to investigate its target in correlation with fibrotic ILD.

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